Visualization of the moonlighting protein CD26DPPIV
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Visualization of the moonlighting protein CD26/DPPIV

E.P. Boonacker
VISUALIZATION OF THE MOONLIGHTING PROTEIN
CD26/DPPIV

ACADEMISCH PROEFSCHRIFT

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CHAPTER 1

General introduction
General introduction

Complex scientific questions concerning functioning of biological organisms are usually approached as a series of subquestions that are easier to define and to investigate. When these subquestions have been solved, they provide insights, which illuminate the complexity of the entire organism. This approach has been successful so far and has led to the identification of the function of many proteins and other macromolecules. However, this approach does not allow elucidation of the simultaneous complex interactions that occur in living cells, tissues and organisms. The genome of man and other organisms has been sequenced, we know in principle which proteins are involved, but this information does not help us in the understanding of the functioning of these proteins. Furthermore, many macromolecules in cells are not encoded for, such as lipids and sugars. These molecules play essential but often unknown roles in cellular processes as well. So, the intact biocomplex entity such as a cell functions by virtue of a multitude of interacting molecular events. External stimuli or intracellular changes in gene expression modulate these interactions. Recently, it has become clear that transcription of proteins is clustered in the so-called transcriptome (Caron et al. 2001). Moreover, proteins function in complexes which determine how the individual proteins act (Hehre 2001; Miklós and Maleszka 2001; Schwille and Kettling 2001; Gavin et al. 2002) and on top of that, many proteins are multifunctional.

Multifunctionality of proteins may have been introduced by the fusion of genes, thereby limiting the number of genes, but maintaining the different functions (Davidson et al. 1993). Multifunctionality of proteins may be a common phenomenon and is called moonlighting (Jeffery 1999). It adds another dimension to cellular complexity, from which cells can benefit in various ways. Therefore, elucidation of the functional roles of moonlighting proteins and their regulation will become pertinent in cell biology as part of the functional proteomics approach (Perham 2000; Miklós and Maleszka 2001).

Complex interactions between macromolecules also play an important role in regulating metabolic activity in vivo. However, metabolic processes are usually studied in homogenates, in which the complex interactions are lost (Van Noorden and Jonges 1995; Stoward et al. 1998). A logical next step to overcome these limitations is the use of molecular tools to study metabolic processes under controlled conditions in living systems (Haugland 1995; Sarneni et al. 2000; Boonacker and Van Noorden 2001; Hahn and Toutchkine 2002; Patton and Beechem 2002). Studies with the use of intact biological systems have taught us thus far that metabolic processes are heavily regulated posttranslationally by molecular interactions (Ovadi and Srere 2000). For example, enzymes are liable to rapid variations in kinetic parameters, that are regulated by interactions with other (macro)molecules (Swezey and Epel 1986; Rees et al. 1996). As it turns out, a wide variety of cellular proteins, from oligomeric enzymes to crystallins and ion channels, switches partners and are then involved in different functions when their aggregation state or intracellular and/or extracellular localization is changed (Jeffery 1999; Ejiri 2002). These variations that occur in vivo are not found when cells and tissues are (chemically) fixed or their integrity has
been destroyed. To clarify how metabolic processes proceed in vivo, enzyme reactions have to be monitored in living cells, tissues or organisms. One approach that is very attractive for this purpose is the quantitative microscopical analysis of enzyme reactions in time and space (4D-analysis) in living cells using fluorogenic or chromogenic substrates and digital microscopy (Haugland 1995; Boonacker and Van Noorden 2001; Patton and Beechem 2002). A different approach to unravel the functioning of enzymes in living cells is the detection of processing of natural substrates.

The complexity of metabolism in a living cell

Studies with purified enzymes have provided insight that enzymes which were generally considered to be soluble and freely diffusible, such as glycolytic enzymes, are in fact organized in multi-enzyme complexes or so-called molecular machines (Perham 2000). It has become clear that well-defined intracellular compartments, such as cytosol and mitochondrial matrix, cannot simply be regarded as bags containing homogeneous solutions of enzymes, but are rather well organized. Furthermore, the role of water in metabolic processes is an interesting but largely ignored aspect in cell biology. Usually enzyme reactions are measured in dilute solutions of cell or tissue extracts, whereas enzymes in vivo function in high (protein) concentrations in cells (Srere 1967, 1980; Fulton 1982; Srivastava and Bernhard 1987; Aragon and Sols 1991). Cells contain 20-40% proteins by weight. In this rich solution, interactions between enzymes and their cellular microenvironment are completely different from those in dilute solutions (Masters 1981; Fulton 1982). Homologous (Hulme and Tipton 1971; Minton and Wilf 1981) and heterologous (Wiame 1971; Srivastava and Bernhard 1987) molecular interactions have been described to occur in vivo. Homologous interactions are considered to be changes in enzymes from a monomer form to a polymer form, such as the dimerisation of CD26/dipeptidyl peptidase IV (DPPIV), the moonlighting protein under study in the present thesis. Heterologous interactions are associations between enzymes and other proteins such as structural elements of the cell, e.g. membrane-associated structures or cytoskeletal components (Pette and Brandau 1962; Arnold and Pette 1968; Sigel and Pette 1969; Swezey and Epel 1986; Rogalski et al. 1989; Minaschek et al. 1992). Heterologous interactions of CD26/DPPIV occur with the mannose-6-phosphate receptor (M6P) for transport and with another protease, Fab-a (Rettig et al. 1993, 1994; Scanlan et al. 1994). These interactions may effect the catalytic activity considerably. Furthermore, enzyme complexes enable the product of the first enzyme to be utilized by a second enzyme of a metabolic pathway. Channeling of metabolic intermediates within these complexes has been postulated to lead to increased efficiency of substrate fluxes by coupling of reactions in a metabolic pathway. Channeling can also lead to sequestration of intermediates, so that flux into other pathways that compete for the same substrates and/or intermediate products is restricted (Ovadi and Srere 2000). This mechanism may be called preferential pathway because it explains how a substrate that can be used in different pathways is channeled in one that is relevant for an organism at a certain point in time. This is discussed recently for NADPH in livers of flatfish and explains why female flatfish and not male flatfish develop liver cancer when living in a chemically-polluted
environment (Koehler and Van Noorden 2003). NADPH is a major metabolite in livers used in detoxification processes and biosynthetic processes such as the production of vitellogenin needed for oocytes. In females, NADPH is channeled in the preferential pathway of biosynthesis when oocytes are generated and thus not available for detoxification at that time. As a consequence, females are exposed to toxicants in those periods and may develop liver cancer. It is an example of evolutionary laws that the offspring and not the individual is important for a species to survive. It is obvious that spatial crowding of enzymes in living cells has definitive advantages over an at-random distribution of enzymes in a cellular organelle or the cytoplasm. Nevertheless, our understanding of the formation of these complexes, its regulation and the metabolic consequences of these molecular interactions, is limited due to our lack of understanding of the intracellular environment of enzymes in terms of (free) substrate and effector concentrations and the interactions with other enzymes and macromolecules (Aragon and Sols 1991). Interactions are often transient and based on weak protein-protein interactions. These interactions have been implicated to be important in metabolic flux control (Casecante et al. 2002) but are most of the time ignored in any model based on the kinetic properties of purified enzymes. For example, kinetic parameters of CD26/DPPIV have been determined in situ along villi of rat and human jejunum (Gutschmidt and Gossrau 1981). Differences in $V_{\text{max}}$ and $K_m$ were found in apical and basal villi related with the differentiation of the epithelial cells. These findings suggest that fluxes through this enzyme may differ strongly at the different sites of the villi. CD26/DPPIV shows the lowest $V_{\text{max}}$ and highest $K_m$ at the base of the villi where epithelial cells differentiate, whereas highest $V_{\text{max}}$ and lowest $K_m$ values were found at the apex of the villi where the oldest cells are present.

Besides in vitro experiments to study flux control in metabolic pathways (Groen et al. 1983; Westerhoff et al. 1984; Ovadi 1988; Cascente et al. 2002), there is a need to study enzymes directly in functionally intact living cells to address questions concerning the relevance of enzyme complexes and interactions with other molecules for metabolic processes and other functional processes and the multifunctionality or moonlighting of proteins.

**Metabolic regulation by macromolecular interactions**

Interactions of an enzyme with other macromolecules may be involved in the regulation of activity of that enzyme. Two examples are given here to illustrate this type of (rapid) regulation of activity at the posttranslational level.

Studies by Wang et al. (1997) and Kusakabe et al. (1997) have shown that specific molecular interactions between aldolase, a glycolytic enzyme, and actin is responsible for the functioning of aldolase. Most of the aldolase is preferentially localized on stress fibers and in close vicinity of active ruffles of cells (Kusakabe et al. 1997). This relative subcellular enrichment of aldolase is explained by its interactions with actin that can be modulated by physiological effectors such as insulin, calcium and anoxia. In the turtle brain, increased aldolase binding has been observed during anoxia-induced metabolic arrest (Duncan and Storey 1992). The physiological relevance of binding of aldolase to the cytoskeleton is still not clear, but it has been postulated that aldolase has a dual role as enzyme and as structural part of the cytoskeleton. Binding of aldolase to the F-
actin core of microfilaments inhibits competitively the enzymatic conversion of the substrate of aldolase, fructose-1,6-biphosphate.

Another example of posttranslational regulation of metabolism by macromolecular interactions is the activity of glucose-6-phosphate dehydrogenase (G6PDH). Swezey and Epel (1986), who described the enzyme G6PDH in sea urchin eggs and found that in the unfertilized sea urchin eggs, 60% of G6PDH is bound to structural elements and then has a relatively low \( V_{\text{max}} \) and high \( K_m \) whereas, within a few seconds after fertilization, G6PDH has a distinctly higher \( V_{\text{max}} \) and lower \( K_m \). Because G6PDH is released and produces large quantities of NADPH needed for hardening of the membrane (synthesis of the fertilization membrane) to prevent polyspermy and thus a lethal embryo, underlines the importance of rapid posttranslational metabolic regulation in living cells (Shapiro 1991; Rees et al. 1996).

**Moonlighting proteins**

The function of a moonlighting protein (Jeffery 1999; Ejiri 2002) can vary as a consequence of changes in cellular localization, cell type, oligomeric or polymeric state, and intracellular concentrations of ligand, substrate, cofactor or product. Moonlighting proteins can switch between functions in a number of ways as is demonstrated here with a few examples.

The *Escherichia coli* PutA protein has both proline dehydrogenase activity and pyrroline-5-carboxylate dehydrogenase activity when it is associated with the plasma membrane, but lacks enzymatic activity when it binds to DNA as a transcriptional repressor (Ostrovsky de Spicer and Maloy 1993; Muro-Pastor et al. 1997). Its localization switches on the basis of the amount of available substrate, ligand or cofactor. The PutA protein binds to the plasma membrane when substrate concentrations are high, but binds to DNA when substrate concentrations are low.

Proteins can also have different functions when they are present intracellularly or extracellularly. For instance, phosphoglucoisomerase is an ubiquitous cytosolic enzyme and catalyzes the second step of glycolysis. However, it is also secreted by cells and then it has at least four additional functions. Phosphoglucoisomerase can act as neuroleukin, which is both a cytokine that causes B cells to mature into antibody-secreting cells and a nerve growth factor that promotes the survival of embryonic spinal neurons and sensory nerves (Gurney et al. 1986). In addition, phosphoglucoisomerase/neuroleukin is also known as an autocrine motility factor, which is a cytokine that stimulates cell migration. Finally, the enzyme is a differentiation and maturation mediator that induces differentiation of human myeloid leukemia cells.

A protein can also perform different functions when it is expressed in different cell types. Neuropilin is a cell surface receptor on endothelial cells for vascular endothelial growth factor (Soker et al. 1998). In axons, it serves as a receptor for another ligand, semaphorin III, which guides axons to find their destination during outgrowth.

Some proteins have different functions when they are present as monomer or multimer. For example, the monomer 37-kDa subunit of human glyceraldehyde-3-phosphate dehydrogenase acts as uracil-DNA glycolase in the nucleus (Meyer-Siegler et al. 1991), whereas the tetramer
converts glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate.

**Aim of the study**

Functioning of a protein in a complex environment is not easy to understand. Visualization of the various functions of a protein in living cells in 4D can therefore be a powerful tool to improve our understanding of moonlighting properties of proteins. In the present thesis, principles of metabolic mapping in living cells and tissues with the use of digital microscopy, natural and synthetic substrates are discussed (chapter 2). This approach enables the study of macromolecular interactions that regulate posttranslationally cellular metabolism. We have focussed on CD26/dipeptidyl peptidase IV (DPPIV) as a moonlighting protein that changes its function on the basis of these interactions (chapter 8). Two of its functions, intracellular signaling and proteolysis, have been studied by digital microscopy and flow cytometry of living cells using fluorescent synthetic substrates (chapters 3, 4, 5 and 6) and natural substrates (chapter 7), to understand how different functions of this moonlighting protein are regulated separately and what natural substrates are of DPPIV.

**References**


CHAPTER 2

Enzyme cytochemical techniques for metabolic mapping in living cells, with special reference to proteolysis

Emil Boonacker and Cornelis J.F. Van Noorden

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REVIEW

Enzyme Cytochemical Techniques for Metabolic Mapping in Living Cells, with Special Reference to Proteolysis

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SUMMARY Specific enzymes play key roles in many pathophysiological processes and therefore are targets for therapeutic strategies. The activity of most enzymes is largely determined by many factors at the post-translational level. Therefore, it is essential to study the activity of target enzymes in living cells and tissues in a quantitative manner in relation to pathophysiological processes to understand its relevance and the potential impact of its targeting by drugs. Proteases, in particular, are crucial in every aspect of life and death of an organism and are therefore important targets. Enzyme activity in living cells can be studied with various tools. These can be endogenous fluorescent metabolites or synthetic chromogenic or fluorogenic substrates. The use of endogenous metabolites is rather limited and nonspecific because they are involved in many biological processes, but novel chromogenic and fluorogenic substrates have been developed to monitor activity of enzymes, and particularly proteases, in living cells and tissues. This review discusses these substrates and the methods in which they are applied, as well as their advantages and disadvantages for metabolic mapping in living cells. (J Histochem Cytochem 49:1473-1486, 2001)

KEY WORDS

synthetic substrates
living cells
enzyme histochemistry
metabolic mapping
fluorogenic substrates
chromogenic substrates

SPECIFIC ENZYMES play key roles in many pathophysiological processes. In particular, proteases are enzymes that are responsible for essential steps in many diseases (Roose and Van Noorden 1995). Of the genomes that have been completely sequenced, 2% of the gene products encode proteases (Barrett et al. 1998). For understanding of the function of an enzyme in a pathophysiological process, it is not sufficient to localize mRNA with in situ hybridization (ISH) or its protein with immunocytochemistry (IHC). It is essential to localize and quantify its activity, preferentially under conditions that reflect the in vivo situation (Van Noorden and Jonges 1995a,b). In recent studies in which the localization of mRNA, protein, and activity of an enzyme has been compared directly, it appeared that mRNA and protein distribution patterns are of only limited use because the activity of an enzyme is often highly regulated at the post-translational level and it is the activity that represents the function of an enzyme (Biagiotti et al. 2000; Bleeker et al. 2000; Hazen et al. 2000).

Many enzymes, and particularly proteases, are present in cells and tissue compartments in an inactive form because they are either synthesized as precursors that have little if any catalytic activity and need post translational activation or they are bound to an endogenous inhibitor. The inactive enzyme can be converted to its active form by proteolytic processing by specific proteases, autocatalysis, or by binding of cofactors or removal of inhibitors. Hence, large amounts of inactive and therefore not functional enzyme can be accumulated in a tissue compartment. However, the enzyme can become activated rapidly on demand. This can be achieved, for example, by an amplification loop, in which a small amount of the active protease can directly or indirectly activate its inactive precursor in a defined cell or tissue compartment, resulting in an exponential rate of activation to ensure that the protease can accomplish its function locally when required. Endogenous inhibitors are present in tissues to establish a threshold that regulates the concentration...
of active proteases in cells and tissues, thus keeping proteolysis under control (Thornberry and Lazebnik 1998). It is the balance between post-translational activation and inhibition that determines the activity of a (proteolytic) enzyme. Therefore, histochemical or cytochemical localization of the activity of an enzyme (also called catalytic histochemistry and cytochemistry, or metabolic mapping) is a powerful approach to study whether an enzyme is functionally involved in a pathophysiological process because it links the enzyme activity to cell and tissue structure.

Localization of the activity of an enzyme is traditionally performed at substrate concentrations that produce maximal amounts of colored or fluorescent final reaction product. These concentrations are usually high so that the maximal velocity of the enzyme ($V_{max}$) is obtained. However, these high substrate concentrations are seldom present in vivo. Moreover, the affinity of an enzyme for its substrate(s) can also be under post-translational control (Jonges et al. 1992; Swezey and Epel 1986; Van Noorden et al. 1997a), e.g., due to interactions of the enzyme with other macromolecular structures. Variations in $V_{max}$ and $K_m$ greatly affect substrate conversion by an enzyme at physiological concentrations (Van Noorden and Jonges 1995a). Because of these considerations, metabolic mapping becomes more and more focused on the visualization of enzyme reactions in living cells and tissues at substrate concentrations that are physiological. When metabolic mapping is performed quantitatively with digital microscopy (Chieco et al. 2001), substrate concentrations can be varied and local $V_{max}$ and $K_m$ values can be determined easily in distinct tissue compartments or cell populations on the basis of the amount of colored or fluorescent final reaction product generated (Jonker et al. 1993). In this way, metabolic mapping provides many possibilities to link the actual functioning of an enzyme with pathophysiological alterations so that, for example, specific inhibitors can be tested for their therapeutic use.

Localization and quantification of the activity of enzymes in living cells and tissues can be performed by analysis of either the production or the consumption of fluorescent endogenous molecules, such as NADPH and NADH, or the formation of colored or fluorescent products generated from synthetic chromogenic or fluorogenic substrates using digital microscopy or flow cytometry. This setup allows quantitative monitoring of enzyme reactions in cells and tissues in time and space while the reaction proceeds. Although endogenous fluorescent molecules such as NAD(P)H, flavins, and porphyrins can be useful indicators of enzyme activity, such endogenous metabolites are limited in number and are not specific for a single enzyme. These molecules are both produced and consumed permanently in various enzymatic processes. Therefore, monitoring of these molecules in cells and tissues is not a good parameter of the activity of a specific enzyme. Consequently, the design, synthesis, and application of synthetic chromogenic and fluorogenic substrates are indispensable to identify specific enzymatic reactions in vivo experiments. These substrates must meet a series of criteria for the application to living cells and tissues, such as the following.

- **First**, substrates must have access to the enzyme under study such that the physiological and structural integrity of cells or tissue compartments is maintained. Preferably, these substrates diffuse freely into intact cells without the aid of loading procedures. In general, lipophilic substrates can be introduced into cells by simply adding a small amount of a concentrated solution of substrate, dissolved in a solvent such as dimethylformamide or dimethylsulfoxide, to the incubation medium. The concentration of the solvent should be less than 1% in the final incubation medium to avoid adverse effects of the solvent on the living cells or tissues. However, for quantitative purposes an important question is the exact (intracellular) concentration of the substrate at the site of the active enzyme.

- **Second**, the substrate must be selectively converted by the target enzyme.

- **Third**, fluorescence or absorption of the enzyme product must be sufficiently strong to permit detection at physiological substrate concentrations. When high concentrations of substrates are used, activity can be visualized and increases in absorbance or fluorescence can be readily detected, but then the enzyme is active at non-physiological concentrations of the substrate.

- **Fourth**, in the case of synthetic substrates, conversion by the target enzyme should be similar to that of the natural substrate(s) of the enzyme, so that increase in absorbance or fluorescence in time is a proper reflection of physiological substrate fluxes. In other words, the energy differences between synthetic substrate and product should be similar to that of the natural substrate(s) and product(s) of that enzyme.

- **Fifth**, products of synthetic substrates must accumulate at the site of enzyme activity and the enzyme product should not diffuse away from the site of enzyme activity. Chemical characteristics of the enzyme product are often the cause of a higher affinity for cellular compartments other than those of the active target.

- **Finally**, synthetic substrates should be nontoxic for cells and tissues.

When enzyme reactions are analyzed quantitatively, one should realize that kinetic parameters of
enzymes can be seriously affected by various properties of the synthetic chromogenic or fluorogenic substrates. A number of these pitfalls are listed below.

- Affinity of the enzyme for its synthetic substrate is too low. However, affinity can be manipulated by chemical substitution of side groups in synthetic substrates.
- Synthetic substrates contain more than one site of action for an enzyme. For example, fluorogenic protease substrates often contain two groups that can be cleaved off. Enzymatic hydrolysis of the first group yields a fluorescent half-product and fluorescence increases after hydrolysis of the second group.
- More than one enzyme can convert the substrate.
- Permeability barriers for the substrate in intact cells limit the availability of the synthetic substrate for the enzyme and are rate-limiting, rather than the enzyme reaction itself.
- The product inhibits the enzyme reaction or the product diffuses away from the site of the active enzyme.

All these phenomena can affect the kinetics of enzymes and can therefore complicate the analysis of the activity of enzymes by metabolic mapping. For example, for accurate determination of enzyme activity with substrates containing two sites of action for an enzyme, the two-step catalysis, the channeling effect of intermediate products and the intracellular substrate concentrations should be considered, as described by Huang (1991).

This review discusses the advantages and disadvantages of fluorescent metabolites and chromogenic and fluorogenic substrates for their use in metabolic mapping in living cells and tissues on the basis of these criteria and considerations.

### Metabolic Mapping in Living Cells by Use of Endogenous Fluorescent Metabolites

A number of cellular metabolites are fluorescent. These fluorescent endogenous metabolites can be used to monitor enzyme reactions in living cells and tissues (Table 1). However, most of these metabolites must be excited with ultraviolet (UV) light, which rapidly damages living cells and tissues (König 2000). Furthermore, autofluorescence from molecules other than those under study can mask fluorescence of the specific metabolites. We discuss here characteristics of autofluorescence produced by endogenous metabolites, measurements of concentrations of endogenous fluorescent metabolites, and possible damage that can occur in cells that are irradiated with UV light.

The characteristics of autofluorescence are as follows:

- Autofluorescence of cells is composed of at least four distinct excitation and emission maxima: the tryptophan peak (290-nm excitation, 330-nm emission), the NAD(P)H peak (350-nm excitation, 450-nm emission), the riboflavin (FAD) peak (450-nm excitation, 530-nm emission), and a yet unidentified peak (500-nm excitation, 530-nm emission) (Heintzelman et al. 2000).
- Autofluorescence produced by fluorescent metabolites is mainly found in discrete cytoplasmic vesicles.
- Autofluorescence varies strongly in living cells. For example, it is low in freshly prepared cells and increases with time during culture until a plateau is reached.

Comparison of spectra of intact cells with spectra of known cell metabolites indicates that autofluorescence in cells arises mainly from intracellular NADH and riboflavin, flavin co-enzymes, and flavoproteins present in mitochondria. Co-enzymes fluoresce when in the reduced state (NAD(P)H) and do not fluoresce in the oxidized state (NAD(P)), whereas flavins fluoresce when in the oxidized state (FAD) and fluorescence disappears during reduction (FADH2). It is not known why autofluorescence in living cells varies so widely but, to a certain extent, intensities of autofluorescence reflect intracellular concentrations of NADH and FAD (Aubin 1979).

The spectra of the components NADH and riboflavin compare well with the spectra of autofluorescent cells and the metabolic activity in these cells. Ramanujam et al. (1994) demonstrated that NADH fluorescence increases when tissue progresses from normal to dysplastic, which might be explained by the fact that abnormal tissues have an increased metabolic rate and, therefore, increased concentrations of the fluorescent electron carriers NADH and FAD and decreased concentrations of the nonfluorescent NAD and FADH2. A complicating factor involved in the autofluorescence of NADH and FAD is that the protein-

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**Table 1**  **Endogenous metabolites for metabolic mapping in living cells**

<table>
<thead>
<tr>
<th>Type of enzyme</th>
<th>Enzyme</th>
<th>Substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Redox state&quot;</td>
<td>?</td>
<td>FAD</td>
<td>Thorell 1983</td>
</tr>
</tbody>
</table>
bound and therefore also enzyme-bound forms of these compounds exhibit fluorescence emission maxima that are shifted to shorter wavelengths by 20 nm and 5 nm, respectively. Furthermore, nonspecific binding of NADH to serum components such as albumin has also been shown to enhance its fluorescence, with a shift of the emission spectrum to shorter wavelengths (Canessa-Fischer and Davis 1966). Such nonspecific binding may contribute to autofluorescence when serum proteins in culture media remain bound to the surface of cells after their removal from culture. These different excitation and emission spectra of components such as NADH and FAD lead to wavelength shifts and band broadening in spectra, which makes (quantitative) analysis difficult. The use of fluorescent properties of NAD(P)H and FAD to monitor enzyme reactions in living cells was performed in the 1960s and 1970s by pioneers of living cell cytochemistry, such as Kohen and Thorell and co-workers (Table 1; for review see Van Noorden and Bticher 1991), because they were not able to use tools of modern cytochemistry such as synthetic substrates. However, as stated above, NAD(P)H is produced by a series of enzymes and is used by many others involved in various metabolic processes such as the respiratory chain, biosynthesis, and detoxification. Therefore, the approach to analyzing autofluorescence for metabolic mapping in living cells and tissues has become obsolete.

Experiments with HeLa cells transfected with GFP-tagged histone 2B clearly demonstrated the phototoxic effects of excitation light. It was expected that a beta sheet surrounding the GFP fluorochrome would prevent energy transfer to surrounding molecules to enhance the quantum efficiency and thus would restrict phototoxic effects. However, transfected cells appeared to be much more vulnerable to phototoxic effects than untransfected cells, which resulted in cell-cycle arrest or cell death. These living cells can be imaged only during the entire cell cycle, when the total amount of excitation light is kept to an absolute minimum of approximately 10 J cm\(^{-2}\) (E. Manders, personal communication). Manders’ experiments demonstrated the dose-dependent relationship between the amount of excitation light and cell damage. However, the type of fluorophore also plays an important role.

Exposure of cells to stressful conditions, such as excitation, triggers stress responses. Solar UV light is a major source of environmental stress for mammalian cells (Tyrrell 1996a, b; König 2000). UV-C (200–290 nm) is strongly absorbed by nucleic acids and causes DNA damage by inducing the formation of pyrimidine dimers, resulting in mutations and eventually carcinogenesis (Hall et al. 1988). UV-A (320–380 nm) is less strongly absorbed by nucleic acids but causes the production of a variety of reactive oxygen species (ROS), including superoxide and lipid peroxides (Jukiewicz and Buttner 1996; Scharfetter-Kochanek et al. 1997). UV-A irradiation can produce lipid peroxidation when superoxide anion and hydrogen peroxide form hydroxyl radicals in the presence of iron. Hydroxyl radicals can then react with polyunsaturated fatty acids to abstract hydrogen, forming lipid hydroperoxyl radicals with a half-life of seconds, which are then able to diffuse over significant distances before detoxification. Propagation and diffusion allow a peroxidative chain reaction to spread through membranes, generating new radical species as the reaction proceeds and greatly amplifying the damage produced (Petkau 1986; Vlaminrov 1986; Nishi et al. 1990). Intermediate UV-B (290–320 nm) is absorbed by nucleic acids but also contains an oxidative component (Tyrrell 1996a). UV irradiation also triggers the activation of surface death receptors, such as epidermal growth factor (EGF) receptor, CD95/Fas, or certain members of the tumor necrosis factor (TNF-\(\alpha\)) receptor family (Rosette and Karin 1996), the src family of tyrosine kinases (Devary et al. 1992), and phosphatidyl-inositol 3-kinase (PI-3) (Kabuyama et al. 1998). These are believed to activate protein phosphorylation cascades, resulting in mitogen-activated protein (MAP) kinase activation. The various MAP kinases are activated in a wavelength-specific manner by UV light (Kabuyama et al. 1998). Furthermore, UV irradiation results in the activation of various genes that are involved in the regulation of cell proliferation, such as tumor suppressor p53, which acts as a cell-cycle checkpoint. UV irradiation causes cells to be arrested in G1-phase, as was observed in experiments that followed cells in time under UV exposure (Maltzman and Czyzylko 1984; Hall et al. 1993; Di Leonardo et al. 1994). Expression of bax is enhanced by p53 after transcriptional activation (Miyashita et al. 1994; Selvakumaran et al. 1994; Miyashita and Reed 1995; Hansen and Braithwaite 1996). Bax induces cytochrome c release from mitochondria and activation of caspase-9, which activates caspase-3 and induces apoptosis. Vitamin E, a lipid-soluble antioxidant that scavenges ROS and singlet oxygen, inhibits activation of various MAP kinases. In conclusion, metabolic mapping in living cells and tissues on the basis of endogenous fluorescent metabolites has only limited applications. Concentrations of these metabolites do not reflect the activity of a specific enzyme and these metabolites must be excited with UV light, which is detrimental to living cells and tissues. Excitation light of short wavelengths is far more damaging to cells because the energy content is higher than that of light of longer wavelengths. Moreover, UV light can induce activation of signal transduction pathways within minutes, leading to profound alterations in cell metabolism.
Synthetic Chromogenic Substrates for Metabolic Mapping in Living Cells

Tetrazolium salt methods are established methods for the localization of the activity of dehydrogenases, reductases, and oxidases. Enzyme-catalyzed oxidation of the substrate liberates protons that are subsequently transferred to a tetrazolium salt such as (tetra)nitro BT as final electron acceptor. In this way, a water-insoluble formazan is produced (Lojda et al. 1976; Seidler 1991; Van Noorden and Frederiks 1992). This method has been applied to quantify glucose-6-phosphate dehydrogenase (G6PDH) activity in living hepatocytes of marine fish (Winzer et al. 2001a; Table 2). Formazan was localized exclusively in the cytoplasm of cells, leaving the nucleus unstained (Figure 1). Polyvinyl alcohol was used in the incubation medium as an additive to stabilize the tetranitro BT in the incubation medium due to the low amounts of polyvinyl alcohol present, a final concentration of 1 mM instead of 5 mM was used. Under these conditions, capturing of electrons is just as efficient as at high tetrazolium salt concentrations (Van Noorden 1988). The intact plasma membrane did not appear to be a barrier to substrate, co-enzyme and dye molecules necessary to detect intracellular G6PDH activity. Nevertheless, the authors mention that quantitative histochemical data obtained with the use of cryostat sections were higher than those obtained with intact hepatocytes, possibly caused by the process of penetration of components into the living cells.

D.T-diaphorase that can use either NADH or NADPH as substrate was demonstrated in living fish hepatocytes as well (Winzer et al. 2001b). Activity in hepatocytes appeared to be lower when NADPH was used as substrate. The method was also adopted for the demonstration of activity of aldehyde dehydrogenase in living fish hepatocytes (Table 2).

Reduction of tetrazolium salts has been used as test for viability of eukaryotic and prokaryotic cells. Although this approach is not specific for a particular dehydrogenase or reductase, it has been used successfully for subcellular localization of reducing enzyme systems in intact human hepatoma cells (Bernas and Dobrucki 1999).

In conclusion, chromogenic substrates thus far have been only rarely exploited for metabolic mapping in living cells and tissues, but this approach is promising, especially for high-throughput screening of effects of potential drugs on living cells, as absorbance measurements are simpler than fluorescence measurements.

Table 2 Chromogenic substrates for metabolic mapping in living cells

<table>
<thead>
<tr>
<th>Type of enzyme</th>
<th>Enzyme</th>
<th>Substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydrogenases</td>
<td>Glucose-6-phosphate dehydrogenase (1.1.1.49)</td>
<td>Glucose-6-phosphate and NADP</td>
<td>Winzer et al. 2001a</td>
</tr>
<tr>
<td></td>
<td>Aldehyde dehydrogenase (1.2.1.3)</td>
<td>Propionaldehyde</td>
<td>Winzer et al. 2001b</td>
</tr>
<tr>
<td></td>
<td>Succinate dehydrogenase (1.3.99.1)</td>
<td>MTT/XTT</td>
<td>Scudiero et al. 1988</td>
</tr>
<tr>
<td>Reductases</td>
<td>D.T-diaphorase (1.6.99.2)</td>
<td>NADH or NADPH</td>
<td>Winzer et al. 2001b</td>
</tr>
<tr>
<td>Redox state</td>
<td>—</td>
<td>—</td>
<td>Bernas and Dobrucki 1999</td>
</tr>
</tbody>
</table>

Synthetic Fluorogenic Substrates for Metabolic Mapping in Living Cells

Synthetic fluorogenic substrates can be used for determination of viability of cells, but also for metabolic mapping. Various fluorogenic substrates have been developed, especially for hydrolytic enzymes such as proteases. Here we discuss synthetic fluorogenic substrates that have been applied to living cells and tissues (Table 3).

Fluorochromes Used in Synthetic Fluorogenic Substrates

Fluorogenic substrates are usually esters or ethers of phenolic fluorophores; 7-hydroxy-4-methylcoumarin (β-methylumbelliferone) and its analogues fluorescein and resorufin are the most common examples of fluorophores used in fluorogenic substrates (Haugland 1995). Other fluorophores for fluorogenic substrates of hydrolytic enzymes are rhodamine 110 (Leytus et al. 1983a,b) and cresyl violet (Van Noorden et al. 1997b). These synthetic substrates are small molecular substrates, consisting of amino acids attached to a fluorochrome. Internally quenched fluorogenic substrates (Matayoshi et al. 1990) and, in the future, fluorescence resonance energy transfer (FRET)-based substrates will become increasingly available. These types of substrates are macromolecular substrates, consisting of fluorochromes attached to proteins. Spectral characteristics of the various fluorochromes used in fluorogenic substrates are presented in Table 4.

Formation of esters or ethers of phenolic fluorophores results in a shift to shorter wavelengths of absorbance and either partial or total quenching of the long-wavelength fluorescence of the fluorophore. Fluorescein-based substrates are not fluorescent because quenching is complete as the dye is converted into a non-fluorescent colorless lactone by the formation of two ether or ester bonds. Therefore, these substrates do not cause background fluorescence and are among the most sensitive fluorogenic substrates known. Fluorescein is water-soluble, and free fluorescein can be re-
Metabolic Mapping in Living Cells

tained in living cells for at least a few minutes at room temperature (Rotman and Papermaster 1966; Sengbusch et al. 1976; Watson et al. 1977; Dolbeare and Phares 1979; Dolbeare 1990). Fluorescein accumulation was claimed to be linear up to 80 min of the reaction to detect β-galactosidase activity in living fibroblasts (Jongkind et al. 1986). Afterwards, intracellular accumulation of fluorescence levels off as a result of both product inhibition and fluorescein diffusion out of the cells (Kohen et al. 1973a,b; Watson et al. 1977).

Cooling of cells on ice after loading with substrate slows leakage of fluorescein (Nolan et al. 1988), but this prevents metabolic mapping under physiological conditions for most organisms.

Cytochrome P450 activity in living cells can be analyzed using the 7-ethoxycoumarin-O-deethylase (EROD) assay based on the formation of fluorescence of resorufin (Burke and Mayer 1974). The assay has been modified by Behrens et al. (1998) for study of living trout hepatocytes. Enzyme activity can also be assessed by using 7-ethoxycoumarin-O-deethylase (ECOD) as substrate (Scholz and Segner 1999).

Activity of cathepsin K was localized by Tepel et al. (2000) using the substrate benzoyloxycarbonyl-glycylprolyl-arginine-4-methoxy-β-naphthylamide (Bz-Gly-Pro-Arg-4MBA) in combination with nitrosalicylaldehyde, which couples directly with the proteolytically released 4MBA to produce a yellow fluorescent final reaction product that can then be visualized by fluorescence microscopy (Van Noorden et al. 1987). Living thyroid epithelial cells showed the precipitate to be mainly present at the cell surface, indicating the inability of these living cells to internalize either the substrate or the coupling reagent.

A unique fluorogenic substrate for phosphatases, 2-(5'-chloro-2-phosphoryloxyphenyl)-6-chloro-4(3H)-quinazolinone or ELF-97-phosphate, has recently been developed (Paragas et al. 1997). The ELF-97-alcohol that results after liberation of the phosphate group by phosphatase activity forms a bright yellow-green fluorescent precipitate at the site of the activity (Figure 2). Fluorescence of ELF-97 product is very stable; there is minimal diffusion of the reaction product from the site of enzymatic activity because it is water-insoluble and it provides high spatial resolution (Breininger and Baskin 2000). The ELF-97 phosphatase substrate contains only a single phosphate group, which makes it suitable for simple kinetic analysis of phosphatase activity. Another advantage is its large Stoke's shift. It is maximally excited at 345 nm and emits at 530 nm (yellow; Figure 2). The substrate itself is weakly blue fluorescent, unfortunately in the region of autofluorescence. Therefore, it is not possible to use the blue fluorescence of this synthetic substrate to determine its intracellular concentration. In that case, ratio images of both substrate and product could be made and concentration of both substrate and product determined. A major disadvantage of the ELF-97 product is the short excitation wavelength in the range of UV-A, which makes it largely unsuitable for metabolic mapping in living cells. Moreover, the substrate can, in principle, be cleaved by every phosphatase in a living cell, thus providing little specificity. In conventional enzyme histochemistry, the composition of the incubation medium determines which phosphatase is detected (e.g., acid pH for acid phosphatase and alkaline pH for alkaline phosphatase), but in a living cell each phosphatase is present in its own natural environment.

Rhodamine-based fluorogenic dipeptidyl substrates have been synthesized by Leytus et al. (1983a,b). The non-fluorescent substrate diffuses readily into living cells, in which it can be hydrolyzed into a strongly fluorescent product that is entrapped within cells because of its positive charge (Assafi-Machleidt et al. 1992). Activity of cysteine proteinases has been detected in

Figure 1 Localization of dipeptide 6-phosphate dehydrogenase activity in living isolated hepatocytes of European flounder. The colored final reaction product, formazan, is localized in the cytoplasm, leaving the nucleus unstained. This approach is fast and simple and is therefore suitable for high-throughput screening of effects of drugs or for biomonitoring in environmental research. After Winzer et al. (2001a). Bar = 15 μm.

Figure 2 Gallery of images of a living human fibroblast incubated in a medium containing ELF-97 phosphate. First image is taken at 30 sec after the incubation was started and each subsequent image at intervals of 15 sec. Yellow fluorescence is produced by phosphatases, mostly in lysosomes, as most ELF-97 product is generated in intracellular granular form. Bar = 2 μm.

Figure 3 Localization of dipeptidyl peptidase IV (DPPIV) protein (also known as CD26) using an antibody coupled with FITC (A) and DPPIV activity using (Ala-Pro)-(cys)yl violet substrate (B). Combination of images A and B shows that the clustered CD26 molecules are “islets” (yellow) in small “lakes” of cysyl violet (red) what they have produced during an incubation of 60 sec (C). Some inactive CD26 clusters remain green in C. Bar = 10 μm.

Figure 4 Caspase activity in living but apoptotic thymocytes as demonstrated by incubation with CaspaLux-6 substrate for caspase 6 (VEIDase) and PhiPhiLux substrate for caspases 3 and/or 7 (DEVDase). Combination of these images (BOTH) shows distribution patterns of caspase 6 activity and activity of caspases 3 and/or 7 in the same cells. Images were taken after 140-min incubation. The Nomarski image is at lower right. The dark images of cells in each fluorescence image correspond to those cells in which intracellular caspase activation is not yet detectable. Bar = 20 μm. From Komoriya et al. (2000), with permission.
Table 3 Fluorogenic substrates for metabolic mapping in living cells

<table>
<thead>
<tr>
<th>Type of enzyme</th>
<th>Enzyme</th>
<th>Substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycosidases</td>
<td>β-Galactosidase (3.2.1.23)</td>
<td>Di-β-D-galactopyranoside</td>
<td>Jongkind et al. 1986; Nolan et al. 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S-Dodecanoylamino-fluorescein-di-β-D-galactopyranoside</td>
<td>Rotman and Pappenmaster 1966</td>
</tr>
<tr>
<td>Phosphatases</td>
<td>Acid (3.1.3.2) and alkaline (3.1.3.1) phosphatase</td>
<td>ELF-97</td>
<td>Paragas et al. 1997</td>
</tr>
<tr>
<td>Proteases</td>
<td>Aminopeptidase (3.4.11.10)</td>
<td>(Leu)yl-rhodamine 110</td>
<td>Bedner et al. 1998</td>
</tr>
<tr>
<td></td>
<td>DPP IV (3.4.14.5)</td>
<td>(Ala-pro)yl-cresyl violet</td>
<td>Van Noorden et al. 1997b</td>
</tr>
<tr>
<td></td>
<td>Cathepsin B (3.4.22.1)</td>
<td>Xaa-pro-rhodamine 110-Y</td>
<td>Lorey et al. 2000</td>
</tr>
<tr>
<td></td>
<td>Cathepsin D (3.4.23.5)</td>
<td>(Z-arg)yl-cresyl violet</td>
<td>Van Noorden et al. 1998a</td>
</tr>
<tr>
<td></td>
<td>Cathepsin H (3.4.22.16)</td>
<td>(CPGC) (Cy5.5)-Pl-methoxy-polyethylene glycol 92</td>
<td>Leytus et al. 1983a,b</td>
</tr>
<tr>
<td></td>
<td>Cathepsin K (3.4.22.38)</td>
<td>(Z-leu-arg)yl-rhodamine 110</td>
<td>Mahmood et al. 1999</td>
</tr>
<tr>
<td>Proteases</td>
<td>Aminopeptidase (3.4.11.10)</td>
<td>(Ala-pro)yl-cresyl violet</td>
<td>Jones et al. 1997</td>
</tr>
<tr>
<td></td>
<td>DPPIV (3.4.14.5)</td>
<td>Xaa-pro-rhodamine 110-Y</td>
<td>Jones et al. 1997</td>
</tr>
<tr>
<td></td>
<td>Cathepsin 8 (3.4.22.1)</td>
<td>(CPGC) (Cy5.5)-Pl-methoxy-polyethylene glycol 92</td>
<td>Jones et al. 1997</td>
</tr>
<tr>
<td></td>
<td>Cathepsin D (3.4.23.5)</td>
<td>(Z-arg)yl-cresyl violet</td>
<td>Jones et al. 1997</td>
</tr>
<tr>
<td></td>
<td>Cathepsin H (3.4.22.16)</td>
<td>(CPGC) (Cy5.5)-Pl-methoxy-polyethylene glycol 92</td>
<td>Tung et al. 1999</td>
</tr>
<tr>
<td></td>
<td>Cathepsin K (3.4.22.38)</td>
<td>(Z-leu-arg)yl-rhodamine 110</td>
<td>Mahmoud et al. 1999</td>
</tr>
<tr>
<td></td>
<td>MMP-2 (3.4.24.24)</td>
<td>(Z-arg)yl-rhodamine 110</td>
<td>Mahmoud et al. 1999</td>
</tr>
<tr>
<td>Lipases</td>
<td>Glucocerebrosidase</td>
<td>Lysamin rhodamine-12-glucosyleramide</td>
<td>Claveau et al. 2000</td>
</tr>
<tr>
<td>Esterases</td>
<td></td>
<td>Fluorescein diacetate (FDA)</td>
<td>Reis et al. 2000</td>
</tr>
</tbody>
</table>

“Detoxification” | Cytochrome P450 (1.4.14.1) | EROD | Tepel et al. 2000 |

Living human monocytes and rat macrophages with the use of flow cytometry. Rhodamine production was completely inhibited by specific inhibitors of cysteine proteinases such as E-64 and Z-Phe-Ala-CHN2. Rhodamine 110 is a diamino analogue of fluorescein that exhibits properties similar to those of fluorescein (Table 4). When amino groups of rhodamine 110 are blocked by acetylation, the intensely colored dye is converted into a colorless and non-fluorescent compound, implying that the conjugation of the chromophore is interrupted (Drexhage 1976). Therefore, rhodamine-based substrates are highly reactive because loss of acetylation is accompanied by a large increase in the degree of conjugation and hence a large increase in stability. (Cbz-Arg-NH2)-rhodamine 110 is a stable compound that does not exhibit spontaneous hydrolysis in aqueous solutions during assays. Like fluorescein-based substrates, rhodamine 110-based bisubstituted substrates have low fluorescence coefficients that increase when the bisubstituted derivatives are converted into their corresponding mono- 

substituted compounds and increase again after conversion of the monosubstituted compounds into their corresponding unsubstituted compounds. Nevertheless, this does not complicate the interpretation of kinetic data providing that less than 15% of the substrate is hydrolyzed during an assay (Leytus et al. 1983b). Under these conditions, the increase in fluorescence is solely due to the production of the monosubstituted (Cbz-Arg-NH2)-rhodamine 110 compound. Bisubstitution of (Cbz-Arg-NH2)-rhodamine 110 also has certain advantages. The effective concentration of susceptible amide bonds is twice the substrate concentration, thus lowering the Km. Furthermore, the presence of two amino acid residues per rhodamine moiety enhances the water-solubility of the substrate.

In contrast, coumarin-based substrates are less useful than rhodamine-based substrates because suboptimal conditions for detection of the fluorophore must be used to maximize spectral differences between substrate and product. Furthermore, a structural change...
in the aminocoumarin moiety does not take place when the substrate is cleaved and, therefore, coumarin-based substrates are less useful than rhodamine-based substrates (see also below). Recently, a new type of fluorogenic substrate for proteases has been synthesized based on the leaving group cresyl violet (Van Noorden et al. 1997b). Cresyl violet has been used in histology for over a century because of its metachromatic properties. In the early days it was employed for Nissl staining of neural tissue and is excellent for staining connective tissue. Furthermore, it stains nuclei violet, cytoplasm blue, and amyloid, mucin, and mast cell granules red. It has also been used for vital staining of white blood cells and for staining of cancer cells in biopsies. Cresyl violet is also a fluorescent molecule. It can be used in combination with other fluorescent dyes, such as fluorescein, or green fluorescent protein. Two amino-acid side chains can be bound by peptide bonds to the two amide groups in the fluorophore. At present, a bifunctional synthetic substrate for dipeptidyl peptidase IV (DPP IV) (Ala-Pro)_{2}–cresyl violet (Van Noorden et al. 1997b), and one for cathepsin B (Z-Arg)_{2}–cresyl violet (Van Noorden et al. 1998a,b), are available. In Figure 3, activity of DPP IV is demonstrated on the membrane of a living human T-helper-cell and is co-localized with the DPP IV protein (also known as CD26), showing the good localization properties of fluorescent cresyl violet.

Casein conjugates of two BODIPY dyes, one named BODIPY fluorescein (FL) and the other BODIPY Texas red (TR), have been developed by Jones et al. (1997) as fluorogenic protease substrates. The basic structure of the BODIPY fluorophore is 4,4’-difluoro-4-bora-3a,4a-diaza-s-indacene. Solutions of the alkyl-substituted derivates have a green fluorescein-like fluorescence, but when substituents that yield additional conjugation are added to the molecule, both absorption and emission shift to longer wavelengths. Conjugates are labeled to such an extent that the dyes are efficiently quenched in the protein, yielding virtually non-fluorescent substrate molecules. These fluorogenic substrates release highly fluorescent BODIPY-labeled peptides, and this release is proportional with protease activity. These quenched substrates are suitable for continuous assay of enzymatic activity in living cells and tissues, particularly in fluorescence microscopy readers using either fluorescein excitation and emission wavelengths to measure BODIPY FL casein hydrolysis or Texas Red wavelengths to detect proteolysis of BODIPY TR casein. Compared with the HTT–casein assay, the BODIPY-labeled protease assay is simple and precise and has greater sensitivity and a broader dynamic range of detection. In this way, it is possible to detect the activity of a wide variety of proteolytic enzymes with high sensitivity (Jones et al. 1997).

DQ-BSA is also based on the BODIPY fluorochrome. BODIPY FL is conjugated to BSA at a high molar ratio. The resulting conjugate is self-quenched by fluorescence energy transfer between neighboring BODIPY molecules. Although this substrate is not membrane-permeable, it can be internalized by forming a complex with anti-BSA that is taken up by the Fc receptor of macrophages (Reis et al. 1998). DQ-ovalbumin is also nonpermeable to cell membranes but can be internalized by mannose receptor-mediated endocytosis by antigen-presenting cells (Rodriguez and Diment 1995).

The fluorogenic substrates for caspases in Table 3 are based on peptides of 18 amino acids containing caspase cleavage sequences, with two identical fluorophores covalently attached near their termini. Such substituted peptides are assumed to have an oval-shaped structure in solution due to the formation of intramolecular excitonic H-dimers between the fluorophores (Packard et al. 1996, 1997). In such rhodamine-derivatized dimers, the fluorophore fluorescence is quenched for 90–99%. When a protease cleaves the peptide backbone of this complex, the cyclic structure incorporating the fluorophores is broken and two highly fluorescent substituted peptide fragments are generated (Figure 4). Whereas normal peptides of 18 amino acids do not enter cells without a specialized means of transport, the caspase substrates of this design are permeable to cells (Komoriya et al. 2000).

Substrates have been designed for specific enzymes, such as cathepsin D, which is overexpressed in a number of cancers (Rochefort and Liaudet-Coopman 1999). The Cy5.5 fluorochrome can be excited in the near-infrared region and has been attached to the amino terminal of a sequence of 11 amino acids that is specifically recognized by cathepsin D. The peptides were subsequently attached to a synthetic graft polymer for efficient tumor delivery (Tung et al. 1999). The spatial proximity of the fluorochromes results in quenching of fluorescence in the bound state. A 350-fold-higher fluorescence signal was observed after
cleavage in vitro. Cell culture experiments using a rodent tumor cell line stably transected with human cathepsin D confirmed enzyme-specific production of fluorescence. This sequence, but not a scrambled control sequence, resulted in specific production of fluorescence due to enzyme activity in vitro. The same type of substrate has also been used to demonstrate matrix metalloproteinase (MMP)-2 activity. The substrate consists of three elements: the quenched Cy5.5 fluorochrome, an MMP-2-sensitive peptide sequence, and a poly-L-lysine backbone. In theory, this design has the following advantages: (a) the closely packed fluorochromes in the substrate are quenched; (b) specificity for the enzyme is dictated by the conjugated peptide substrates; (c) the loading capacity of the substrate is high; (d) cleavage of the multiple probes by a single enzyme is repetitive; and (e) efficient delivery in vivo is still possible because of a long-circulating half-life and the use of a non-immunological backbone that serves as a delivery vehicle (Bremer et al. 2001).

The small molecular substrates listed in Table 3 are not very fluorescent themselves, but the internally quenched macromolecular substrates in Table 3 may have the advantage that fluorescence is negligible and therefore sensitivity of the enzymatic assay is higher due to an increased signal-to-noise ratio. On the other hand, when the substrate is also fluorescent but with spectral characteristics that are different from those of the product, measurement of intracellular substrate concentrations is possible.

Enzyme products that can be excited in the red or near-infrared region of the spectrum are, in principle, the best for in vivo imaging of enzyme activity because the excitation light is less harmful than light of shorter wavelengths.

In the near future, frequency resonance energy transfer (FRET)-based substrates will become available for analysis of enzyme activity in living cells. FRET-based substrates should be synthesized in such a way that two different fluorophores, of which the emission peak of one overlaps with the excitation peak of the other, are located in close proximity at opposite sides of a bond susceptible to enzymatic cleavage. Preferably, these two fluorophores have a large Stokes' shift. Excitation of the fluorochrome with excitation and emission peaks at shorter wavelengths can then, in theory, result in enhanced fluorescence of the second fluorochrome, with excitation and emission peaks at longer wavelengths when the substrate is not enzymatically processed. When the substrate is cleaved, the FRET phenomenon disappears and fluorescence of the fluorochrome with excitation and emission peaks at shorter wavelengths appears. For example, when the fluorophores Alexa Fluor 488 and rhodamine are combined in such a FRET-based substrate, excitation of the Alexa Fluor 488 fluorophore in the intact substrate results in enhanced emission of rhodamine substrate results in enhanced emission of rhodamine fluorescence, which is a measure for the local (intracellular) substrate concentration. Alexa Fluor 488 fluorescence itself is a measure of the amount of substrate processed. Therefore, FRET-based substrates will enable measurements of both the amount of product generated by enzymatic activity and the intracellular concentration of the substrate, even in subcompartments of cells. The use of this type of substrate would solve the problem of estimating local substrate concentrations in cells or cell compartments to calculate accurately Vmax and Km values for enzymes in living cells. Labeling peptide sequences with two different dyes has been described by Bark and Hahn (2000).

The FRET phenomenon can also be useful to demonstrate specificity of a substrate for an enzyme. When the enzyme of interest is tagged with, e.g., green fluorescent protein (GFP) by transfection, co-localization of the enzyme and the enzyme product containing a fluorophore that has an excitation peak that overlaps the emission peak of GFP may result in the FRET phenomenon.

In conclusion, fluorophores with high fluorescence quantum yield should be selected for incorporation into synthetic fluorogenic enzyme substrates to obtain sufficient sensitivity to analyze enzyme reactions at physiological substrate concentrations. Substrates that contain fluorophores with excitation peaks in the red or infrared region of the spectrum are the substrates of choice. Moreover, kinetic parameters of the enzyme for the synthetic fluorogenic substrate should resemble that for its natural substrate(s), as explained below. In addition to small molecular fluorogenic substrates, macromolecular substrates containing quenched fluorophores are useful for analysis of specific activity of enzymes in living cells and tissues, and the concept of FRET-based fluorogenic substrates is intriguing.

Reactivity of Synthetic Fluorogenic Substrates

The ability of an enzyme to discriminate among many potential substrates is an important factor in maintaining organization of most biological functions in the biocomplexity of cells and tissues. Although substrate selection can be regulated at many levels in a biological context, such as spatial and temporal localization of enzyme and substrate, concentrations of enzyme and substrate, and requirement of co-factors, substrate specificity at the enzyme active site is the overriding principle that determines the turnover of a substrate (Harris et al. 2000). The effectiveness of the conversion of a substrate by an enzyme can be defined as the product of the second-order rate constant Kcat/ Km or specificity constant (Knight 1977; McRae et al. 1981) and the relative detectability of the fluorescent leaving group given by the molar fluorescence coefficient. On the basis of this criterion (Cbz-Arg-NH2-
rhodamine 110 is a substrate with higher effectiveness than 7-(N-Cbz-L-argininamido)-4-methylcoumarin for bovine trypsin by a factor of 280, for human and dog plasmin by a factor of 200, and for human thrombin by a factor of 50. The differences between the individual $K_{cat}$ and $K_m$ values for (Cbz-Arg-NH$_2$)-rhodamine 110 and 7-(N-Cbz-L-argininamido)-4-methylcoumarin are consistent with observations that the nature of a chromophoric or fluorophoric leaving group can significantly affect interactions between a synthetic substrate and the active site of an enzyme (Chase and Shaw 1969). The larger $K_{cat}$ values for (Cbz-Arg-NH$_2$)-rhodamine 110 suggest either that rhodamine is a better leaving group than methylcoumarin, because the rhodamine-based substrate is intrinsically more reactive, or that the rhodamine moiety influences the orientation of the substrate in the active site of the enzyme, allowing a more efficient catalysis, or both. Furthermore, rhodamine 110 is four- to fivefold more fluorescent than 4-methylcoumarin and cleavage bonds in (Cbz-Arg-NH$_2$)-rhodamine 110 are more reactive than those in coumarin-based substrates. The larger degree of conjugation in the monoamide product of hydrolysis relative to the enhanced reactivity of the amide bonds in the substrate offers higher sensitivity and selectivity.

Rhodamine-based substrates exhibit a wide range of specificity constants. Amino acids in the $P_2$ position in dipeptide substrates determine specificity in a large part. Comparison of the kinetic constants of plasmin or thrombin for the best dipeptide substrates with those for (Cbz-Arg-NH$_2$)-rhodamine 110 indicates that the large increases in $K_{cat}/K_m$ obtained by extending the single amino acid substrate with an appropriate $P_2$ residue in a dipeptide substrate are primarily the result of a large increase in $K_{cat}$ and a decrease in $K_m$. Therefore, the specificity of proteases for synthetic substrates depends to a great extent on interactions between amino acids in the active site of a protease and amino acid residues in the peptide substrate. Because occupation of the $P_2$ position does not increase specificity of coumarin thiolester-based substrates as much as it increases specificity of rhodamine-based substrates, selectivity can be much greater with rhodamine-based substrates. Therefore, rhodamine-based substrates are in principle more useful to detect selectively protease activity in living cells and tissues.

In conclusion, characterization of substrate specificity of an enzyme provides useful information for the dissection of complex biological pathways and also provides the basis for the design of selective substrates and potent inhibitors to study enzyme activity.

Localization of Final Fluorescent Reaction Product

When a fluorescent final reaction product of an enzyme accumulates at a certain site, this does not automatically imply that it has been produced in that site. Chemical properties of the fluorophore have effects on its intracellular localization. The charge of a fluorophore, such as rhodamine, can lead to accumulation in mitochondria. However, the charge of a molecule depends on its $pK$ value. For example, rhodamine 110 has a $pK_a$ of 4.3 at physiological pH 7.2, and one in a thousand rhodamine molecules is in the protonated acidic state with a positive charge. In the protonated state, rhodamine 110 accumulates in mitochondria as a result of the intramitochondrial electric potential (Jeannot et al. 1997). Other factors, such as lipophilicity or hydrophobicity, are of crucial importance, not only for the uptake of these substrates by living cells but also for final localization of the fluorescent product. Therefore, it appears attractive to develop fluorochromes with a chemical anchor (Lorey et al. 2000) or to use a trapping agent to stop fluorochromes from diffusing into cell compartments on the basis of their charge, lipophilicity, or hydrophobicity, or the pH of intracellular compartments. In this way, diffusion of the fluorescent enzyme product from the site of enzyme activity can be limited.

In conclusion, chemical properties of fluorogenic substrate and fluorescent product must be taken into account for proper localization of enzyme activity in living cells and tissues.

Conclusions

The present overview of methods for detection of enzyme activity in living cells and tissues indicates our limited knowledge of molecular interactions that take place during incubation and recording of the formation of a colored or fluorescent reaction product. Metabolic mapping in living cells and tissues on the basis of endogenous fluorescent metabolites has only limited applications. Concentrations of these metabolites do not reflect the activity of a specific enzyme and these metabolites must be excited with UV light, which is detrimental to living cells and tissues. Excitation light of short wavelengths is far more damaging to cells because the energy content is higher than that of longer wavelengths. Moreover, UV light can induce activation of signal transduction pathways within minutes, leading to profound alterations in cellular metabolism.

Chromogenic substrates thus far have rarely been exploited for metabolic mapping in living cells and tissues, but this approach is promising, especially for high-throughput screening of effects of potential drugs on living cells, because absorbance measurements are far more simple than fluorescence measurements.

Important criteria for the selection of fluorophores to incorporate into synthetic fluorogenic substrates are a high-fluorescence quantum yield to obtain suffi-
cient sensitivity to analyze enzyme reactions at physiological substrate concentrations. Substrates that contain fluorophores with excitation peaks in the red or infrared region of the spectrum are the substrates of choice. Moreover, kinetic parameters of the enzyme for the synthetic fluorogenic substrate should resemble those for its natural substrate(s). In addition to small molecular fluorogenic substrates, macromolecular substrates containing quenched fluorophores are useful for analysis of specific activity of enzymes in living cells and tissues, and the concept of FRET-based fluorogenic substrates is promising.

Intrinsic chemical properties of fluorophores in synthetic substrates have a strong effect on their detection and also on the reactivity of the substrate. These phenomena may be due to many factors, such as steric hindrance or different chemical properties of the fluorophores used. The specificity exhibited by many enzymes depends, to a large extent, on the interaction of subsite amino acids in the active site of the enzyme. Specificity can be characterized with the use of synthetic substrates by studying variations in the specificity constant on substitution or alteration of single amino acid residues in the substrates. Furthermore, localization of fluorophores in living cells at the site of enzyme activity is a major issue. Addition of chemical anchors to a fluorophore may improve localization properties.

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Metabolic Mapping in Living Cells


Boonacker, Van Noorden


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Fluorogenic Substrate Ala-Pro-Cresyl Violet But Not Ala-Pro-Rhodamine 110 Is Cleaved Specifically by DPPIV activity: A Study in Living Jurkat Cells and CD26/DPPIV-transfected Jurkat Cells

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SUMMARY Fluorogenic substrates [Ala-Pro]-cresyl violet and Ala-Pro-rhodamine 110 have been tested for microscopic detection of protease activity of dipeptidyl peptidase IV (DPPIV) in living cells. DPPIV activity is one of the many functions of the multifunctional or moonlighting protein CD26/DPPIV. As a model we used Jurkat cells, which are T-cells that lack CD26/DPPIV expression, and CD26/DPPIV-transfected Jurkat cells. Ala-Pro-rhodamine 110 is not fluorescent, but after proteolytic cleavage rhodamine 110 fluoresces. [Ala-Pro]-cresyl violet is fluorescent by itself but proteolytic cleavage into cresyl violet induces a shift to longer wavelengths. This phenomenon enables the simultaneous determination of local (intracellular) substrate and product concentrations, which is important for analysis of kinetics of the cleavage reaction. [Ala-Pro]-cresyl violet, but not Ala-Pro-rhodamine 110, appeared to be specific for DPPIV. When microscopic analysis is performed on living cells during the first minutes of the enzyme reaction, DPPIV activity can be precisely localized in cells with the use of [Ala-Pro]-cresyl violet. Fluorescent product is rapidly internalized into submembrane granules in transfected Jurkat cells and is redistributed intracellularly via internalization pathways that have been described for CD26/DPPIV. We conclude that [Ala-Pro]-cresyl violet is a good fluorogenic substrate to localize DPPIV activity in living cells when the correct wavelengths are used for excitation and emission and images are captured in the early stages of the enzyme reaction. (J Histochem Cytochem 51:1111-1120, 2003)

KEY WORDS
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Now that genomes of species are becoming elucidated and proteomic analyses in health and disease are under way, analysis of functions of proteins is becoming more important than ever (Jessani et al. 2002). Biocomplexity demands functional analysis in living cells and tissues because interactions between macromolecules and post-translational modifications regulate functions of many macromolecules. This is especially true for enzymes, because their activity and/or their kinetic parameters often depend on the microenvironment of the enzyme (Swezey and Epel 1986; Jonges et al. 1992; Van Noorden et al. 1997a, 2002; Bleeker et al. 2000). Therefore, metabolic mapping in living cells and tissues and especially of proteolytic enzymes has gained enormous attention in recent years (Haugland 1995; Perham 2000; Sameni et al. 2000; Boonacker and Van Noorden 2002; Miklos and Maleszka 2001; Hahn and Toutchkine 2002; Patton and Beechem 2002).

Various types of synthetic fluorogenic substrates are available for detection of activity of proteases in living cells (for review see Boonacker and Van Noorden 2001). Usually the substrate consists of one or more sequences of amino acids and a leaving group. The amino acid sequence(s) in the substrate de-
termine(s) to a large extent the specificity for a protease on the basis of interactions between the amino acid sequence and the active site of the enzyme. Specificity of synthetic substrates is obtained by studying variations in the specificity constant on substitutions or alterations of single amino acids in the substrate (Leytus et al. 1983b). Figure 1 shows two examples of fluorogenic substrates to detect dipeptidyl peptidase IV (DPPIV) activity. Substrate specificity is based on the amino acid sequence alanine-proline (Ala-Pro) (Demuth and Heins 1995) and the leaving groups are either rhodamine 110 (Figure 1A) or cresyl violet (Figure 1B). Rhodamine 110 is not fluorescent when amino acids are attached, as it is completely quenched (Leytus et al. 1983a) and becomes fluorescent after proteolytic release, whereas cresyl violet is always fluorescent even when amino acid sequences are attached but fluorescence is shifted to a longer wavelength when the amino acid sequences are removed (Boonacker and Van Noorden 2001). However, the amino acid sequence does not completely determine substrate specificity because intrinsic chemical properties of fluorophores in synthetic substrates may also affect the reactivity of the substrate with the protease (Chase and Shaw 1969; Boonacker and Van Noorden 2001; Lorey et al. 2002). This phenomenon can be due to sterical hindrance or to particular chemical properties of the fluorophore. Assay conditions also determine reactivity of a synthetic substrate with the enzyme. When intact cells or tissues are used, homologous proteases may interfere with the cleavage of the synthetic substrate.

Proline is a unique amino acid because of its cyclic structure. This specific conformation of proline imposes many restrictions on cleavage of peptides and proteins that contain proline. A large series of physiologically important biomolecules contain proline in the penultimate position and their biological properties are highly regulated by this proline motif. Only a limited number of proteases can cleave proline-containing peptides (Yaron and Naider 1993; Demuth and Heins 1995). Until recently, it was believed that CD26/DPPIV was one of the very few proteases that can cleave off a terminal dipeptide from proteins with proline in the penultimate position, but it has been recently shown that this is not the case (Boonacker and Van Noorden 2003). It appeared that a series of DPPIV homologues exist. For example, DPPII acts preferentially at acidic pH (Lojda et al. 1976) and prolyl carboxypeptidase shows similar activity as CD26/DPPIV (Rawlings and Barrett 1996; Sedo and Malik 2001). Recently, it was found that specific inhibitors of post-proline-cleaving proteases cause apoptosis in quiescent lymphocytes independently of CD26/DPPIV. These results lead to the discovery of another proline-specific peptidase, quiescent cell proline dipeptidase (Chiravuri et al. 1999; Underwood et al. 1999). Quiescent cell proline dipeptidase was cloned from a Jurkat T-cell line that lacks CD26/DPPIV expression. The putative active site residues serine, aspartate, and hist-
To test specificity of substrates, samples containing soluble fluid were run on polyacrylamide gels and submitted to CD26/DPPIV (sCD26/DPPIV) derived from human seminal Western Blotting and Zymography of DPPIV Activity. Suspensions of 4 x 10^6 cells/ml were used. Per-activity measurements, cells were washed twice in cold PBS (three times, 5 sec each). Mcabilization of cells was performed by ultrasonic treatment. Intact cells were kept on ice before mixing with the incubation medium of quiescent cell proline dipeptidase shows an ordering of the catalytic triad similar to that in the post-proline-cleaving exopeptidases prolyl carboxypeptidase and CD26/DPPIV (David et al. 1993; Hooper et al. 2001). DPPIV homologues are listed in Sedo and Malik (2001) and in Boonacker and Van Noorden (2003). To study activity of DPPIV and its homologues in a specific manner, the characteristics of fluorogenic substrate have to be known.

In this study we compared the reactivity of two synthetic substrates that contain the same amino acid sequence but different fluorogenic leaving groups, cresyl violet (Van Noorden et al. 1997b) and rhodamine 110 (Lehtus et al. 1993a,b; Figure 1), in the analysis of DPPIV activity after purification and in living Jurkat cells lacking CD26/DPPIV and CD26/DPPIV-transfected Jurkat cells (Hegen et al. 1993a,b; Tanaka et al. 1993) to establish to what extent the Ala-Pro-containing substrates are specific for DPPIV. We found that the two substrates show different specificities towards DPPIV-like proteases, depending on the leaving group. [Ala-Pro]_2-cresyl violet is specific for DPPIV activity whereas Ala-Pro-rhodamine 110 is not. We concluded that [Ala-Pro]_2-cresyl violet is the preferred substrate for microscopic analysis of DPPIV activity in living cells. Because of the highly dynamic character of (enzymatic) processes in living cells and to avoid diffusion of cresyl violet from sites in cells where it is formed, microscopic images have to be taken during the first few minutes of incubation of cells.

**Materials and Methods**

**Jurkat Cell Lines**

Jurkat cells (clone E6-1; American Type Culture Collection, Manassas, VA), which lack CD26/DPPIV expression, were used as well as Jurkat cells transfected with CD26/DPPIV (Hegen et al. 1993a,b). This model system enables the determination of specificity for substrates of DPPIV. Clone E6-1 was cultured in Iscove’s modified Dulbecco’s medium (IMDM; Bio-Whitaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS), whereas the CD26/DPPIV transfectants were grown in Dulbecco’s modified medium supplemented with 10% FCS, containing glutamine (1 mM) and Gentamicin G418 (1 mg/ml; Invitrogen, Carlsbad CA) to maintain the selection for the CD26/DPPIV construct (Hegen et al. 1993a,b; Tanaka et al. 1993). Before DPPIV activity measurements, cells were washed twice in cold PBS. Intact cells were kept on ice before mixing with the incubation medium. Suspensions of 4 x 10^6 cells/ml were used. Permeabilization of cells was performed by ultrasonic treatment (three times, 3 sec each).

**Western Blotting and Zymography of DPPIV Activity**

To test specificity of substrates, samples containing soluble CD26/DPPIV (sCD26/DPPIV) derived from human seminal fluid were run on polyacrylamide gels and submitted to Western blotting or were incubated in the presence of a series of synthetic DPPIV substrates. sCD26/DPPIV was enriched by isolating prostasomes from human seminal fluid, as described by Ronquist and Brody (1985). The pelleted prostasome fraction was resuspended in 20 mM Tris-HCl (pH 7.4) containing 1% Triton X-100 for 1 hr at 4°C. Samples were treated ultrasonically three times for 5 sec each. Subsequently, 3 x Laemmli buffer, consisting of 150 mM Tris-HCl (pH 6.8), 50% glycerol, 6% SDS, and 0.3% bromophenol blue, was added and the samples were heated to 37°C for 5 min. Equal amounts of proteins were transferred onto the gels. After electrophoresis at 20 mA per gel at 4°C, gels were washed twice at room temperature (RT) with PBS containing 2.5% Triton X-100 for 30 min to remove SDS. DPPIV activity was detected with the following substrates: 20 μM [Ala-Pro]_2-cresyl violet (Enzyme Systems Products and Protopetek, Livermore, CA; Van Noorden et al. 1997b), 20 μM Ala-Pro- AFC (Enzyme Systems Products; Smith et al. 1997), or 20 μM Ala-Pro- MNA (Enzyme Systems Products; Smith et al. 1997) in 100 mM cacodylate buffer (pH 7.4), or 20 μM Ala-Pro-rhodamine 110 (Molecular Probes, Eugene, OR; Lehtus et al. 1983a,b) in 10 mM Tris-HCl buffer (pH 7.5). In the case of Ala-Pro-MNA, a coupling agent was added as well, either 1 mM nitrosalicylaldehyde (Merck, Darmstadt, Germany; Van Noorden and Frederiks 1992) or Fast Blue B (FBb; Serva, Heidelberg, Germany; Van Noorden and Frederiks 1992). After 20 min of incubation, DPPIV activity was determined using a STORM Fluor-imager (Molecular Dynamics; Sunnyvale, CA) and Image Quant Software Package (Molecular Dynamics) or, in the case of the chromogenic substrates, scanned on a flatbed scanner using white light.

Samples were also subjected to Western blotting to determine CD26/DPPIV protein expression. Proteins were blotted at 4°C and 100 V to nitrocellulose paper for 1 hr. After washing in PBS containing 0.05% Tween, blots were blocked overnight with 5% BSA in PBS. Blots were incubated with anti-CD26 antibody Tα1 (Central Laboratory for Blood Transfusion; Amsterdam, The Netherlands) at a dilution of 1:100 in the blocking buffer containing 0.05% Tween for 1 hr, then washed twice in PBS containing 0.05% Tween, followed by 1-hr incubation with monoclonal horseradish peroxidase-conjugated goat anti-mouse IgG (Nordic; Tilburg, The Netherlands) in a dilution of 1:200 in blocking buffer. Then blots were washed again in PBS. Finally, after 10 min of incubation with Lum- Light Western blotting substrate (Roche Diagnostics; Mannheim, Germany), chemiluminescence was detected by the Lum-Imager (Roche Diagnostics).

**Fluorescence Spectra of [Ala-Pro]_2-Cresyl Violet Substrate and Cresyl Violet Product**

Fluorometric analysis of concentrations of [Ala-Pro]_2-cresyl violet and cresyl violet acetate (Enzyme Systems Products) was performed on a LS 50 fluorescence spectrometer (Perkin-Elmer; Gouda, The Netherlands). [Ala-Pro]_2-cresyl violet (20 μM) and cresyl violet acetate (20 μM) were measured separately and mixed in 1:1 and 1:4 ratios. Excitation was performed at 496 nm and 591 nm and fluorescence emission was monitored at wavelengths ranging from 500 nm to 700 nm.
Thin-Layer Chromatography of [Ala-Pro]2-Cresyl Violet Substrate and Cresyl Violet Product

To demonstrate fluorescence components in batches of substrate and product, separation by thin-layer chromatography (TLC) was performed, using silica gel-coated TLC plates (Merck; Darmstadt, Germany) as the stationary phase and methanol as the mobile phase. Equal amounts of substrate and product molecules were dissolved in methanol and applied to TLC plates. The plates were placed upright in running fluid and ran until the front of the running fluid had reached the end of the plate. The plates were dried and stored for further analysis. Images of the plates were made using white light and a digital camera (Coolpix; Nikon, Tokyo, Japan) to demonstrate the color change from orange to violet as is also observed with the naked eye when living cells are incubated with the substrate in a cuvette. Then the plates were illuminated with UVA light (320-380 nm), and photographed with a UV-blocking filter (>300 nm) using the same camera.

Fluorospectrometric Analysis of DPPIV Activity

Living Jurkat cells and CD26/DPPIV-transfected Jurkat cells were harvested and DPPIV activity was determined by fluorospectrometry. Before DPPIV activity measurements, cells were washed twice in cold PBS. Intact cells were kept on ice before mixing with the enzyme incubation media. Parts of the cells were lysed by ultrasonic treatment three times for 5 sec. Incubations were started at t=0 by suspending Jurkat cells or transfected Jurkat cells in prewarmed PBS supplemented with 1.7 mM CaCl2 and 1 mM MgCl2 at 37°C containing 0-40 μM of the DPPIV substrate [Ala-Pro]2-cresyl violet or Ala-Pro-rhodamine 110. Actual amounts of ester bonds that are cleaved are twice as high as that of the free fluorochrome in the case of [Ala-Pro]2-cresyl violet, because two amino acid sequences are spliced off per cresyl violet fluorochrome (Figure 1). Incubations were carried out at 37°C by using prewarmed PBS to which the substrate was added just before the start of the incubation. The cell suspension was kept on ice before the incubation and added 30 sec after the start of recording. For each measurement, 4 × 10^6 living cells, or its equivalent in cell lysates, were incubated in a final volume of 1200 μl prewarmed PBS containing 0-40 μM of [Ala-Pro]2-cresyl violet or Ala-Pro-rhodamine 110 substrate. Fluorometric analysis was performed on an LS 50 fluorescence spectrometer under continuous magnetic stirring to keep cells in suspension. Cuvettes with an excitation light path of 1 cm and an emission light path of 4 mm were used. Excitation for cresyl violet was performed at 591 nm with a slit width of 10 nm and emission was detected at 628 nm with a slit width of 10 nm (Boonacker and Van Noorden 2001). Rhodamine 110 was excited at 491 nm with a slit width of 10 nm and emission was detected at 529 nm with a slit width of 10 nm (Boonacker and Van Noorden 2001). Fluorescence was measured continuously during the first 4 min of incubation. Because both synthetic substrates are not completely stable in aqueous solution, a spontaneous small but continuous increase of fluorescence is detected when the substrates are incubated in an aqueous solution. By starting the incubation with the medium containing the substrate only, fluorescence of Ala-Pro-cresyl violet and due to spontaneous formation of product (both cresyl violet and rhodamine 110) was measured. These values were subtracted from fluorescence values obtained after the cells were added to the medium, thus correcting for the spontaneous nonspecific increase in fluorescence. Fluorescence values were plotted against time.

Confocal Microscopic Analysis of DPPIV Activity in Living Cells

Confocal analysis was performed to localize DPPIV-like activity in living Jurkat and transfected Jurkat cells on a Leica SP2 AOBS confocal microscope (Leica Microsystems; Mannheim, Germany). In case of the use of [Ala-Pro]2-cresyl violet, fluorescence of both substrate and product was analyzed with settings of the AOBS for 488-nm excitation of the substrate and 594-nm for excitation of the product. Fluorescence was measured at 515-576 nm and 613-734 nm for substrate and product, respectively. Each focal plane was scanned in a sequential manner in time, x, y, or in volume, z, for end-point images. In case of the use of Ala-Pro-rhodamine 110, excitation was performed at 496 nm and emission was measured at 530-580 nm. Living Jurkat cells were incubated on the stage of a confocal microscope in Dulbecco's modified medium containing 0.1 mg/ml Geneticin G418 and 1 mM glutamine and 10% FCS. This medium allowed prolonged incubations for longer periods of time without any significant cell damage. The suspended cells were incubated in 1 ml incubation medium on the microscope stage on a y-irradiated glass bottom in a poly-l-lysine-coated petri dish (MarTek; Ashland, MA). Nonadherent blood cells tend to stick after a certain period of time to the poly-l-lysine-coated glass bottom. After allowing the cells to adhere for a few minutes, cells were selected in the transmission image mode using white light and brought into focus, and transmission images were made to check whether cells were not moving any more. Then substrate was added by carefully dropping the substrate on top of the cells, preventing the cells to drift out of focus or the field of observation. Then the substrate was added as 10 μl of a 100 × concentrated stock solution to the medium. The petri dishes were mounted in the specimen clamp on the stage of the microscope with the use of a circular rubber self-constructed O-ring. This enables the administration of the substrate directly on top of the cells, while the cells can be imaged continuously. The first fluorescence images were captured at 30 sec after the substrate was added in a single focal plane at a rate of 1.8 sec per scan. In total, 200 images were made in 6 min. Sequential scanning was performed to minimize crosstalk.

Results

Reactivity of the various Ala-Pro-containing synthetic substrates with CD26/DPPIV was demonstrated with the use of an enriched fraction of sCD26/DPPIV that was submitted to native gel electrophoresis and Western blotting. Figure 2 demonstrates a similarly stained banding patterns with two major bands of DPPIV activity obtained with all substrates tested. Western blotting after staining with the anti-CD26/DPPIV anti-
DPPIV Activity in Living Jurkat Cells

Figure 2 DPPIV activity as detected after gel electrophoresis of sCD26/DPPIV enriched from proteasomes in human seminal fluid with different synthetic substrates for DPPIV (Lanes 1–5) and CD26/DPPIV protein after Western blotting (Lane 6). DPPIV activity is detected in Lane 1 with [Ala-Pro]_2-cresyl violet (CV), in Lane 2 with Ala-Pro-AFC (AFC), in Lane 3 with Ala-Pro-rhodamine 110 (R 110), in Lane 4 with Ala-Pro-MNA in combination with NSA as coupling agent (MNA-NSA), and in Lane 5 with Ala-Pro-MNA in combination with FBB* as coupling agent (MNA-FBB). Molecular weight markers are presented at right. Native CD26/DPPIV is located just above the arrow of 120 kD, which is in agreement with the molecular weight of 140 kD of the native protein. Due to the limited number of excitation wavelengths on the storm scanner, emission intensities cannot be directly compared with respect to activity.

Figure 3 Emission spectra of [Ala-Pro]_2-cresyl violet and its cleavage product cresyl violet. The [Ala-Pro]_2-cresyl violet shows maximal absorbance at 488 nm and an emission maximum at 570 nm (A). Cresyl violet shows an absorbance maximum at 591 nm and an emission maximum at 628 nm (B). Both in A and B, spectra were collected using excitation light of 480, 500, 520, 540, 560, and 580 nm. Excitation stray light peaks are present in both figures. Although the spectra are not corrected for excitation light energy, maxima of emission were consequently found at 570 nm for substrate and at 628 nm for product. (C) Mixtures of substrate and product in 1:1 and 1:4 ratios at suboptimal excitation of wavelengths of 480 nm and 540 nm that are available on commercial confocal microscopes. It shows that specific product fluorescence is hard to detect when the wrong excitation is used. 1, Emission spectrum of 20 μM [Ala-Pro]_2-cresyl violet when excited at 540 nm. 2, Emission spectrum of 20 μM cresyl violet when excited at 480 nm. 3, Emission spectrum of [Ala-Pro]_2-cresyl violet when excited at 480 nm. 5, Emission spectrum of a 1:1 mixture of [Ala-Pro]_2-cresyl violet and cresyl violet when excited at 480 nm. 6, Emission spectrum of a 1:4 mixture of [Ala-Pro]_2-cresyl violet and cresyl violet when excited at 540 nm. 7, Emission spectrum of a 1:4 mixture of [Ala-Pro]_2-cresyl violet and cresyl violet when excited at 480 nm. (D) TLC analysis of [Ala-Pro]_2-cresyl violet and cresyl violet. Lane 1 shows the orange-red color of [Ala-Pro]_2-cresyl violet substrate as detected by illumination with white light. Lane 2 shows fluorescence of [Ala-Pro]_2-cresyl violet substrate as detected with UVA light of 320–380 nm and a longpass filter of >500 nm. Lane 3 shows the deep red color of cresyl violet as detected by illumination with white light. Lane 4 shows fluorescence of cresyl violet as detected with UVA light illumination and a longpass filter.
cent, whereas the DPPIV reaction product, rhodamine 110, has an excitation peak at 491 nm and an emission peak at 529 nm (Leyrus et al. 1983a,b).

Comparison of [Ala-Pro]²-cresyl violet and Ala-Pro-rhodamine 110 as substrates for CD26/DPPIV was demonstrated in intact and permeabilized wild-type Jurkat cells and CD26/DPPIV-transfected Jurkat cells.

Table 1  Kinetic parameters of cleavage of [Ala-Pro]²-cresyl violet (CV) and [Ala-Pro]-rhodamine 110 (Rho 110) by intact and permeabilized Jurkat cells and CD26/DPPIV-transfected Jurkat cells

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<th>Intact cells</th>
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<td>CV Rho 110</td>
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<tr>
<td>[Ala-Pro]²-CV</td>
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<td>3.7</td>
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<tr>
<td>[Ala-Pro]-Rho</td>
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<td>3857</td>
<td>8416</td>
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<td>K_{m} (µM)</td>
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*nd, not detectable.

**Units, arbitrary fluorescence units per 4 x 10⁶ cells/ml."
DPPIV Activity in Living Jurkat Cells

Figure 5 Galleries of confocal images of fluorescence in time in optical sections of CD26/DPPIV-transfected intact living Jurkat cells using 20 μM [Ala-Pro]²-cresyl violet synthetic substrate to localize DPPIV activity. The three galleries are sets of images of the same cells captured at every 30 sec after substrate was added to the incubation medium. (A) Accumulation of [Ala-Pro]²-cresyl violet fluorescence (excitation at 494 μM, emission at 550–580 nm) is shown in green and generation of cresyl violet fluorescence (excitation at 594 nm, emission at ~620 nm) in red. Co-localization of substrate and product is shown in yellow. (B) Fluorescence images in A are superimposed on transmission images of cells to demonstrate cellular sites of substrate accumulation and product formation. Note that substrate accumulates especially in cells that do not show product formation. These cells are most likely dead cells. (C) Fluorescence of cresyl violet only is superimposed on transmission images of cells to show exact localization of DPPIV activity. Bar = 20 μm.

transfected Jurkat cells by incubating the cells for 4 min in the presence of both substrates (Figure 4). A small increase in fluorescence over time was observed when both synthetic substrates, but particularly [Ala-Pro]²-cresyl violet, were incubated in the aqueous medium in the absence of cells, indicating spontaneous disintegration of the substrates. The increase in fluorescence over time in wild-type Jurkat cells was similar to the spontaneous disintegration of [Ala-Pro]²-cresyl violet, indicating that other DPPIV-like proteases did not cleave the substrate. CD26/DPPIV-transfected Jurkat cells produced significantly higher amounts of fluorescence at 628 nm in the presence of [Ala-Pro]²-cresyl violet. Figure 4B shows a similar production of fluorescence at 628 nm in intact and permeabilized transfected Jurkat cells against 20 μM [Ala-Pro]²-cresyl violet, again indicating that intracellular DPPIV-like proteases do not cleave the substrate.

Wild-type Jurkat cells lacking CD26/DPPIV process Ala-Pro-rhodamine 110 at a considerable rate, but the reaction velocity in CD26/DPPIV-transfected Jurkat cells against 20 μM Ala-Pro-rhodamine 110 was considerably higher than in Jurkat cells lacking the enzyme (Figure 4C). Permeabilization increases the reaction rate in both wild-type Jurkat cells and transfected Jurkat cells, indicating that intracellular DPPIV-like proteases cleave the rhodamine 110-containing substrate (Figure 4D). Please note that inner filter effects did not play a significant role because all plots are linear with time, showing that saturation does not occur due to inner filter effects. Kₘ values of cleavage of [Ala-Pro]²-cresyl violet and Ala-Pro-rhodamine 110 by intact living and permeabilized wild-type Jurkat cells and CD26/DPPIV-transfected Jurkat cells were similar in the range of 3-10 μM (Table 1). Remarkably, Vₘₐₓ values of Ala-Pro-rhodamine cleavage were
similar in wild-type Jurkat cells and transfected Jurkat cells, and permeabilization of the cells increased $V_{\text{max}}$ almost threefold (Table 1). Please note that $V_{\text{max}}$ values are given in arbitrary fluorescence values, so the values for rhodamine 110 can be directly compared but not rhodamine 110 values with cresyl violet values.

Confocal microscopical analysis of cleavage of [Ala-Pro]$^2$-cresyl violet and Ala-Pro-rhodamine 110 in living wild-type Jurkat cells and CD26/DPPIV-transfected Jurkat cells is shown in Figures 5 and 6. Accumulation of cresyl violet in time was observed only in transfected Jurkat cells (Figure 5) and not in Jurkat cells lacking CD26/DPPIV (Figure 6) in small vesicles near the plasma membrane during the first few minutes of incubation. After longer incubation periods, the fluorochrome was transported to other organelles in transfected Jurkat cells (Figure 7), mimicking the pathways of internalization that have been described for CD26/DPPIV (Boonacker and Van Noorden 2003).

When Ala-Pro-rhodamine 110 was used as substrate, fluorescence occurred in both transfected and wild-type living Jurkat cells. Although fluorescence appeared to be more diffuse, accumulation was also observed in small vesicle-like structures near the plasma membrane (Figure 7).

**Discussion**

For visualization of specific enzyme reactions in living cells in general and activity of CD26/DPPIV in particular, it has to be established whether other enzymes interfere in the reaction and whether substrate is available at all sites of the enzyme (Boonacker and Van Noorden 2001). Therefore, we compared synthetic fluorogenic substrates that are considered to be specific for DPPIV activity (Lojda et al. 1976). These synthetic substrates differ in fluorochrome and the number of peptides attached. The rhodamine-containing substrate is monosubstituted, whereas the cresyl violet-containing substrate is bisubstituted.

Surprisingly, the two substrates behaved in rather different ways with respect to specificity. Ala-Pro-rhodamine appeared to react with other DPPIV activity homologues, such as lysosomal DPPIII, DPPVIII, and attractin, whereas [Ala-Pro]$^2$-cresyl violet reacted with DPPIV only. The most likely explanation is that the catalytic cleft of DPPIV only is suitable to give access to cresyl violet. The 3D configuration of the tunnel in the molecule of CD26/DPPIV and activity homologues that gives access to the active site is rather tight (Brandt 1997; Gorrell et al. 2000) and must provide steric hindrance for the cresyl violet-based substrate in the DPPIV activity homologues. As both substrates are built up rapidly in T-helper cells, it is unlikely that differences in permeability of the cells for the substrates cause the differentiation in specificity (Boonacker and Van Noorden 2001).

Excitation and emission spectra of cresyl violet and rhodamine 110 are not the same. Therefore, differences in absolute amounts of product formation on the basis of increases in fluorescence cannot be com-
Figure 7 Higher magnification of CD26/DPPIV-transfected intact living Jurkat cells incubated for 5 min in a medium containing 20 μM [Ala-Pro]²-cresyl violet to demonstrate DPPIV activity. (A) Accumulation of substrate in the cells in green (excitation at 494 nm, emission at 550-580 nm). (B) Product formation in the cells in red (excitation at 594 nm, emission at >620 nm). (C) Combination of images in A and B to show granular intracellular localization of cresyl violet in yellow. Bar = 10 μm.

pared. Probably, rhodamine 110 is excited more intensely than cresyl violet, because light of 488 nm is more intense than light at 591 nm. The illumination source is not similarly intense throughout the spectrum, as light (lasers) does not produce similar amounts of photons at each wavelength. Furthermore, hydrolysis of monosubstituted and bisubstituted fluorochromes is not comparable either, especially when over 15% of the substrate is hydrolyzed during an assay (Leytus et al. 1983b). Lastly, fluorescence generated during incubation can differ on the basis of the microenvironment of the fluorophore.

With respect to cytotoxicity, it can be stated that low amounts of excitation light should be used to avoid damage to the cells. Preferentially, laser power lower than 10 mW/cm² should be used (Manders, personal communication). Illumination by laser light is more toxic for cells containing fluorochromes than cells without fluorochromes. Because of the lower energy of light at longer wavelengths, [Ala-Pro]²-cresyl violet is also to be preferred over Ala-Pro-rhodamine because cresyl violet has to be excited at 594 nm and rhodamine at 488 nm.

A disadvantage of the cresyl violet-based substrate over the rhodamine-based substrate is the rather high instability of the former in aqueous solutions. However, the great advantage of [Ala-Pro]²-cresyl violet is its specificity for DPPIV activity. Wild-type Jurkat cells do not produce more fluorescence than incubation medium only, whereas CD26/DPPTV-transfected Jurkat cells produce distinctly more fluorescence. The same incubations were also performed on permeabilized cells. After sonification, cells were incubated as described above. Permeabilization of wild-type Jurkat cells and of CD26/DPPTV-transfected Jurkat cells did not lead to an additional increase in fluorescence. Apparently, [Ala-Pro]²-cresyl violet does not react with other DPPIV activity homologues.

In intact cells, green fluorescence of the [Ala-Pro]²-cresyl violet substrate is converted into red fluorescence of the liberated cresyl violet. Strikingly, damaged or apoptotic cells showed hardly any enzymatic activity but did accumulate [Ala-Pro]²-cresyl violet rapidly, as if the enzyme was already inactivated.

Although [Ala-Pro]²-cresyl violet is specific for DPPIV, localization of the cleavage product is another matter. Accumulation of the fluorochrome at first in small submembranous granules and later in larger intracellular compartments may be due to lipophilicity, the charge of cresyl violet, or transport in intact CD26/DPPIV-expressing cells, because intracellular localization patterns are in agreement with the recycling pathway of the M6P receptor, which can bind CD26/DPPIV and molecules associated with it (Boonacker and Van Noorden 2003).

Ala-Pro-rhodamine 110 also shows some spontaneous hydrolysis when dissolved in incubation medium. When incubations were performed with intact living Jurkat cells lacking CD26/DPPIV, hydrolysis of Ala-Pro-rhodamine 110 was distinctly higher, demonstrating its lack of specificity. CD26/DPPIV-positive transfected Jurkat cells produced an additional increase of fluorescence over time. Permeabilization of cells almost doubled the production of fluorescence, indicating that diffusion into the cells of the rhodamine 110-based substrate is a limiting step in the accessibility of this substrate for intracellular proteases other than CD26/DPPTV. Therefore, we conclude that the nature of the fluorophore significantly affects interactions of these synthetic substrates with the active site of DPPIV as has been demonstrated before (Chase and Shaw 1969; Wong and Shaw 1976; Castillo et al. 1979; Lokey et al. 2002).

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CHAPTER 4

Determination of reactions of enzymes and their kinetic parameters in living cells by flow cytometry

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Determination of reactions of enzymes and their kinetic parameters in living cells by flow cytometry

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Quantitative enzyme histochemical methods are applied to determine enzyme reactions and kinetic parameters of enzymes in intact cryostat sections of tissues and cell preparations to understand how enzymes function in vivo [1]. Activity reflects an enzyme's physiological function and is the ultimate outcome of regulation at transcriptional, translational and posttranslational levels. However, these methods were traditionally applied to frozen and, thus, dead tissues and cells. So, dynamic interactions between enzymes and macromolecular and structural elements as occur in vivo are lost. These interactions are part of posttranslational control of the activity of enzymes [2,3]. Therefore, we developed methods to analyze enzyme activity in living cells and used flow cytometry for rapid measurements of the enzyme reactions in individual cells [4].

Since enzyme activities often display a heterogeneous character in cell populations, flow cytometry is an ideal tool to study quantitatively enzyme activity in individual living cells. Furthermore, activity can be related to other relevant parameters such as the amount of enzyme molecules present as detected immunocytochemically, for example to determine posttranslational control [5,6].

In contrast to fluorescence microscopy in which time lapse series of digital images can be made of living cells during incubation to determine activity of a specific enzyme [4], each cell is measured only once in flow cytometry. In the latter case, fading of fluorescence is of negligible influence on the measurements, but on the other hand, enzyme reactions cannot be determined in time per individual cell. We solved this problem by analysis of enzyme reactions in time in large numbers of cells while the cells are incubated as shown in Fig. 1.

Proteases are a class of enzymes that play essential roles in health and disease, for example in the turnover of extracellular matrix components, activation of the immune system, apoptosis, arthritis, and metastasis of cancer. To visualize protease activity in individual living cells, a new class of synthetic substrates containing the fluorescent group cresyl violet has been synthesized [4,7].
Cresyl violet has a different fluorescence spectrum when amino acids are attached, but that changes after proteolytic liberation of the amino acids. To specifically detect liberated cresyl violet, excitation at 591 nm and emission at 628 nm is needed [6].

The new synthetic substrates were used for the subcellular localization of cathepsin B activity in cancer cells [7] and activity of dipeptidyl peptidase IV (CD26/DPPIV) in hepatocytes [4] and T helper cells [6].

DPPIV is an ectopeptidase that is present on the plasma membrane of many different types of cells. It is present on brush border membranes of intestine and kidney, where it is involved in the digestion of polypeptides to provide substrates for peptide and amino acid transport systems [8]. Furthermore, DPPIV is homologous with CD26, which has a costimulatory function in T helper cell activation. So, the CD26 molecule has at least 2 functions in T cells: a proteolytic function that is involved in activation of procytokines or inactivation of cytokines and a signal transduction function [9]. We investigated kinetic parameters of DPPIV/CD26 in individual living rat hepatocytes and in polarized human T helper 1 and T helper 2 cells by incubating cells with different concentrations of ala-pro-cresyl violet as substrate and analysis of the liberation of cresyl violet by flow cytometry. In that way, we found that there is a strong and dynamic posttranslational regulation of the kinetics of DPPIV in both cell types. This regulation strongly determines its physiological function. For example, in rat hepatocytes the affinity of DPPIV is high (low Km) when few enzyme molecules are present (low Vmax) and vice versa [4]. On the other hand, T helper 1 cells contain 10-fold more CD26 molecules than T helper 2 cells but DPPIV activity in the latter cells shows a Vmax and Km that are both 2-fold lower [6]. As a result DPPIV activity in T helper 1 and T helper 2 cells at physiological substrate concentrations is roughly the same despite the 10-fold difference in the number of enzyme molecules present per cell. This phenomenon of posttranslational regulation becomes apparent only when activity is analyzed in living cells.

Protocols for detection of DPPIV activity in living cells

A: Protocol for detection of DPPIV activity in living rat hepatocytes

Hepatocytes are isolated by collagenase perfusion of livers of Wistar rats as described by Caro et al. [10]. Hepatocytes (5-10 mg dry mass/ml) are kept in Krebs-Henseleit bicarbonate medium containing 1.3 mM Ca²⁺, 10 mM Hepes (pH 7.4), 20 mM glucose, and 1 mM octanoate on ice until enzyme assays are performed. Prior to flow cytometric analysis, hepatocytes are stained with the DNA dye, Hoechst 3.3.3.4.2 (1 µg/ml; Hoechst, Amsterdam, The Netherlands), for 30 min at 0°C. Hepatocytes are suspended 1:10 v/v in Krebs-Henseleit buffer.

Hepatocytes are mixed with a solution of the synthetic substrate, ala-pro-cresyl violet (Enzyme Systems Products, Livermore CA, USA). A stock solution of 1 mM ala-pro-cresyl violet in
Ringer is made. Analysis is started by establishing forward scatter, and Hoechst and cresyl violet fluorescence. Viable cells have a high forward scatter and low Hoechst fluorescence, whereas cells with high fluorescence and low forward scatter are not likely to be intact or are loose nuclei (Fig. 1). Viable hepatocytes are selected by gating the population with high forward scatter and low Hoechst fluorescence (Fig. 1A).

The substrate is added in various concentrations (0-100 μM) to the cells in a tube and mixed quickly and the sample differential pressure is boosted to prevent a lag phase in the kinetic measurements. Then, the cells are analyzed for the amount of liberated cresyl violet per hepatocyte in time (Fig. 1B).

**Fig. 1A.** Flow cytometric analysis of Hoechst 33342 fluorescence (DNA dye) versus forward scatter (FCS). A selection of intact living rat hepatocytes was made on the basis of low fluorescence and high forward scatter, indicated by the oval window.

**Fig. 1B.** Generation of cresyl violet fluorescence by DPPIV activity as a function of time in rat hepatocytes that were gated in Fig. 1A with the oval window, using 10 μM ala-pro-cresyl violet as substrate, which was added at t=0 as indicated by arrow.

**B: Protocol for the simultaneous detection of CD26 expression and DPPIV activity on living human T helper cells**

Living T cells are harvested at different time points after stimulation and analysed for their CD26 expression and DPPIV activity by means of flow cytometry. For CD26 detection, cells are incubated for 30 min at 0°C with FITC-conjugated anti-human CD26 monoclonal antibody Tal 1 (1:80 dilution of a stock solution of 0.2 mg/ml) and washed twice in cold phosphate buffered saline (PBS), pH 7.4. Cells were kept on ice prior to mixing with the enzyme incubation medium. Incubations were started at t=0 by suspending T cells in PBS containing 10 μM of the DPPIV substrate ala-pro-cresyl violet (Enzyme Systems Products). Enzyme reactions are carried out at 20°C.
Flow cytometric analysis of CD26 expression and formation of cresyl violet on living T helper cells are performed on a FAC-star plus (Becton and Dickinson, Mountain View CA, USA), using the software program CellQuest version 3.2. Analysis was started by establishing forward scatter and then adding the substrate at t=0. Analysis is performed at a rate of 200 cells/sec. The parameters measured are time, forward scatter, side scatter, FITC fluorescence representing CD26 expression, and fluorescence of liberated cresyl violet formation (excitation at 591 nm and emission at 628 nm). The increase in fluorescence is measured during 4 min (Fig. 2A) and substrate specificity of DPPIV activity is tested in the presence of a specific inhibitor (Fig. 2B). Fluorescence values are plotted against substrate concentrations. A hyperbolic curve is fitted to the data with the use of a curve fitting program (Mac Curve Fit; Apple, Cupertino CA, USA) and Vmax and Km values are calculated for living T helper cells according to [11].

![Fig. 2A. Generation of cresyl violet fluorescence by DPPIV activity on living human T helper I cells, as a function of time, using 25 μM ala-pro-cresyl violet as synthetic substrate.](image1)

![Fig. 2B. Inhibition of DPPIV activity by preincubation with 10 μM inhibitor P34201 (Enzyme Systems Products) to demonstrate specificity of DPPIV for the synthetic substrate ala-pro-cresyl violet which was added in a concentration of 25 μM.](image2)

References


CHAPTER 5

Ala-Pro-cresyl violet,
a synthetic fluorogenic substrate for the analysis of
kinetic parameters of dipeptidyl peptidase IV (CD26) in
individual living rat hepatocytes

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A new type of fluorogenic substrates for proteases based on the leaving group cresyl violet has been synthesized. Cresyl violet is not fluorescent when amino acids or peptide groups are attached but becomes highly fluorescent after proteolytic liberation. Its fluorescence shows linearity with concentration and barely any fading. The properties of Ala-Pro-cresyl violet as substrate for dipeptidyl peptidase IV (DPPIV) (CD26) for localization and quantification of its activity in individual freshly isolated living rat hepatocytes were investigated using confocal microscopy, image analysis, and flow cytometry. DPPIV activity was localized exclusively in patches at plasma membranes likely being bile canalicular domains. Activity was analyzed quantitatively in individual cells by capturing series of images in time. Production of fluorescence was analyzed on the basis of the series of digital images and it appeared to be nonlinear with time. By calculation of the initial velocity at time zero, activity of DPPIV per individual hepatocyte was calculated. Cresyl violet-dependent fluorescence appeared in a similar way when cells were analyzed by flow cytometry. A dipeptide phosphonate inhibitor inhibited production of fluorescence competitively with a $K_i$ of 7 $\mu$M. $K_m$ values in individual hepatocytes varied in the range of 6–22 $\mu$M depending on the individual rat from which the hepatocytes were obtained, whereas the $V_{max}$ values per individual rat were inversely correlated indicating posttranslational regulation of the kinetic parameters of DPPIV. This relationship was lost when membrane fractions of the same hepatocyte suspensions were analyzed. It is concluded that cresyl violet-based protease substrates are the compounds of choice to localize and quantify protease activity in living cells and tissues. 

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Quantitative enzyme histochemical methods have been applied to determine kinetic parameters of enzymes in intact unfixed tissue sections to obtain information on behavior of enzymes in their own cellular environment and zonal differences in their function within a tissue (1–6). These studies demonstrated that both variations and regional differences in the kinetic parameters of several enzymes occur which partly explain the enormous plasticity of tissues to adapt to alterations in the environment. Although unfixed cryostat sections is one step closer to the in vivo situation than homogenates, they still do not provide information on how enzymes behave in vivo. We want to establish the exact role of proteases in physiological and pathophysiological processes. Examples are turnover of collagen (7–9), activation of the immune system (10), arthritis (11, 12), and metastasis of cancer (13, 14). For such studies, methods to measure protease reactions in individual living cells are needed. To visualize protease activity in single cells, a new class of fluorogenic substrates for proteases containing cresyl violet was synthesized. This is a highly fluorescent leaving group after proteolytic cleavage of the amide bonds. Dipeptidyl peptidase IV (DPPIV) is an ectopeptidase present at the plasma membrane of many cell types. It is a transmembrane glycoprotein with a short cytoplasmic tail, one hydrophobic transmembrane segment, and a large extracellular domain (15). DPPIV is involved in activation of bioactive molecules such as cytokines (16, 17) and it participates in the extracellular digestion of polypeptides to provide substrates for peptide and amino acid reabsorption (18, 19). In hepatocytes, the enzyme is present at the apical bile canalicular mem-

Abbreviations used: DPPIV, dipeptidyl peptidase IV; $V_{max}$, initial reaction rate.
brane and exerts its function in the lumen of bile canaliculi (20). DPPIV is homologous with CD26 (21, 22) which has a receptor function for T cell activation and can bind to collagen (23–26). The CD26 molecule can become heavily glycosylated and sialylated and this regulates its receptor function strongly. For example, it is involved in several immune-mediated diseases, including AIDS. It can act as a binding protein for HIV (27) but only when it is heavily sialylated (28). Inhibition of DPPIV activity by specific tripeptides has an immunosuppressive effect in vivo (29).

We investigated kinetic parameters of DPPIV in individual living hepatocytes to establish the possibilities of the use of fluorogenic cresyl violet-based substrate in combination with digital imaging techniques for in vivo analysis of enzyme function. Images were captured continuously in time while the reaction and thus generation of fluorescence in the cells proceeded. Analysis of series of these images provided quantitative information of the enzyme reaction as a function of time per individual cell.

MATERIALS AND METHODS

Synthesis of [Ala-Pro]2-Cresyl Violet

Z-Alanyl-proline dicyclohexylamine salt (5 g, 10 mmol) was suspended in 80 ml dimethylformamide/pyridine (1:1, v/v) and cooled to 0°C. Then, 1-(3-methylaminopropyl)-3-ethylcarbodiimide hydrochloride (2 g, 10.4 mmol) was added. After 20 min at 0°C, cresyl violet hydrochloride (1 g, 3.2 mmol) was added. The reaction mixture was allowed to stir 18 h while the temperature was raised slowly to room temperature. The solvents were removed at 50°C and the residue was dissolved in 300 ml ethyl acetate. The solution was washed twice with 100 ml 1 N aqueous hydrochloric acid, once with 50 ml saturated aqueous brine, twice with 100 ml saturated aqueous sodium bicarbonate, and once with 100 ml saturated aqueous brine. The ethyl acetate solution was dried over anhydrous magnesium sulfate, filtered, and dried under high vacuum. The crude product (1.5 g) was chromatographed on silica gel using 5% (v/v) methanol in chloroform. The purified product, [Ala-Pro]2-cresyl violet, weighed 0.9 g (33%). It was then treated at room temperature for 1 h with 10 ml of 30% (w/v) hydrogen bromide in acetic acid. The reaction mixture was allowed to stir 18 h while the temperature was raised slowly to room temperature. The solvents were removed at 50°C and the residue was dissolved in 300 ml ethyl acetate. The solution was washed twice with 100 ml 1 N aqueous hydrochloric acid, once with 50 ml saturated aqueous brine, twice with 100 ml saturated aqueous sodium bicarbonate, and once with 100 ml saturated aqueous brine. The ethyl acetate solution was dried over anhydrous magnesium sulfate, filtered, and dried under high vacuum. The crude product (1.5 g) was chromatographed on silica gel using 5% (v/v) methanol in chloroform. The purified product, [Ala-Pro]2-cresyl violet, weighed 0.9 g (33%). It was then treated at room temperature for 1 h with 10 ml of 30% (w/v) hydrogen bromide in acetic acid. The reaction mixture was added to 200 ml diethyl ether. The precipitated substrate, [Ala-Pro]2-cresyl violet dihydrobromide, was filtered, washed with ether, and dried under high vacuum. The yield was 0.84 g. Thin-layer chromatography on silica gel with butanol:acetic acid:water (4:1:1) showed a single brown spot at Rf = 0.05 that was not fluorescent by itself but was red fluorescent after strong heating. The chemical structures of the nonfluorescent substrate, the fluorescent product, cresyl violet, and the cleavage products for DPPIV are shown in Fig. 1 (U.S. patent pending, Enzyme Systems Products, Dublin, CA).

Isolation of Hepatocytes

Hepatocytes were isolated by collagenase perfusion of livers of male Wistar rats (200–250 g; HSD Animal Farm, Zeist, The Netherlands) after 24 h of starvation as described previously (30). The animals were exposed to a controlled dark–light cycle (light: 7:00 AM to 7:00 PM) throughout the acclimatization period of at least 1 week. Before starvation, animals had free access to food (standard chow diet; Hope Farms, Woerden, The Netherlands) and water. The animals had always free access to water. During operation, the animals were under Nembutal anesthesia. Animal care was performed according to the guidelines of the University of Amsterdam. Hepatocytes (5–10 mg dry mass/ml) were kept in Krebs–Henseleit bicarbonate medium containing 1.3 mM Ca2+, 10 mM sodium Hepes (pH 7.4), 20 mM glucose, and 1 mM octanoate on ice until enzyme assays. Homogenates were prepared by freezing cell suspensions in liquid nitrogen and subsequent thawing. One volume of homogenates and 10 vol of 20 mM Tris–HCl buffer (pH 7.4) containing 1% (v/v) Triton X-100 were mixed and incubated at 4°C under constant stirring for 60 min. One part of these homogenates was used for the determination of DPPIV activity. Another part was centrifuged (40,000g, 4°C, 20 min) and the supernatants were used as membrane fractions for the determination of DPPIV activity.

Analysis of DPPIV Activity

DPPIV activity was determined in hepatocytes using six approaches. Activity was determined in living hepatocytes with confocal scanning laser microscopy, image processing and analysis, flow cytometry, and fluorometry. Fluorometry was also used for the determination of activity in homogenates of hepatocytes and in membrane fractions of hepatocytes. Incubations were started at t = 0 by adding an aliquot of 60 μl hepatocytes to 3 ml Krebs–Henseleit medium containing 0–50 μM [Ala-Pro]2-cresyl violet in the presence or absence of 0–50 μM Ala-Pip4(OPh-4-Cl)2 (Enzyme Systems Products), which is a selective DPPIV inhibitor (31, 32). Substrate and inhibitor were dissolved first in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the incubation medium was 0.5% (v/v). Incubations were carried out at 20°C. Confocal scanning laser microscopy was performed after 100 μl of an assay medium containing hepatocytes was brought into a well formed by a stainless-steel ring attached to a glass slide (33). Images of cells were captured in time with a Leica confocal scanning laser microscope fitted to a Leica Fluovert inverted microscope equipped with a PL APO oil-immersion objective (63×, NA 1.40). Excitation was performed at 568 nm and fluorescence was captured at >595 nm. The
pinhole aperture was set to provide an optimized image (34). Cells were subjected to optical serial sectioning after each 60 s of incubation up to 300 s. Images in the X–Y plane were recorded with intervals of 0.5 μm in the Z direction. Each optical section was averaged eight times. Images were recorded in a 512 × 512 pixel format. The size of each pixel represented 0.015 μm² in the object. Confocal data stacks of 20–30 optical sections were processed with standard Leica software package.

Image analysis was performed by transferring an aliquot of 100 μl of an incubation medium containing hepatocytes into an incubation chamber that consisted of an object glass and a cover glass separated by a spacer made of adhesion tape with a thickness of 40 μm. The object glass was set on the stage of a fluorescence microscope (Leitz Dialux 20, Wetzlar, Germany) with a ×25 objective (NA 0.75). A drop of medium was placed beside the cover glass and the chamber became filled by capillary forces in a few seconds. The first image was captured at 15–30 s after the reaction was started using a CCD camera with an 8-bit resolution (Cohu 4910; San Diego, CA), frame grabber (LG-3, Scion; Frederick, MD), and a Power Macintosh 8100/110 computer (Apple, Cupertino, CA), using the public domain NIH imaging software program (version 1.57; written by W. Rasband and available via Internet by anonymous ftp from zippy.nimh.nih.gov). Settings of camera and frame grabber were according to Jonker et al. (35). A parabolic curve was fitted to the data per hepatocyte over time using a least-squares curve-fitting method (Mac Curve Fit 1.2d4 program; MCF, Shareware by K. Raner, Internet: kraner@aslinc.net.au) (4, 33). In this function, \( f(t) = at^2 + bt + c \), coefficient \( a \) represents the time-dependent deviation from the initial reaction rate, \( b \) represents the initial reaction rate (\( V_{\text{max}} \)), and \( c \) represents the fluorescence at \( t = 0 \). \( V_{\text{max}} \) values were used as measure of DPPIV activity. These values were plotted against substrate concentration. A hyperbolic curve was fitted to the data with the use of the MCF program and \( V_{\text{max}} \) and \( K_m \) values were determined.

Calibration was performed by measuring fluorescence of a series of solutions of 0–10 μM cresyl violet in Krebs–Henseleit medium. These solutions were also used to test the properties of cresyl violet for quantitative purposes. The depth of the incubation chamber was 24 μm and the size of the area measured 240 × 170 μm. The total volume that was measured was thus 0.001 μl. The amount of fluorescence captured by image analysis was linearly related with concentrations up to at least 10 μM when measured in a thin film of 24 μM which approximates the diameter of cells (Fig. 2A). Fading did not occur to any significant extent during measurements in time as is shown in Fig. 2B. Thus, cresyl violet has fluorescence properties that are necessary for quantitative fluorescence microscopy (36). On the basis of Fig. 2A, it was calculated that 100 gray values corresponded with 6.7 fmol cresyl violet. From these data, DPPIV activity could be calculated in absolute enzyme units (1 U = 1 μmol cresyl violet produced per minute). Flow cytometric analysis of production of cresyl violet in hepatocytes was performed with a FACStar Plus (Becton and Dickinson, San Jose, CA) using the software program CellQuest (version 1.0: Becton and Dickinson). Prior to analysis, cells were stained with the DNA dye Hoechst 3.3.3.4.2 (36 μg/ml; Hoechst Holland, Amsterdam, The Netherlands) for 30 min at 0°C. Analysis was started at a flow rate of 200 cells/s. The parameters measured were time, forward scatter, fluorescence of Hoechst 3.3.3.4.2 dye (excitation at 350 nm, emission at 485 nm with a band width of 20 nm), and fluorescence of cresyl violet (excitation at 514 nm, emission at 630 nm with a band width of 22 nm). Filters were obtained from Becton and Dickinson. The power of the lasers was set at 200 W. Fluorometric analysis with time was carried out with a Perkin–Elmer LS 50 fluorescence spectrometer (Perkin–Elmer, Gouda, The Netherlands) under continuous magnetic stirring. Culture medium was equilibrated with a light path of 1 cm was used. Excitation was performed at 591 nm with a slit width of 10 nm and emission was measured at 628 nm (slit width 10 nm). The first measurement was made at 5 s after mixing cells and incubation medium.

**FIG. 1.** Chemical structures of the fluorogenic substrate, [Ala-Pro]⁺ cresyl violet, and the fluorescent product, cresyl violet, after cleavage by DPPIV (large arrows).
FIG. 2. Relationship between fluorescence (gray values) and concentration of cresyl violet (µM) in a 24-µm-thick incubation chamber (A) and between fluorescence and incubation time (B) as determined by image analysis.

RESULTS AND DISCUSSION

The localization properties of cresyl violet in individual cells were investigated using confocal microscopy. Figure 3 is a 3D representation of individual living hepatocytes incubated for 5 min in Krebs-Henseleit buffer containing 10 µM substrate. The fluorescence is localized only in patches at the plasma membrane. Because DPPIV is an ectoproteinase that is localized at the apical bile canalicular membrane, these patches are likely bile canalicular membrane domains. It shows that cresyl violet fluorescence represents sites of activity of enzymes in intracellular domains. This good localization in combination with the fact that excitation can be performed at a relatively long wavelength (568 nm) to avoid photochemical damage to the living cells make cresyl violet-based fluorogenic substrates excellent tools for living cell cytochemistry. The only other type of synthetic protease substrates that has been applied so far to living cells are rhodamine-based (37, 38). These substrates have several disadvantages in comparison with cresyl violet-based substrates due to the low water solubility (39, 40) and the tendency of the rhodamine leaving group to accumulate in mitochondria of living cells after proteolytic cleavage (41) which limit their usefulness for living cell cytochemistry. Furthermore, rhodamine-based substrates for cathepsin B are not very specific because of the large size of the rhodamine leaving group (39, 40). Rhodamine-based substrates are suitable for flow cytometric analysis of protease activity in living cells (17, 40, 42, 43) or virus particles (44) provided low substrate concentrations are used which hampers the determination of kinetic parameters of enzymes (39, 40).

The exact intracellular localization (Fig. 3) in combination with the quantitative properties (Fig. 2) prove that cresyl violet has excellent properties as a leaving group for quantitative cytochemical analysis using confocal microscopy (Fig. 3), image analysis (Figs. 4–7), and flow cytometry (Fig. 8). Prolonged periods of incubation resulted in diffusion of cresyl violet from the cells but the first 5 min of incubation could be used safely for intracellular localization and quantification without introducing errors by diffusion. We also tried to employ cresyl violet-based substrates for visualization of protease activity in cells after freezing and thawing and in unfixed cryostat sections but these efforts were without success due to rapid diffusion of cresyl violet from the sites where it was produced. Apparently, the high-quality localization characteristics of the fluorescence is localized only in patches at the plasma membrane. Because DPPIV is an ectoproteinase that is localized at the apical bile canalicular membrane, these patches are likely bile canalicular membrane domains. It shows that cresyl violet fluorescence represents sites of activity of enzymes in intracellular domains. This good localization in combination with the fact that excitation can be performed at a relatively long wavelength (568 nm) to avoid photochemical damage to the living cells make cresyl violet-based fluorogenic substrates excellent tools for living cell cytochemistry. The only other type of synthetic protease substrates that has been applied so far to living cells are rhodamine-based (37, 38). These substrates have several disadvantages in comparison with cresyl violet-based substrates due to the low water solubility (39, 40) and the tendency of the rhodamine leaving group to accumulate in mitochondria of living cells after proteolytic cleavage (41) which limit their usefulness for living cell cytochemistry. Furthermore, rhodamine-based substrates for cathepsin B are not very specific because of the large size of the rhodamine leaving group (39, 40). Rhodamine-based substrates are suitable for flow cytometric analysis of protease activity in living cells (17, 40, 42, 43) or virus particles (44) provided low substrate concentrations are used which hampers the determination of kinetic parameters of enzymes (39, 40).

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FIG. 4. (A) Formation of cresyl violet fluorescence in a single hepatocyte as a function of time in the presence (○) or absence (●) of 10 μM Ala-Pro-cresyl violet as measured with image analysis and in suspensions of hepatocytes (B) or membrane fractions (C) as measured with fluorometry. Cells and substrate were mixed at t = 0 as indicated by arrow. The derivative at t = 0 of the quadratic function representing the reaction rate yielded initial velocity (V_{ini}).

cresyl violet are very much dependent on the vital status of cells. We have also applied cresyl violet-based substrates to visualize protease activity in cultured tissues and these efforts were very promising (V. Everts and C. J. F. Van Noorden, unpublished data) again indicating that the viability of cells is essential for a successful application of cresyl violet-based substrates. Cresyl violet-based substrates penetrate living cells as was shown in parallel experiments using [Z-Arg]^{2}-cre-

FIG. 5. Relationships between initial velocity of DPPIV (V_{ini} expressed in nU activity) in living hepatocytes and the Ala-Pro-cresyl violet concentration in the incubation medium for 5 individual rats. Each measuring point represents the mean V_{ini} of 10 individual hepatocytes as determined with image analysis.

FIG. 6. Characteristics of inhibition of DPPIV activity in individual living hepatocytes by the selective competitive inhibitor Ala-Pip'(OPh-4Cl), as determined by image analysis. Each measuring point is the mean value of 10 individual hepatocytes.
syl violet to demonstrate lysosomal cathepsin B activity in living hepatocytes and colon cancer cells (13, 45). On the basis of these studies of intralysosomal cathepsin B activity and Fig. 3, it can be concluded that production of fluorescence on the plasma membrane of hepatocytes incubated in the presence of [Ala-Pro]_2-cresyl violet represents the exact localization of DPPIV activity.

When 2D images of cells were captured in time and formation of fluorescence was analyzed, plots like the one in Fig. 4A were obtained. There was no signal obtained when cells were incubated in the absence of substrate. Autofluorescence was very low or even absent because emission was captured at >595 nm. These plots enabled the calculation of initial velocities (V_{init}) as described previously for quantitative chromogenic enzyme cytochemical methods (4, 33). When the reactions were analyzed fluorometrically using suspensions of living hepatocytes or membrane fractions of hepatocytes, similar plots were obtained (Figs. 4B and 4C).

V_{max} was taken as a measure of DPPIV activity in cells. Variation of the substrate concentration revealed Michaelis–Menten kinetics of DPPIV in individual hepatocytes (Fig. 5). The intercellular variation of DPPIV activity in an individual rat was rather constant (the standard error of the mean was in the range of 10–20% irrespective the substrate concentration. This variation is visualized in Fig. 8B (see below)).

The effects of the selective competitive dipeptide phosphonate inhibitor are shown in Fig. 6. It indicates the selective visualization of DPPIV activity with Ala-Pro-cresyl violet as substrate. The K_{i} was 7 µM. When calculating V_{max} and K_{m} values from the data in Fig. 5, it appeared that there was a strong inverse correlation between K_{m} and V_{max} per individual rat (Fig. 7). When the V_{max} was low, the K_{m} was high and vice versa. This phenomenon has important implications for the activity of DPPIV at physiological substrate concentrations. The conversion rate in the rat with the highest V_{max} and lowest K_{m} can be 10- to 20-fold higher than that in the rat with the lowest V_{max} and highest K_{m}. It suggests that there is posttranslational regulatory control of DPPIV activity in hepatocytes. Flow cytometric analysis of the enzyme reaction in individual hepatocytes is shown in Fig. 8. Figure 8A is a plot of Hoechst fluorescence versus scatter. This allowed us to select the population of living cells. The reaction of DPPIV in time in the selection of living hepatocytes is shown in Fig. 8B. This figure indicates the intercellular variation of activity.

The present study shows that (a) cresyl violet-based substrates have great potential to visualize and quantify activity of proteases in individual living cells. These substrates allow a very precise localization of active fractions of proteases in intracellular compartments when applying confocal microscopy. Quantitative determination of the active fraction in individual cells can be ob-

**FIG. 7.** Inverse relationship between K_{m} in µM and V_{max} in nU of DPPIV in individual living hepatocytes of 5 individual rats.
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CD26/DPPIV signal transduction function, but not proteolytic activity, is directly related to its expression level on human Th1 and Th2 cell lines as detected with living cell cytochemistry.

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CD26/DPP IV Signal Transduction Function, but Not Proteolytic Activity, Is Directly Related to Its Expression Level on Human Th1 and Th2 Cell Lines as Detected with Living Cell Cytochemistry


SUMMARY CD26/DPP IV is a cell surface glycoprotein that functions both in signal transduction and as a proteolytic enzyme, dipeptidyl peptidase IV (DPP IV). To investigate how two separate functions of one molecule are regulated, we analyzed CD26 protein expression and DPP IV enzyme activity on living human T-helper 1 (Th1) and Th2 cells that express different levels of CD26/DPP IV. DPP IV activity was specifically determined with the synthetic fluorogenic substrate ala-pro-cresyl violet and CD26 protein expression was demonstrated with an FITC-conjugated CD26-specific antibody. Fluorescence of liberated cresyl violet (red) and FITC (green) was detected simultaneously on living T-cells using flow cytometry and spectrofluorometry. Th1 cells expressed three- to sixfold more CD26 protein than Th2 cells. The signal transduction function of the CD26/DPP IV complex, tested by measuring its co-stimulatory potential for proliferation, was directly related to the amount of CD26 protein at the cell surface. However, DPP IV activity was similar in both cell populations at physiological substrate concentrations because of differences in \( K_m \) and \( V_{max} \) values of DPP IV on Th1 and Th2 cells. Western blotting and zymography of Th1 and Th2 whole-cell lysates demonstrated similar patterns. This study shows that two functions of one molecule can be controlled differentially. (J Histochem Cytochem 50:1169-1177, 2002)

CD26/DPP IV is a 110-kD cell surface glycoprotein that is mainly expressed on mature thymocytes, activated T-cells, B-cells, NK-cells, macrophages, and epithelial cells of the small intestine, kidney, and liver. It has at least two functions, a signal transduction function and a proteolytic function (Morimoto and Schlossman 1998). CD26 interactions with molecules such as adenosine deaminase (ADA) (Kameoka et al. 1993; Dong et al. 1996,1997) and CD45 (Koretzky et al. 1990; Torimoto et al. 1991) initiate signal transduction (Munoz et al. 1992) and modulate co-stimulation and proliferation of activated T-cells (Hegen et al. 1997). In addition, FcR-mediated crosslinking of CD26 by a CD26-specific antibody induces activation of human T-lymphocytes (Dang et al. 1990).

DPP IV proteolytic activity can modify proteins with the dipeptide sequences X-ala or X-pro at the N-terminal position. Examples of these proteins include cytokines, such as interferon-\( \gamma \) (IFN-\( \gamma \)) and interleukin-2 (IL-2), growth factors, and chemokines, such as granulocyte chemotactic protein-2 (GCP-2) and the C-C chemokine RANTES (Proost et al. 1998,1999; Schols et al. 1998; Iwata et al. 1999; Van Damme et al. 1999). Processing by DPP IV activity either leads to activation of the proforms or is a first step in proteolytic degradation. It has recently been demonstrated that glucagon also is cleaved by DPP IV activity, thereby lowering its affinity for the glucagon receptor (Hinke et al. 2000). Other natural substrates of DPP IV are the hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). The intact forms enhance glucose-stimulated insulin secretion. DPP IV activity can abolish...
this effect in vitro (Marguet et al. 2000). It has been demonstrated that the chemokine eotaxin is an important mediator in allergic reactions because it attracts eosinophils, Th2-lymphocytes, and basophils. Human eotaxin has a pentulismate proline residue and is effectively cleaved by CD26/DPPIV. NH₂-terminal truncation reduces the chemotactic activity of eotaxin as it does for factor-1α (Proost et al. 1998) and the monoocyte chemotactic proteins 1 and 2 (Proost et al. 1999). This physiological processing may be an important downregulatory mechanism, limiting the eotaxin-mediated inflammatory response (Struyf et al. 1999).

CD26/DPPIV is involved in the pathology of a variety of diseases, such as AIDS (Blazquez et al. 1992; Vanham et al. 1993; Schols et al. 1998), Graves' disease (Eguchi et al. 1989; Nishikawa et al. 1995), and cancer (Steece et al. 1997).

Protective immunity against different types of pathogens requires polarization of the immune response (Kapsenberg et al. 1991; Romagnani 1994). Th1 cells produce high levels of IFN-γ, which is instrumental in the cellular immune response to intracellular pathogens, whereas Th2 cells produce interleukin 4 (IL-4), which is needed for the humoral immune response to extracellular pathogens (O'Garra 1998). However, chronic activation of Th1 or Th2 effector cells may lead to autoimmune disorders or allergy, respectively (Kapsenberg et al. 1991; Romagnani 1994). Human Th1 cell lines are known to express higher levels of CD26 protein than Th2 cell lines (Willheim et al. 1997). Thus far, it is unknown how CD26 protein expression and DPPIV activity are regulated after T-cell activation and whether both functions of the molecule contribute to differences in Th1 and Th2 cell function. To investigate the coordinate regulation of the two functions of this molecule, we applied a model system of human T-cells that lack CD26/DPPIV expression.

Materials and Methods

Generation of Polarized Human Th1 and Th2 Cell Lines

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB); Amsterdam, The Netherlands) by density gradient centrifugation on Lymphoprep (Nycomed; Torshov, Norway). From this material, CD4⁺ CD45RA⁺ naive Th cells were isolated to high purity through one-step high-affinity negative selection columns (R&D Systems; Abingdon, UK) according to the manufacturer's instructions. These purified naive Th cells (normally >98%) were stimulated as described (Hilkens et al. 1997) in 96-well culture plates (Costar, Cambridge, MA; 10⁴ cells/well) with immobilized CD3 MAb (CLB-T3/3; 1 μg/ml) and soluble CD28 MAb (CLB-28.1; 2 μg/ml), both obtained from CLB. Cells were cultured for 10 days in the presence of rIL-2 (10 U/ml; Chiron, Emeryville, CA) and either rIL-4 (1000 U/ml; Pharmacia Biotechnology, Hannover, Germany) for Th2-polarizing conditions, or rIL-12 (100 U/ml; gift from Dr. Gately, Hofman-La Roche, Nutley, NJ) for Th1-polarizing conditions. The resulting Th1 and Th2 cell lines were tested for IL-4 and IFN-γ production by intracellular staining and fluorescence microscopy to confirm polarization. Aliquots of polarized cells were frozen and stored at —80°C.

For each experiment, Th1 and Th2 cell lines were thawed and restimulated with phytohemagglutinin (10 μg/ml; Difco, Detroit, MI) as described previously (Wierenga et al. 1990) in the presence of rIL-2 (10 U/ml) and 3000-rad irradiated feeder cells (PBMCs from two different donors) and in the additional presence of either rIL-12 (100 U/ml) + neutralizing anti-IL-4 (gift from Dr. C.T.M. van de Pouw Kraan; CLB) to maintain Th1 polarizing conditions, or rIL-4 (1000 U/ml) + neutralizing anti-IL-12 (gift from Dr. P.H. van der Meide; U-cytech, Utrecht, The Netherlands) to maintain Th2 polarization conditions. All cell cultures were performed in Isco's modified Dulbecco's medium (IMDM; Bio-Whitaker, Walkersville, MD) supplemented with 5% pooled C-inactivated normal human serum (Bio-Whittaker) and gentamicin (80 μg/ml; Duchefa, Haarlem, The Netherlands) and were incubated at 37°C in a humidified atmosphere containing 5% CO₂. For CD26/DPPIV analyses on living cells, activated Th cells were harvested at day 3 by density centrifugation (20 min at 800 × g) on Lymphoprep to separate them from dead feeder cells. Living T-cells were collected from the interface, washed, and diluted to 2 × 10⁶ cells/ml for all experiments. As a negative control, Jurkat cells (clone E6-1; American Type Culture Collection, Manassas, VA) were used, which lack CD26/DPPIV expression.

Polarization Studies

Changes in CD26/DPPIV expression during differentiation of T-cells were established during restimulation of Th1 and Th2 cells under different conditions. On day 3 of restimulation, CD26 expression and DPPIV activity were measured when differences in CD26/DPPIV expression between Th1 and Th2 cells are most profound (Kähne et al. 1996; Willheim et al. 1997).

Detection of Intracellular Cytokines by Flow Cytometry

Cytokine production was determined by intracellular staining using the method described by Jung et al. (1998). Briefly, Th1 and Th2 cells were cultured for 10 days as described
Regulation of CD26 Expression and DPPIV Activity

above. After removal of dead feeder cells, Th cells were restimulated for 6 hr in 24-well culture plates (Costar; 10² cells/well) with phorbol 12-myristate 13-acetate (10 ng/ml; Sigma, St Louis, MO) and ionomycin (1 µg/ml; Sigma) and the last 5 hr in the presence of brefeldin A (10 µg/ml; Sigma). Cells were then washed in PBS. The supernatant was discarded and the pelleted cells resuspended, fixed in 4% cold paraformaldehyde (Merck; Darmstadt, Germany), and gently vortexed. Fixation was performed at RT for 15 min. The cells were washed for a second time in PBS, followed by a wash step in PBA [PBS + 0.05% (w/v) BSA + 0.05% (w/v) sodium azide; Roche, Mannheim, Germany]. The fixed cells were stained by addition of 50 µl of antibody solution in saponin buffer (PBA + 0.5% saponin; Sigma) to 10⁷ cells and incubation for 30 min at 4C. Anti-human IL-4-phycocerythrin (PE) and anti-human IFN-γ-FITC, both from Becton-Dickinson (Mountain View, CA), were used according to the manufacturer’s recommendations. The cells were washed in saponin buffer, followed by a second wash in PBA. The supernatant was discarded, and the pelleted cells were resuspended in 0.2 ml PBA and stored at 4C until analyzed. Flow cytometric analysis of IL-4 and IFN-γ production in living T-helper cells was performed on a FAC-star plus (Becton-Dickinson) using the software program CellQuest, version 3.2. Analyses were performed at a rate of 200 cells/sec. PE fluorescence was determined as a measure of IL-4 production (excitation at 543 nm and emission at 575 nm). FITC fluorescence was determined as a measure of IFN-γ production (excitation at 488 nm and emission at 530 nm). Cells were gated only on the basis of forward and side scatter.

Fluorescence Microscopy

A mixture of Th1, Th2, and Th0 cells was stained for intracellular IL-4 and IFN-γ production. Cells were then stained for CD26 expression as described below in detail. Triple-stained cells were mounted on an object glass coated with poly-L-lysine to immobilize the cells. Fluorescence microscopy was performed on a Leica DMRA HC “upright” microscope (Leica; Wetzlar, Germany), using a KX series imaging system (Apostec Instruments; Logan, UT) and imaging software Image Pro Plus (Media Cybernetics; Silver Spring, MD).

T-cell Proliferation Assay

ELISA plates (Costar) were coated for 2 hr at 37C with a goat anti-mouse IgG antibody (Zymed, San Francisco, CA; 1:1000). Then the protein binding sites were saturated for 30 min with IMDM containing 10% FCS. After blocking, the plates were washed with IMDM + 5% human serum. The plates were then incubated for 60 min at 37C with 1:3 serial dilution of anti-human CD3 antibody (CLB-T3/3; CLB) starting at 1:4000 (0.75 µg/ml), in the presence or absence of anti-human CD26 (Ta1; CLB) starting at a dilution of 1:200 (1 µg/ml). Finally, the plates were washed with IMDM + 5% human serum. Polarized Th1 and Th2 cells were seeded onto the coated plates (2 X 10⁵ cells/well) in a final volume of 200 µl. After 24 hr, 20 µl of [³H]-TdR (0.3 µCi; Amersham, Poole, UK) was added to each well for a 16-hr pulse, after which incorporation of radioactivity was determined in a scintillation spectrometer (Biorad; Hercules, CA) as a measure of proliferation.

Analysis of CD26 Expression and DPPIV Activity

Living T-cells were harvested at different time points after stimulation and analyzed for their CD26 expression and DPPIV activity by flow cytometry and fluorospectrometry, respectively. For CD26 detection, cells were incubated for 30 min at 4C with FITC-conjugated anti-human CD26 MAb Ta1 (1:60 diluted stock solution of 0.2 mg/ml) and washed twice in cold PBS. In some cases, CD26 was detected using a two-step incubation with unconjugated Ta1 mAb used in the first step and a Cy5-conjugated goat anti-mouse IgG (Amersham) in the second step (dilution 1:200). Cells were kept on ice before mixing with the enzyme incubation medium. Incubations were started at t=0 by suspending T-cells in PBS containing 20 µM of the DPPIV substrate ala-pro-cresyl violet, which becomes fluorescent after proteolysis (Enzyme Systems Products and Protokit; Livermore, CA; Van Noorden et al. 1997). Incubations were carried out at 37C. Fluorometric analysis was performed on an LS 50 fluorescence spectrometer (Perkin-Elmer; Gouda, The Netherlands) under continuous magnetic stirring. Cuvettes with a light path of 1 cm were used. Excitation was performed at 591 nm with a slit width of 15 nm and emission was detected at 628 nm with a slit width of 15 nm (Boonacker and Van Noorden 2001). The increase in fluorescence was measured over 4 min. Fluorescence values were plotted against substrate concentrations. A hyperbolic curve was fitted to the data with the use of a curve-fitting program (Mac Curve Fit; Apple, Cupertino, CA) and V_max and K_m values were calculated for living Th1 and Th2 cells. Statistics were performed where appropriate, using the one-tailed Student’s t-test for paired observations (Graph Pad Instat version 3.00 for Windows 95; Graph Pad Software, San Diego, CA; www.graphpad.com). Differences were considered significant when p < 0.05.

Flow cytometric analysis of CD26 expression on living T-helper cells was performed on an FAC-star plus (Becton-Dickinson) using the software program CellQuest version 3.2. Analyses were performed at a rate of 200 cells/sec. FITC fluorescence was determined as a measure of CD26 expression (excitation at 488 nm and emission at 530 nm).

Confocal Microscopy

Th1 cells were stained for CD26 expression as described above. Cells were kept on ice to prevent internalization of CD26. Cells were kept in cold PBS using glass-bottomed poly-L-lysine-coated microwell dishes (MatTek, Ashland, MA) during recording. Cells were analyzed with a CLSM SP2 fitted to a Fluovert inverted microscope DM IRB (Leica). Excitation was performed at a wavelength of 488 nm and fluorescence was captured with a bandpass filter (530 ± 15 nm). A confocal data stack of 30 optical sections was processed and a maximal intensity projection over an angle of 6° was calculated with Leica software.

Western Blotting and Zymography of DPPIV Activity

Cells were cultured and washed as described above. At day 3, equal numbers of washed cells were lysed by freezing in liquid nitrogen and membrane fractions were pelleted, resuspended in 25 mM Tris-HCl (pH 7.4) containing 50 mM NaCl and 1% Triton-X100, and kept for 1 hr at 0C. Both Th1 and Th2 samples were ultrasonically shaken three times...
for 15 sec and centrifuged a second time for 10 min at 4C. The pellet was discarded and the membrane proteins in the supernatants were mixed with (5 ×) sample buffer free of β-mercaptoethanol and heated to 37°C for 5 min. Equal amounts of protein were transferred onto 7.5% SDS gels. After electrophoresis, the gels were washed twice with 2.5% Triton-X100 (v/v) at RT for 30 min to remove SDS. Gels were then rinsed three times with PBS and incubated at 37°C in PBS containing 20 μM ala-pro-cresyl violet for up to 12 hr. Zymograms were digitally recorded by analyzing fluorescence directly in the gels using a Storm 860 scanner (Molecular Dynamics; Sunnyvale, CA). To correlate DPPIV activity and CD26 protein expression, samples were also subjected to Western blotting. To this aim, gels were transferred to nitrocellulose filters overnight at 30 mA. The blots were washed in PBS and blocked for 1 hr in 5% Protifar (Nutricia; Zoetermeer, The Netherlands) in PBS containing 0.05% Tween-20. Blots were stained for 1 hr with anti-CD26 antibody Ta1 (1:200 in blocking buffer) to label CD26 protein and washed twice for 15 min in 5% Protifar in PBS containing 0.05% Tween-20. As a secondary antibody, we used monoclonal horseradish peroxidase-conjugated goat anti-mouse IgG in a dilution of 1:2000 (Nordic; Tilburg, The Netherlands) using Lumi-Light Western blotting substrate (Boehringer; Mannheim, Germany). Chemiluminescence was analyzed in the Lumi-Imager (Boehringer).

Electron Microscopy

For electron microscopy, living T-cells were incubated to demonstrate DPPIV activity after washing in phosphate buffer (100 mM, pH 7.4). The incubation lasted for 30 min at 37°C in 100 mM cadocylate buffer, pH 7.4, containing 6% polyvinyl alcohol and 2 mg ala-pro-methoxynaphthalamine (MNA; Enzyme Systems Products) as substrate, which was first dissolved in 20 μl dimethylformamide and as coupling reagent 60 μl/ml hexazoitized pararosanilin as described by Van Noorden and Frederiks (1992). After incubation, cells were washed in phosphate buffer (100 mM, pH 7.4) and fixed in McDowell 1% (w/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 100 mM cacodylate buffer, pH 7.4; fixation lasted for 60 min at 4C. Fixation was followed by centrifugation at 1000 × g at 4C. Cells were rinsed in 100 mM cacodylate buffer, pH 7.4, for 30 min, postfixed in 1% OsO₄ (Drijfhout; Amsterdam, The Netherlands) in 100 mM cacodylate buffer, pH 7.4, for 60 min at 4C, dehydrated, and embedded in epoxy resin Lx-112 (Ladd; Burlington, VT) according to standard procedures. Semithin sections (1-2 μm thick) were cut on a Pyramitome (LK; Uppsala, Sweden) parallel to the surface of the embedded cells and stained with methylene blue to check the quality of preservation of the morphology of cells. Ultrathin sections (50–70 nm thick) were cut on a Ultrotome III ultramicrotome (LK) and studied with an EM-10C transmission electron microscope (Zeiss; Oberkochen, Germany).

Results

Cytokine production by Th1 and Th2 cell lines was analyzed by intracellular labeling and flow cytometry to test for polarization. Double labeling of the hallmark Th1 and Th2 cytokines IFN-γ and IL-4, respectively, confirmed almost complete polarization on neutral stimulation without exogenous IL-4 or IL-12 restimulation on day 11 as determined by FACS analysis (Figure 1) and fluorescence microscopy (Figure 2). Intact living T-helper cells expressed CD26/DPPIV on their plasma membrane, as shown in Figure 3. When intact living Th cells were incubated to localize DPPIV activity, final reaction product was present on the plasma membrane only (Figure 4). Flow cytometric analysis of IL-4- or IFN-γ-producing cells for expression of CD26/DPPIV showed that Th1 cells expressed sixfold more CD26/DPPIV at day 3 after restimulation than Th2 cells (Figure 5). At that stage, the difference in CD26/DPPIV expression between Th1 and Th2 cells is most pronounced (Kahne et al. 1996; Willheim et al. 1997).

When the reactions were analyzed fluorometrically in suspensions of living Th1 and Th2 cells, a linear increase in cresyl violet fluorescence was observed for up to 4 min (Figure 6A). Jurkat cells, which lack CD26/DPPIV expression, did not show any fluorescence formation due to DPPIV activity. Variation of the substrate concentration revealed Michaelis-Menten kinetics of DPPIV activity in both living Th1 and living Th2 cells (Figure 6B). Both Kₘ and Vₘₐₓ values were approximately twofold higher on Th1 cells than on Th2 cells (Th1 Vₘₐₓ = 30.3 ± 10.8 FU/sec and Kₘ = 10.8 ± 1.7 μM; Th2 Vₘₐₓ = 13.3 ± 2.8
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Figure 2 Triple labeling of a mixture of Th0, Th1, and Th2 cells. Green fluorescence (FITC) represents intracellular IFN-γ, blue fluorescence (PE) represents intracellular IL-4, and red fluorescence (Cy5) represents extracellular CD26/DPPIV. High CD26/DPPIV expression (Th1, A) is associated with IFN-γ production, whereas low CD26/DPPIV expression (Th2, B) is associated with IL-4 production. Th0 cells show intermediate CD26/DPPIV expression (C). Bar = 15 μm.

Figure 3 Red and green stereo pair of two maximal intensity projections of a confocal stack at an angle of 6° of a living Th1 cell after staining with an FITC-conjugated anti-human CD26/DPPIV MAb, resulting in a 3-D representation of CD26/DPPIV expression on a living Th1 cell when observed with anaglyph glasses. Bar = 3 μm.

Figure 4 Electron micrograph of a Th1 cell that has been incubated with ala-pro-MNA and hexaazotized pararosanilin as coupling reagents to detect DPPIV activity. Final reaction product is present only at the plasma membrane (arrow). Bar = 3 μm.

FU/sec and $K_m = 6.3 \pm 2.1 \mu M; n=3; p<0.005$). This means that the cells with the lowest number of proteolytically active CD26 molecules had the highest affinity for the synthetic substrate. Despite the sixfold difference in CD26 expression, the capacity of Th1 and Th2 cells to cleave the synthetic substrate was hardly different, as shown on the basis of the calculation of virtual fluxes (Figure 7) using the formula

$$\phi = \frac{V_{\text{max}} \times [S]}{K_m + [S]}.$$

A series of differently restimulated Th1 and Th2 cell lines all showed the same phenomenon at day 3 of re-stimulation. CD26/DPPIV expression on Th1 cells was threefold higher than on Th2 cells, whereas DPPIV activity per CD26/DPPIV molecule was twofold higher on Th2 than on Th1 cells (Table 1).

To demonstrate specificity of the synthetic substrate for DPPIV activity, native samples of total cell lysates were made and separated by gel electrophoresis. Western blotting (Figure 8A) confirmed the sixfold difference in CD26/DPPIV expression levels between Th1 and Th2 cell lines, which is in agreement with the flow cytometric data (Figure 5). Similarly, zymography with the use of 20 μM ala-pro-cresol violet revealed a major band of active protein in both samples (Figure 8B), which corresponded with the molecular weight of the native CD26/DPPIV protein (140 kD), and a faint band of the dimerized protein. Th1 cells showed twofold higher activity against 20 μM substrate than Th2 cells, which is in agreement with the spectrofluorometric analysis shown in Figure 6B.

To test the signal transduction function of CD26/DPPIV, its co-stimulatory function in anti-CD3-induced proliferation was monitored by CD26 cross-linking and [3H]-TdR incorporation, applying a threefold serial dilution of anti-CD3 in the absence or presence of anti-CD26. Figure 9 shows a six- to ninefold higher sensitivity of Th1 cells than of Th2 cells for CD26 crosslinking, which is consistent with the sixfold difference in CD26 expression between Th1 and Th2 cells. This correlation between CD26 expression (Th1, A) is associated with IFN-γ production, whereas low CD26/DPPIV expression (Th2, B) is associated with IL-4 production. Th0 cells show intermediate CD26/DPPIV expression (C). Bar = 15 μm.
ated per sec (FU/sec). Both \( V \) and \( K_m \) values are approximately twofold higher on Th1 than on Th2 cells.

![Graph](image)

\[ V_{max} \] and [S] in \( \mu M \)

Figure 7 Virtual flux of DPPIV on living Th1 and Th2 cells. Flux, \( V_{max} \) ([S]/K_m), was calculated on the basis of the data in Figure 6. Differences in both \( V_{max} \) and K_m values of DPPIV on Th1 (•) and Th2 (○) cells resulted in a similar capacity to process the synthetic substrate ala-pro-cresyl violet at physiologial substrate concentrations.

Figure 8 Western blot of CD26 and zymogram of DPPIV activity on Th1 and Th2 cells. (A) CD26 localization by indirect immunolabeling on a Western blot using the Lumi-Light substrate. Native CD26 was located just above the arrow indicating 120 kD, which is in agreement with the molecular weight of 140 kD of the native CD26 protein. The amount of CD26 on Th1 cells was sixfold higher than on Th2 cells. (B) DPPIV activity as demonstrated by zymography using the fluorescent substrate ala-pro-cresyl violet (20 \( \mu M \)) in PBS, showing a similar banding pattern as CD26 protein in A; the activity in Th1 cells against 20 \( \mu M \) substrate was twofold higher.

**Discussion**

The signal transduction function and proteolytic activity of CD26/DPPIV were studied on activated living T-cells to investigate whether or not the regulation of enzymatic DPPIV activity depends on the regulation of CD26 protein expression. We studied simultaneously expression of the protein and activity of the proteolytic enzyme in the two different types of polarized Th cells that are involved in cellular (Th1) and humoral (Th2) immune responses and are known to express different levels of CD26 (Willheim et al. 1997). We found that the proteolytic activity of CD26/DPPIV is kept at a steady functional level despite a three- to six-fold difference in CD26 expression.

Relatively few enzymes have the ability to cleave proline-containing peptide bonds and might contribute to the proteolytic activity observed in the Th1 and Th2 cells. These include peptidases such as dipeptidyl

**Table 1 Effects of culture conditions on CD26/DPPIV expression and activity on Th1 and Th2 cell lines**

<table>
<thead>
<tr>
<th>Clone (stimulus)</th>
<th>Th1 (CD3/28)</th>
<th>Th2 (CD3/28)</th>
<th>Th1 (CD3/28, IL-12/anti-IL-4)</th>
<th>Th2 (CD3/28, IL-4)</th>
<th>Th1 (Feedermix, anti-IL-12)</th>
<th>Th2 (Feedermix, IL-4/anti-IL-12)</th>
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<th>Mean Th2</th>
<th>p value</th>
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<tr>
<td>( K_m )</td>
<td>10.7</td>
<td>8.9</td>
<td>8.5</td>
<td>6.8</td>
<td>7.1</td>
<td>3</td>
<td>6.8</td>
<td>5.6</td>
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<tr>
<td>( V_{max} )</td>
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<td>60</td>
<td>65</td>
<td>38</td>
<td>49</td>
<td>20</td>
<td>67</td>
<td>30</td>
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<tr>
<td>CD26 exp</td>
<td>381</td>
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<td>199</td>
<td>613</td>
<td>124</td>
<td>468</td>
<td>165</td>
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<td>Flux/CD26</td>
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<td>2.86</td>
<td>1.29</td>
<td>2.41</td>
<td>0.99</td>
<td>0.03</td>
<td>1.84</td>
<td>2.75</td>
<td>0.035</td>
</tr>
</tbody>
</table>

\( \times 10^{-2} \) \( \times 10^{-2} \)

*Under all conditions, polarization to the Th1 phenotype resulted in higher CD26/DPPIV expression but lower DPPIV activity per molecule than polarization to the Th2 phenotype, as demonstrated by the virtual flux per CD26 molecule at a substrate concentration of 1 \( \mu M \). Statistical analysis was performed by one-tailed Student's t-test for paired observations.*
Regulation of CD26 Expression and DPPIV Activity

![Graph showing proliferation assay using [H]-TdR incorporation. Crosslinking of CD3 only (● Th1, ○ Th2 cells) or of both CD3 and CD26 (□ Th1, ▲ Th2) in a serial dilution (each step is a threefold dilution) demonstrating the co-stimulatory effect of CD26 ligation on T-cell proliferation. Additional CD26 crosslinking to CD3 resulted in a six- to ninefold stronger proliferative response in Th1 cells than in Th2 cells.](image)

Our data indicate that differences in signal transduction activity of CD26 do not lead to differences in activation or inactivation of bioactive peptides. Apparently, DPPIV activity is kept constant, possibly by regulation at the post-translational level. Therefore, variation of kinetic properties of DPPIV is an adaptational mechanism that keeps DPPIV activity constant, whereas CD26 protein expression is regulated transcriptionally. Post-translational regulation of enzyme activity by variation of kinetic parameters as an adaptational mechanism has been described previously for other enzymes, such as glucose-6-phosphate dehydrogenase and glucose-6-phosphatase (for review see Van Noorden and Jonges 1995). How DPPIV activity is modulated post-translationally is not yet understood and subject to further study. One explanation may be variation in sialylation or glycosylation of the molecule. For example, it is known that HIV infection causes hypersialylation of CD26 (Smith et al. 1998). Sialic acids, by virtue of their negative charge, can bind non-covalently to exposed cationic sites of a peptide. This phenomenon not only may play a role in the binding of positively-charged substrates but also may influence the affinity of DPPIV for its substrates and thereby regulate enzymatic activity (Gutheil et al. 1994).

DPPIV is also necessary for T-cells to proliferate because its activity is involved in the transition from G1- to S-phase, as was demonstrated with Jurkat cells transfected with CD26 without DPPIV activity. CD26+/DPPIV– transfected Jurkat cells proliferated poorly unless soluble active CD26/DPPIV was added (Tanaka et al. 1994). Normally, DPPIV activity enhances T-cell responses to external stimuli via CD26 and the TCR/CD3 complex (Tanaka et al. 1993). Therefore, it appears that DPPIV activity also has a role in T-cell activation, although it was demonstrated that most of the extracellular part of CD26 can be deleted without affecting its co-stimulatory function, implying that the proximal glycosylation-rich region is sufficient to transduce co-stimulatory signals (Huhn et al. 2000).

In conclusion, our findings indicate that the signal transduction function, but not the proteolytic function of CD26/DPPIV, depends on the expression level of CD26, suggesting that different functions of one and the same molecule can be regulated differentially. This implies separate post-translational regulation of DPPIV activity on top of transcriptional and/or translational regulation of CD26 expression during T-helper cell activation. As far as we know, this is the first report on two functions of one and the same protein that are differentially expressed.

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Regulation of CD26 Expression and DPPIV Activity


CHAPTER 7

Rapid assay to detect natural protease substrates in living cells

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Rapid assay to detect natural protease substrates in living cells

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Key words: fluorogenic substrates - fluorescence - Magic Red - rhodamine 110 - β-casomorphin_{1,5} - CD26/DPPIV - metabolic mapping - live cells

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Summary

Proteolysis is a regulatory step in many physiological processes, but which proteases in what cellular sites are involved in activation or degradation of which peptides is not well known. We developed a rapid assay consisting of living cells and fluorogenic protease substrates to determine which bioactive peptides are natural substrates of a specific protease with the multifunctional or moonlighting protein, CD26/dipeptidyl peptidase IV (DPPIV) as model. CD26/DPPIV catalyzes cleavage of peptides from the amino terminus of peptides with proline at the penultimate position. Many biologically active peptides, such as β-casomorphin_{1,5}, contain proline in the penultimate position. We incubated Jurkat cells,
which are T cells that lack CD26/DPPIV, and CD26/DPPIV-transfected Jurkat cells in the presence of the fluorogenic substrate [Ala-Pro]³-cresyl violet (Magic Rec) and β-casomorphin. Fluorescent cresyl violet was generated by CD26/DPPIV-transfected Jurkat cells but not by wild-type Jurkat cells with a $K_m$ of 3.7 μM. β-Casomorphin appeared to be a natural substrate of CD26/DPPIV, because it inhibited production of fluorescence competitively ($K_i = 60 \mu M$). The assay using living cells and a fluorogenic protease substrate is an efficient system to determine whether specific peptides are natural substrates of a particular protease.

Introduction

Proteolytic activation or degradation of bioactive peptides is a regulatory step in many biological processes but it is not yet exactly known which peptides are cleaved by which proteases in intact complex cellular systems. For the understanding of the regulation of (local) levels of bioactive peptides in tissues or organs and the development of therapeutic strategies in relation to these peptides, it is essential to know which proteases are involved and in what location in a living organism (1).

The ectoprotease CD26/dipeptidyl peptidase IV (DPPIV) cleaves off terminal dipeptides at the amino terminus of a peptide with proline as the penultimate amino acid (2). Many biologically active peptides such as neuropeptides, hormones, cytokines and chemokines (3-5), contain this proline at the penultimate position as a regulatory element in proteolytic processing. Therefore, proline-specific proteases such as CD26/DPPIV are likely to play a role in modulating activity of these bioactive peptides, but which are actually cleaved by DPPIV in vivo is unknown (5).

Second, CD26/DPPIV has been considered for a long time to be the only membrane-bound protease specific for proline in the penultimate position at the amino terminus, but in recent years, a series of CD26/DPPIV activity and/or structure homologues (DASH) has been described that are either ectoenzymes at the cell membrane or intracellular proteases (Table 1; 2,5).
Third, complex formation of CD26/DPPIV with other proteins can modulate the accessibility of the active site and this may differ for the different isoforms of CD26/DPPIV (6,7). These aspects make it difficult to predict which natural substrates are processed by which isoforms of CD26/DPPIV in vivo (5) but demonstrate that the role of proteolysis in the regulation of the activity of bioactive peptides is complex. Understanding proteolysis-based regulation mechanisms in vivo is still in its infancy and is receiving only lately the attention that is needed in this era of functional proteomics (8). Therefore, we have investigated whether a relatively simple assay could be designed for rapid detection of natural substrates of a specific protease, with the example of CD26/DPPIV.

Recently, we have established that two fluorogenic synthetic substrates, [Ala-Pro]²-cresyl violet (Magic Red; 9) and Ala-Pro-rhodamine 110, can be used to determine CD26/DPPIV-like activity in living cells (10). It was shown that specificity of the cresyl violet-based substrate was high for DPPIV activity whereas the rhodamine 110-based substrate was also cleaved by other proteases, most likely DASH, as was predicted by Lorey et al. (11,12). These differences in specificity are likely due to the fact that the leaving group in a substrate can affect interactions between the active site of an enzyme and the substrate and show that not only the amino acid sequence determines the reactivity (7,13-16). The two fluorogenic substrates have been used in combination with living Jurkat cells, which are T cells that lack CD26/DPPIV, and CD26/DPPIV-transfected Jurkat cells (17,18) to investigate whether a potential natural substrate for CD26/DPPIV, β-casomorphin₁₅ (19,20) is a natural substrate of CD26/DPPIV. It was reasoned that when β-casomorphin₁₅ is cleaved specifically by CD26/DPPIV, β-casomorphin₁₅ reduces concentration-dependently the production of fluorescence when incubating CD26/DPPIV-transfected Jurkat cells but not of Jurkat cells in the presence of a fluorogenic substrate. As this appeared to be the case for [Ala-Pro]²-cresyl violet whereas Ala-Pro-rhodamine 110 was cleaved by both transfected and non-transfected Jurkat cells, it is concluded that β-casomorphin₁₅ is a natural substrate for CD26/DPPIV but also for (a number of) DASH proteases. The principle described here can be applied to any protease and any natural substrate to elucidate which substrate is preferentially cleaved by which protease.
Material and Methods

Jurkat cell lines

Jurkat cells (clone E6-1; American Type Culture Collection, Manassas, VA, USA), which lack CD26/DPPIV expression were used as well as Jurkat cells transfected with CD26/DPPIV (17,18). This model system enables the determination of specificity of substrate cleavage by DPPIV activity. Clone E6-1 was cultured in Iscove’s modified Dulbecco’s medium (IMDM; Bio-Whittaker, Walkerville, MD, USA), supplemented with 10% fetal calf serum, whereas the CD26/DPPIV transfectants were grown in Dulbecco’s modified medium supplemented with 10% fetal calf serum, containing glutamine (1 mM) and Geneticin G418 (1 mg/ml; Invitrogen, Carlsbad, CA, USA) to maintain the selection for the CD26/DPPIV construct (17,18,21).

Analysis of DPPIV activity

Living Jurkat cells were harvested and DPPIV activity was determined by means of fluorospectrometry. Before DPPIV activity measurements, cells were washed twice in cold phosphate-buffered saline (PBS). Intact cells were kept on ice prior to mixing with the incubation media. In some cases, cells were permeabilized by ultrasonic treatment, 3 times (5 sec each). Incubations were started at t=0 by suspending Jurkat cells or CD26/DPPIV-transfected Jurkat cells in PBS at 37°C containing 0-40 μM [Ala-Pro]²-cresyl violet (Magic Red; Enzyme Systems Products and Prototek, Livermore, CA, USA; 22) or Ala-Pro-rhodamine 110 (Molecular Probes, Eugene, OR, USA; 23,24). The first substrate is fluorescent by itself but fluorescence is shifted to a longer wavelength when the amino acids are cleaved off from the leaving group, cresyl violet is cleaved off (10), whereas the second substrate does not fluoresce but when the amino acids are removed from the leaving group, rhodamine 110 becomes fluorescent (23,24). Incubations were carried out at 37°C. For each measurement, 4x10⁶ cells, or an equivalent of cell lysate, were incubated in a volume of 1200 μl containing 0-40 μM [Ala-Pro]²-cresyl violet or 0-20 μM Ala-Pro-rhodamine 110 and 0-200 μM β-casomorphin₁₋₅ under continuous stirring. For fluorometric analysis, an LS 50 fluorescence spectrometer (Perkin-Elmer, Gouda, The Netherlands) and cuvettes with a light
path of 1 cm were used. Excitation for cresyl violet was performed at 591 nm with a slit width of 10 nm and emission was detected at 628 nm with a slit width of 10 nm (16). Rhodamine 110 was excited at 485 nm and emission was detected at >535 nm (16). In order to correct for spontaneous breakdown of the synthetic substrates, reaction rates were determined as described in Boonacker et al. (10). Furthermore, increase in fluorescence was measured during 4 min in 96-well plates on a Victor3 1420 multilabel counter (Wallac, Turku, Finland) using Elisa plates. First, wells were filled with a droplet of a synthetic substrate solution and a droplet of a solution of β-casomorphin_{1-5} (Sigma, St. Louis, MO, USA) in opposite corners of the wells, in order to avoid mixing of the substrates before incubation. PBS was added to the wells, and the 96-well plate was placed on a pre-heated stage of the photospectrometer at 37°C. The final volume in the wells was 200 μl and contained 0-20 μM Ala-Pro-rhodamine 110 and 0-200 μM β-casomorphin_{1-5}. Then, 2/3 \times 10^5 cells were added to obtain the same concentration of cells as was used in incubations in cuvettes. Measurements were made each 5 sec. Before and between measurements, the plate was shaken during 1 sec. All reactions were performed in duplicate. In order to reduce the time needed for measurements, plates were only filled with 40 or 50 samples maximally. Linear regression was used to analyze the slope of the increase in fluorescence over time. The increase in fluorescence per unit incubation time was plotted against substrate concentrations. A hyperbolic curve was fitted to the data with the use of a curve fitting program (Mac Curve Fit; Apple, Cupertino, CA, USA) and \( V_{\text{max}} \) and \( K_m \) values were calculated for living Jurkat cells and CD26/DPP IV-transfectant Jurkat cells, both intact and permeabilized (Table 1). Statistics were performed, where appropriate, using one-way ANOVA with a Dunnett’s post test (Graph Pad Instat version 3.00 for Windows 95; Graph Pad, San Diego, CA, USA). Differences were considered significant when \( p<0.05 \).
Results

Fig. 1. Measurements of fluorescence in arbitrary units (AU) in time (sec) generated by cleavage of [Ala-Pro]_2-cresyl violet (A) and Ala-Pro-rhodamine 110 (B) by intact living or permeabilized Jurkat cells and CD26/DPPIV-transfected Jurkat cells.

- - - - , Intact Jurkat cells;
- - - - - , permeabilized Jurkat cells;
- - - - , intact CD26/DPPIV-transfected Jurkat cells;
- - - - - , permeabilized CD26/DPPIV-transfected Jurkat cells;
- - - - - , nonspecific substrate decomposition in the absence of cells.

Detection of DPPIV-like activity in living cells showed differences in reaction rates depending on the fluorogenic substrate used (Fig. 1). First, cleavage rates of [Ala-Pro]_2-cresyl violet were similar in intact and permeabilized cells (Fig. 1A) whereas Ala-Pro-rhodamine 110 was cleaved at a higher rate by permeabilized cells than by intact cells (Fig. 1B). Second, Jurkat cells that lack CD26/DPPIV cleaved Ala-Pro-rhodamine 110 (Fig. 1B) but not [Ala-Pro]_2-cresyl violet (Fig. 1A). These findings indicate that [Ala-Pro]_2-cresyl violet is cleaved specifically by CD26/DPPIV and Ala-Pro-rhodamine 110 is cleaved by both CD26/DPPIV and (intracellular) DASH proteases (Table 1).
Table 1. CD26/DPPIV structure and/or activity homologues (DASH).

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<tr>
<th>Intracellular DASH</th>
<th>Membrane-bound DASH</th>
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<tr>
<td>DPPII (QPP)</td>
<td>Attractin (DPPT-L)</td>
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<tr>
<td>DPPIII</td>
<td>Dipeptidyl aminopeptidase-like protein</td>
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<td>DPPIV</td>
<td>DPPIV</td>
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<tr>
<td>DPP-7</td>
<td>DPPIVβ</td>
</tr>
<tr>
<td>DPPVIII</td>
<td>FAPα (separase)</td>
</tr>
<tr>
<td>PCP</td>
<td>Membrane dipeptidase</td>
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<td>Prolyl oligopeptidase</td>
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<td>Pro-X dipeptidase</td>
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<td>Xaa-Pro dipeptidase</td>
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<td>X-Pro dipeptidase</td>
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Table 2. $K_m$ values (μM) of [Ala-Pro]$^2$-cresyl violet ([A-P]$^2$-CV), and Ala-Pro-rhodamine 110 (A-P-R110) cleavage by intact and permeabilized Jurkat cells (CD26$^+$) and CD26/DPPIV-transfected Jurkat cells (CD26$^-$) and $K_i$ values (μM) of its inhibition by β-casomorphin$_1,3$ (β-cas). N.D., not determined. Please note that CD26$^+$-cells cleaved Ala-Pro-rhodamine 110 substrate but not [Ala-Pro]$^2$-cresyl violet and thus data for CD26$^+$-cells are not available in the case of [Ala-Pro]$^2$-cresyl violet.

<table>
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<tr>
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<th>Intact cells</th>
<th>Permeabilized cells</th>
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<tr>
<td></td>
<td>[A-P]$^2$-CV</td>
<td>A-P-R110</td>
</tr>
<tr>
<td></td>
<td>CD26$^+$</td>
<td>CD26$^-$</td>
</tr>
<tr>
<td>$K_m$</td>
<td>3.7</td>
<td>-</td>
</tr>
<tr>
<td>$K_i$ (β-cas)</td>
<td>60</td>
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When substrate concentrations were varied and intact or permeabilized living CD26/DPPIV-transfected Jurkat cells were used, similar patterns in fluorescence formation were found (Fig. 2).
Fig. 2. Relation between increase in fluorescence per unit time (V) and concentration of [Ala-Pro]$^2$-cresyl violet ([A-P]$^2$-CV; A,B) and Ala-Pro-rhodamine 110 (A-P-R110; C,D) fluorogenic synthetic substrates to detect DPPIV-like activity in intact and permeabilized living CD26/DPPIV-transfected Jurkat cells. Intact cells (●) show a similar reaction rate as permeabilized cells (■) when using [A-P]$^2$-CV (A,B), whereas reaction rates are higher in permeabilized cells than in intact cells when using Ala-Pro-R110 as substrate (C,D). A and B as well as C and D show data obtained in duplicate experiments.

At all concentrations of [Ala-Pro]$^2$-cresyl violet, the reaction rate was similar in intact and permeabilized cells (Fig. 2A,B), whereas rates were higher in permeabilized cells than in intact cells when using Ala-Pro-rhodamine 110 as fluorogenic substrate (Fig. 2C,D). Table 2 shows $K_m$ values of cleavage of both substrates by intact or permeabilized CD26/DPPIV-transfected Jurkat cells and Jurkat cells. The $K_m$ value of cleavage of [Ala-Pro]$^2$-cresyl violet is approx. twice lower than that of Ala-Pro-rhodamine 110, indicating a similar cleavage rate, because [Ala-Pro]$^2$-cresyl violet contains 2 Ala-Pro dipeptides per molecule and Ala-Pro-rhodamine 110 only one (10).
Fig. 3. Example of reaction rate data of DPPIV-like activity in CD26/DPPIV-transfected Jurkat cells as demonstrated in 32 wells of a 96-well plate using the fluorogenic substrate Ala-Pro-rhodamine 110 (Ala-Pro-R110) in the presence or absence of various concentrations (0-200 μM) of β-casomorphin₁₋₅ in living CD26/DPPIV-transfected Jurkat cells. On the X-axis, the concentration of Ala-Pro-R110 (μM) is given and on the Y-axis, the concentration of β-casomorphin₁₋₅ (μM). Incubations were performed in duplicate and fluorescence was measured in time during 240 sec at 37°C using 485 nm excitation light whereas emission was measured at 535 nm.

Fig. 3 shows an example of the detection of DPPIV-like activity by the use of Ala-Pro-rhodamine 110 substrate in the presence of various concentrations of β-casomorphin₁₋₅ (0-200 μM) in a microwell plate. β-Casomorphin₁₋₅ inhibited generation of fluorescence due to hydrolysis of fluorogenic Ala-Pro-rhodamine 110 in a dose-dependent manner. It indicates that both compounds are substrate for the same enzyme(s). Because a microwell plate reader is not available with excitation and emission characteristics to specifically detect cresyl violet, we were unable to perform similar experiments in microwell plates with [Ala-Pro]²-cresyl violet. Therefore, effects of β-casomorphin₁₋₅ on cleavage of [Ala-Pro]²-cresyl violet (Fig. 4) were compared with those on cleavage of Ala-Pro-rhodamine 110 (Fig. 5) in a spectrometer.
Fig. 4. Dixon plots of reaction rates of DPPIV activity in intact living CD26/DPPIV-transfected Jurkat cells in the presence of 2.5 μM (■), 5 μM (▲), 10 μM (▼), and 20 μM (●) [Ala-Pro]$_2$-cresyl violet synthetic substrate. On the X-axis, the concentration of β-casomorphin$_{1-5}$ (μM) is given and on the Y-axis, the inverse reaction rate (1/V). The plot shows that inhibition of β-casomorphin$_{1-5}$ of [Ala-Pro]$_2$-cresyl violet is competitive.

The Dixon plots show that β-casomorphin$_{1-5}$ inhibits cleavage of both synthetic substrates competitively. Table 2 shows $K_i$ values of the competitive inhibition of β-casomorphin$_{1-5}$ on cleavage of the fluorogenic substrates. $K_i$ values of inhibition by β-casomorphin$_{1-5}$ of [Ala-Pro]$_2$-cresyl violet cleavage were 2-fold lower than those of Ala-Pro-rhodamine 110 cleavage in a similar manner as the $K_m$ values. Therefore, it is concluded that the affinity for both fluorogenic substrates and the interference by β-casomorphin$_{1-5}$ are similar in living CD26/DPPIV-transfected Jurkat cells.
Fig. 5. Dixon plots based on reaction rates of DPPIV-like activity in intact (A,B) and permeabilized (C,D) living Jurkat cells in the presence of various concentrations (0-200 μM) of β-casomorphin_{1-5} and 2.5 μM (●), 5 μM (▲), 10 μM (▼), and 20 μM (■) Ala-Pro-rhodamine 110 synthetic substrate. CD26/DPP IV-transfected Jurkat cells (A,C) as well as Jurkat cells lacking CD26/DPP IV (B,D) were used to determine inhibition of fluorescence production by β-casomorphin_{1-5}. On the X-axis, the concentration of β-casomorphin_{1-5} (μM) is given and on the Y-axis, the inverse reaction rate (1/V). The inhibition of cleavage of Ala-Pro-rhodamine 110 is competitive in all cases.

Discussion

The present study shows that the question which bioactive peptides are cleaved by which proteases in vivo can be addressed with a living cell system that expresses a specific protease and a fluorogenic substrate that is cleaved selectively by that protease. It appeared that β-casomorphin_{1-5} is cleaved preferentially by CD26/DPP IV but can also be cleaved by other DASH proteases (Table 1). The combinational use of Jurkat cells and CD26/DPP IV-
transfected Jurkat cells allows direct comparison of cleavage of substrates by cells that lack DPPIV activity and the same cells that have been transfected with the gene that codes for that protease.

It is shown with our living cell system that [Ala-Pro]²-cresyl violet also known by its commercial name “Magic Red” (9) is specifically cleaved by CD26/DPP IV with a $K_m$ of 3.7 μM. β-Casomorphin₁,₅ inhibits cleavage of [Ala-Pro]²-cresyl violet competitively (Fig. 4) with a $K_i$ of 60 μM. These findings indicate that β-casomorphin₁,₅ can be cleaved by CD26/DPP IV. Parallel studies using the substrate Ala-Pro-rhodamine 110 showed that that substrate is less specific and other DASH proteases also cleave it (10). The $K_m$ value of Ala-Pro-rhodamine 110 cleavage in both Jurkat cells and CD26/DPP IV-transfected Jurkat cells is 2- to 3-fold higher than the $K_m$ value of cleavage of [Ala-Pro]²-cresyl violet indicating that the bonds between the dipeptide and the leaving group are cleaved at similar rates because the first substrate contains only one dipeptide per molecule, whereas the second one contains two. Unfortunately, specific detection of fluorescence of cresyl violet is not routine yet due to the rather unusual excitation and emission settings that have to be used (excitation, 591 nm; emission, 628 nm) and these settings are not available on all types of equipment, such as microplate readers, confocal laser scanning microscopes or flow cytometers. The only confocal microscope that is yet available with these excitation and emission settings is the recently-developed AOBS system of Leica (Mannheim, Germany; 10).

Therefore, our attempt to develop a rapid assay to determine whether peptides are natural substrates for specific proteases in vivo with the use of 96-well microplates could not be performed with [Ala-Pro]²-cresyl violet as fluorogenic substrate. The assays were performed with [Ala-Pro]-rhodamine 110 instead, because rhodamine 110 can be visualized using standard fluorescence settings (excitation 488 nm; emission > 535 nm). Fig. 3 shows the results of an experiment and it is clear that the assay is both [Ala-Pro-rhodamine 110]-dependent and [β-casomorphin₁,₅]-dependent.

We selected a living cell system for the determination whether a bioactive peptide (β-casomorphin₁,₅) is a natural substrate for a specific protease (CD26/DPP IV) because the activity of the protease is regulated at the posttranslational level and depends on the microenvironment of the enzyme (8-10,16,25-31). In the past, Lambeir et al. (32) provided
insight in the selectivity of CD26/DPPIV for specific chemokines by ranking the chemokines as substrates on the basis of $k_{cat}/k_m$ values. A huge difference was found between $k_{cat}/k_m$ values for the different chemokines (for example, $5 \times 10^{-6}$M$^{-1}$s$^{-1}$ for SDF-1α versus $0.04 \times 10^{-6}$M$^{-1}$s$^{-1}$ for RANTES). However, the experiments were performed in vitro with purified CD26/DPPIV and it is unknown whether their findings can be transposed directly to the in vivo situation which is manyfold more complex. For example, glucagon$_{1-29}$ is a 29-amino-acid-long peptide hormone that has a half-life of 5-6 min in the circulation and CD26/DPPIV is considered to be at least partly responsible for the activation of glucagon$_{1-29}$ (33). However, it is not known which tissues are responsible for clearance of glucagon from the circulation. One would assume that the kidneys play an important role because CD26/DPPIV is present on the apical surface of epithelial cells of proximal tubules (34) where it can degrade glucagon$_{1-29}$ that is small enough to be filtered in the glomeruli into the primary urine. However, it is not understood why glucagon$_{1-29}$ is not degraded in the circulation (35,36) because CD26/DPPIV is present on the surface of T cells and B cells and as freely-circulating soluble sCD26/DPPIV. This would implicate that different cell types express different isoforms of CD26/DPPIV with different substrate specificities and only certain isoforms of CD26/DPPIV are responsible for degradation of glucagon$_{1-29}$ in vivo (37,38). Therefore, the need for a living cell system to elucidate proteolytic processes in vivo is obvious.

In conclusion, a simple and fast assay has been developed to determine whether or not bioactive peptides are natural substrates for a specific protease in a model system of living cells and synthetic fluorogenic substrates with the example of β-casomorphin$_{1-5}$ and CD26/DPPIV and DASH proteases.

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References


CHAPTER 8

The multifunctional or moonlighting protein
CD26/DPPIV

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The multifunctional or moonlighting protein CD26/DPPIV

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CD26/DPPIV can be considered a moonlighting protein because it is a multifunctional protein that exerts its different functions depending on cell type and intra- or extracellular conditions in which it is expressed. In the present review, we summarize all its known functions in relation to physiological and pathophysiological conditions. The protein is a proteolytic enzyme, receptor, costimulatory protein, and is involved in adhesion and apoptosis. The CD26/DPPIV protein plays a major role in immune response. Abnormal expression is found in the case of autoimmune diseases, HIV-1-related diseases and cancer. Natural substrates for CD26/DPPIV are involved in immunomodulation, psycho/neuronal modulation and physiological processes in general. Therefore, targeting of CD26/DPPIV and especially its proteolytic activity has many therapeutic potentials. On the other hand, there are homologous proteins with overlapping proteolytic activity, which thus may prevent specific modulation of CD26/DPPIV. In conclusion, CD26/DPPIV is a protein present both in various cellular compartments and extracellularly where it exerts different functions and thus is a true moonlighting protein.

Introduction

Since its description in 1966 (Hopsu-Havu and Glenner, 1966), dipeptidyl peptidase IV (DPPIV; EC 3.4.14.5) has been considered to be a unique peptidase that cleaves dipeptides from peptides and proteins containing proline in the penultimate position. It can also cleave dipeptides with alanine in that position. However, proteolysis is only one of the multiple functions that this protein executes. DPPIV, also known as CD26, is involved as well in signal transduction and can bind to a variety of proteins. Due to its multifunctional character and its widespread expression, the exact functions of CD26/DPPIV in vivo have not yet been elucidated. Multifunctional proteins are also called moonlighting proteins (Jeffery, 1999; Ejiri, 2002), and functions of such proteins can vary on the basis of their intracellular or extracellular localization, cell type, oligomeric or polymeric state, and concentrations of ligand, cofactor or product. Numerous cell types express CD26/DPPIV, both intracellularly and extracellularly, thus making it look like an essential moonlighting protein in cell biology. However, Fischer rats that lack CD26/DPPIV have only minor physiological defects, are viable and show no difference in growth rate as compared with control rats (Tiruppathi et al., 1993). Therefore, the functions of CD26/DPPIV are probably not unique and other proteins must be able to perform similar tasks. In the present review, we discuss the multiple cellular processes in which CD26/DPPIV is thought to be involved. Special attention is given to its functioning in the complex microenvironment of the living cell which allows its moonlighting properties to be exerted. Moonlighting of proteins as well as posttranslational modifications of proteins provide an extra dimension to gene expression in the ultimate functioning of cells. Investigations focussed on moonlighting and posttranslational modifications of proteins are only in their infancy yet but are essential for the understanding how a cell functions. Many reviews on specific aspects of CD26/DPPIV have been published recently (Fleischer, 1995; De Meester et al., 1999; Kahne et al., 1999; Hildebrandt et al., 2000; Langner and Ansorge, 2000, 2002; Gorrell et al., 2001) and are referred to for more detailed information on specific aspects of CD26/DPPIV.

Moonlighting properties of CD26/DPPIV

The T cell activation antigen, CD26 or DPPIV (CD26/DPPIV), is a 110-kDa multifunctional moonlighting cell surface protein that is expressed by many cell types. In humans, it is expressed constitutively on epithelial cells of liver, intestine, kidney and in...
a soluble form as sCD26/DPP1V in the circulation. The expression of CD26/DPP1V on T and B cells is regulated (Fox et al., 1984; Srivastava and Bhargava, 1986; Fleischer, 1987; Ulmer et al., 1992; Ansorge et al., 1995; Buhling et al., 1995; Mecoin and Bauvois, 1997; Bauvois et al., 2000).

CD26/DPP1V has at least 5 functions. The protein functions as (1) serine protease, (2) receptor, (3) costimulatory protein, (4) adhesion molecule for collagen and fibronectin, and (5) is involved in apoptosis. Moonlighting of CD26/DPP1V provides cells with a tool that can be used for multiple purposes.

CD26/DPP1V can be considered a housekeeping protein. Hong et al. (1989) have studied expression of CD26/DPP1V mRNA in tissues by means of immunoprecipitation and Northern blot analysis. They detected only one type of CD26/DPP1V mRNA. However, Kahne et al. (1996) showed a broad molecular heterogeneity of the CD26/DPP1V protein by means of isoelectric focussing. These findings indicate that heterogeneity of the molecular forms of CD26/DPP1V is due to posttranslational modifications.

The human CD26/DPP1V gene is located on the long arm of chromosome 2 (2q24.3) and spans approximately 70 kb. It contains 26 exons, ranging in size from 45 bp to 1.4 kb (Abbott et al., 1994). The 5'-flanking domain contains neither a TATA box nor a CAAT box, commonly found in housekeeping genes, but a 300-bp sequence that is extremely rich in C and G which has potential binding sites for several transcription factors, such as NFκB, AP2 or Sp1 (Bohm et al., 1995). These binding sites are features of promoters of genes lacking tissue-specific expression and are also characteristic of a promoter of a housekeeping gene. However, the housekeeping nature of the protein is conflicting with tissue-specific transcription of the CD26/DPP1V gene (Bohm et al., 1995), because CD26/DPP1V is expressed constitutively in liver, kidney and intestine, whereas its expression is regulated in Tand B cells (Boonacker et al., 2002). Bauvois et al. (2000) described that CD26/DPP1V expression is activated by interferons and retinoic acid in chronic B lymphocytic leukemia cells via the signaling pathway involving Stat1α and the GAS response element (TTCnnmGAA) of the CD26/DPP1V promoter.

**Proteolytic function of CD26/DPP1V**

Membrane-bound proteases are found in a wide variety on many cell types. Their expression is usually finely regulated, depending on specific functions of the cell and their engagement in defined physiological pathways. Protein turnover, ontogeny, inflammation, tissue remodeling, cell migration and invasion are some of the many physiological and pathological events in which membrane-bound proteases play a crucial role. They act both as effectors and as regulatory molecules by activation of proforms or degradation of biomolecules by cleavage (Riemann et al., 2001).

CD26/DPP1V was originally believed to be the only membrane-bound protease specific for proline as the penultimate residue at the amino terminus of a peptide chain and to cleave off the terminal dipeptide (Sedo and Malik, 2001). The presence of proline residues gives unique structural features to peptide chains, because it is the only cyclic amino acid substantially affecting the susceptibility of proximal peptide bonds to cleavage (Yaron and Naider, 1993; Demuth and Heins, 1995).

Many biologically active peptides contain this unique evolutionary-conserved proline residue as a regulatory element in proteolytic processing. Therefore, proline-specific proteases play an important role in modulating activity of biologically active peptides such as neuropeptides, hormones, cytokines and chemokines (Yaron and Naider, 1993; Ansorge et al., 1995). Although the natural substrate(s) for CD26/DPP1V remains(s) to be identified, an almost endless list of possible substrates have been proposed, such as substance P, β-casomorphin, kentisn, somatoliberin, RANTES, IL-2, fibrin α-chain, stromal derived factor-1α (SDF-1α), coxatin, and glucagon (Tables I–III). Lambeir et al. (2001b) provided insight in the selectivity of CD26/DPP1V for specific chemokines, by ranking the chemokines as substrates on the basis of their k<sub>t/k</sub><sub>r</sub>, which determines the half-life of the substrate at a given enzyme concentration (Table IV). In general, short peptides such as morphoneptin are considered to be effective substrates for CD26/DPP1V. In contrast, none of the cytokines with higher molecular weights with proline in the penultimate position have been identified as a CD26/DPP1V substrate, whereas smaller fragments of these cytokines up to 24 amino acids long, containing an N-terminal sequence that is similar to that of IL-1β, IL-2 or murine IL-6, are cleaved. The rate of CD26/DPP1V-catalyzed proteolysis is inversely related with the chain length of the protein to be cleaved (Hoffmann et al., 1993; Lambeir et al., 2001b).

Substrate recognition by CD26/DPP1V is defined not only by the size of the substrate, but also by its amino acid sequence. The formalism of Scheckter and Berger (1968) provides a classification system for amino acids in a peptide on the basis of their position in that peptide in relation to the cleavage site: amino acids preceding the scissile bond in the direction of the N-terminus are called P<sub>1</sub>, P<sub>2</sub>, etc., whereas residues into the direction of the C-terminus following the scissile bond are called P<sub>1</sub>′, P<sub>2</sub>′, etc. Proteins containing proline in P<sub>1</sub> usually have large hydrophobic/aromatic side chains. Proteins containing alanine in the P<sub>1</sub>′ position have often aspartate, glutamate, or glutamine in the P<sub>1</sub>′ position. The P<sub>2</sub>′ position is then often occupied by a small polar amino acid (glycine, serine, or alanine). A variety of amino acids with small polar, long aliphatic and positively-charged side chains are often found in the P<sub>1</sub>′ position. A clear pattern has not yet been found for the P<sub>2</sub>′, P<sub>3</sub>′, P<sub>4</sub>′, etc. position of CD26/DPP1V substrates, except for a prevalence of threonine at P<sub>4</sub>′. It indicates that substrate recognition by CD26/DPP1V does not extend much further than the P<sub>4</sub>′ position.

The 3-dimensional structure of CD26/DPP1V has not been elucidated yet on the basis of crystal structures (Ansorge et al., 2000), but computing was used to resolve parts of its structure (Brandt, 1997; Gorrell et al., 2000). Recently, the 3-dimensional structure of the pig serine protease prolyl oligopeptidase has been elucidated and appeared to be homologous to CD26/DPP1V (Fulop et al., 2001). Serine proteases like CD26/DPP1V and prolyl oligopeptidase have the amino acid sequence serine-aspartate-histidine in their catalytic site. This is the reverse sequence found in classical serine proteases that are members of the chymotrypsin and subtilisin families (David et al., 1993; Hooper et al., 2001). CD26/DPP1V and its structural homologues prolyl oligopeptidase and proline imidopeptidase (Table V) contain a similar peptide sequence that forms the last 200 C-terminal residues in their extracellular domain with an α/β-hydrolase fold which contains the catalytic triad serine-aspartate-histidine. The histidine in the catalytic triad of serine
Tab. I. Possible substrates of CD26/DPPIV that are involved in physiological processes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1-Microglobulin</td>
<td>(Nausch et al., 1990)</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>(Nausch et al., 1990)</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>(Kato et al., 1978)</td>
</tr>
<tr>
<td>Chorionic gonadotropin</td>
<td>(Nausch et al., 1990)</td>
</tr>
<tr>
<td>Corticotropin-like intermediate lobe peptide</td>
<td>(Nausch et al., 1990)</td>
</tr>
<tr>
<td>Enterostatin</td>
<td>(Bouras et al., 1995)</td>
</tr>
<tr>
<td>Fibrinogen α-chain</td>
<td>(Heymann and Mentlein, 1984)</td>
</tr>
<tr>
<td>Gastric inhibitory polypeptide</td>
<td>(Mentlein et al., 1993)</td>
</tr>
<tr>
<td>Gastric releasing peptide</td>
<td>(Nausch et al., 1990)</td>
</tr>
<tr>
<td>Growth hormone-releasing factor (GHRF)</td>
<td>(Frohman et al., 1986, 1989; Bongers et al., 1992; Martin et al., 1993)</td>
</tr>
<tr>
<td>Glucagon-like peptide-1 (GLP-1)</td>
<td>(Mentlein et al., 1993; Kieffer et al., 1995; Pauly et al., 1996)</td>
</tr>
<tr>
<td>Glucagon-like peptide-2 (GLP-2)</td>
<td>(Drucker et al., 1997b)</td>
</tr>
<tr>
<td>Glucagon</td>
<td>(Pospisil et al., 2001)</td>
</tr>
<tr>
<td>Glucagon-like insulinotropic polypeptide</td>
<td>(Kieffer et al., 1995; Pauly et al., 1996)</td>
</tr>
<tr>
<td>Glucagon-like insulinotropic polypeptide</td>
<td>(Mentlein et al., 1993)</td>
</tr>
<tr>
<td>Insulin-like growth factor-1</td>
<td>(Mentlein, 1999)</td>
</tr>
<tr>
<td>Morphiceptin</td>
<td>(Truppathi et al., 1990, 1993)</td>
</tr>
<tr>
<td>Monocyte chemotactic protein-1</td>
<td>(Proost et al., 1998a, b, c)</td>
</tr>
<tr>
<td>Monocyte chemotactic protein-2</td>
<td>(Oravecz et al., 1997)</td>
</tr>
<tr>
<td>Monocyte chemotactic protein-3</td>
<td>(Proost et al., 1998a)</td>
</tr>
<tr>
<td>Procalcitonin</td>
<td>(Wrenger et al., 2000)</td>
</tr>
<tr>
<td>Pro-colipase</td>
<td>(Heymann and Mentlein, 1978; Nausch et al., 1990)</td>
</tr>
<tr>
<td>Prolactin</td>
<td>(Heymann and Mentlein, 1978; Kato et al., 1978)</td>
</tr>
<tr>
<td>Substance P</td>
<td>(Nausch et al., 1990)</td>
</tr>
<tr>
<td>Trypsinogen</td>
<td>(Shine et al., 1999)</td>
</tr>
<tr>
<td>Tyr-melanostatin</td>
<td>(Lambeir et al., 2001a)</td>
</tr>
<tr>
<td>Vasoactive intestinal peptide (VIP)</td>
<td></td>
</tr>
</tbody>
</table>

Tab. II. Possible substrates of CD26/DPPIV involved in inflammatory processes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eotaxin</td>
<td>(Struyf et al., 1999)</td>
</tr>
<tr>
<td>IP-10</td>
<td>(Oravecz et al., 1997)</td>
</tr>
<tr>
<td>I-TAC</td>
<td>(Lambeir et al., 2001b)</td>
</tr>
<tr>
<td>Kentsin</td>
<td>(Bueno et al., 1986)</td>
</tr>
<tr>
<td>LD78</td>
<td>(Lambeir et al., 2001b)</td>
</tr>
<tr>
<td>Lymphotixin</td>
<td>(Ansorge et al., 1991)</td>
</tr>
<tr>
<td>MDC67</td>
<td>(Lambeir et al., 2001b)</td>
</tr>
<tr>
<td>MDC69</td>
<td>(Lambeir et al., 2001b)</td>
</tr>
<tr>
<td>Mig</td>
<td>(Lambeir et al., 2001b)</td>
</tr>
<tr>
<td>Monocyte chemotactic protein-1</td>
<td>(Van Collie et al., 1998)</td>
</tr>
<tr>
<td>RANTES</td>
<td>(Oravecz et al., 1997; Iwata et al., 1999)</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>(Proost et al., 1998b)</td>
</tr>
<tr>
<td>SDF-1β</td>
<td>(Shioda et al., 1996)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>(Bauvois et al., 1992)</td>
</tr>
</tbody>
</table>

proteases plays at least two roles in the hydrolysis of peptide bonds. First, it acts as proton acceptor in the formation of an acyl-enzyme intermediate and second, it acts as proton donor in the subsequent deacylation step (Kraut, 1977).

The central tunnel of an unusual seven-blade β-propeller domain covers the catalytic cleft formed by the α/β-hydrolase fold. Both the hydroxide and propeller domain of these proteases are part of the surface of the inner cavity in which catalysis occurs (Abbott et al., 1999). The propeller domain is the reason why these proteases are in fact oligopeptidases because they exclude large peptide sequences from occupying the catalytic pocket. In this fashion, the propeller can protect larger peptides and proteins from proteolysis. The entrance to the catalytic pocket lies in the center of the lower face of the β propeller as a pore-closing domain. It has been suggested that the lining of the opening of this narrow pore allows for specific substrate entry due to charged flexible side chains and that this may be a substrate-mediated event (Fulop et al., 1998). These findings indicate that the catalytic triad of CD26/DPPIV is not so specific at all for proline and for alanine and adjacent amino acids, but rather the molecular environment of the active site in the enzyme molecule defines for a large part substrate specificity.

Substrate-assisted catalysis (SAC) is one of the mechanisms by which substrate specificity is acquired. SAC has been demonstrated for serine proteases as well. When, for example, the serine amino acid in the catalytic triad is replaced by site-
directed mutagenesis, a functional group in the substrate can substitute for the missing catalytic residue. Similarly, replacement of the catalytic histidine by alanine in a substilisin protease reduced the catalytic efficiency by a million-fold. When substrates contain a histidine, they are hydrolyzed much faster than homologous substrates containing alanine or glutamine in the same position. In contrast, the wild-type protease reduced the catalytic efficiency by a million-fold.

When substrates contain a histidine, they are hydrolyzed much faster than homologous substrates containing alanine or glutamine in the same position. In contrast, the wild-type protease reduced the catalytic efficiency by a million-fold.

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When substrates contain a histidine, they are hydrolyzed much faster than homologous substrates containing alanine or glutamine in the same position. In contrast, the wild-type protease reduced the catalytic efficiency by a million-fold.
be determined, such as liver, spleen or kidney. However, the origin of sCD26/DPPIV is still a matter of debate. Shedding or secretion of CD26/DPPIV from different cell types can be responsible for the existence of soluble isoforms of CD26/DPPIV. A correlation between levels of sCD26/DPPIV and specific physiological or pathophysiological processes is shown in Table VI. This observation makes the issue of in vivo processing of molecules such as glucagon by the various isoforms of CD26/DPPIV even more complex.

It has been suggested that CD26/DPPIV is also able to degrade collagen, thereby facilitating cells trafficking through the extracellular matrix. CD26/DPPIV has been reported to be one of the mediators of lymphocyte migration in the thymus from the cortical region to the medulla during maturation (Savinio et al., 1993). Furthermore, gelatin zymography demonstrated gelatinase activity of immunopurified CD26/DPPIV. Gelatinase activity is only possible when the enzyme has endopeptidase activity in addition to its exopeptidase activity. It has been demonstrated in these cells that the receptor for plasminogen type 2 (Pg 2) is CD26/DPPIV. Pg 2 has six glycoforms that differ in their sialic acid content. Only the highly sialylated Pg 2γ, Pg 2β and Pg 2α glycoforms bind to CD26/DPPIV (Gonzalez-Gronow et al., 2001). In human rheumatoid synovial fibroblasts, Pg binding to the Pg 2 receptor and activation of Pg by urokinase-type plasminogen activator induce a significant rise in the cytosolic free Ca²⁺ concentration (Gonzalez-Gronow et al., 1993) via interactions of Pg with CD26/DPPIV associated with the integrin α(IIb)β3 on the cell surface (Gonzalez-Gronow et al., 1994, 1998). Lactose is a sugar which interferes with the binding of Neu5Ac(2→3) or (2→6) residues to sialic acid-binding proteins and inhibits the intracellular Ca²⁺ flux by blocking binding of Pg 2γ, Pg 2β and Pg 2α glycoforms to CD26/DPPIV on the surface of the fibroblasts (Gonzalez-Gronow et al., 1998), suggesting that binding occurs via carbohydrate chains. A similar inhibition of the intracellular Ca²⁺ flux was

### Table VI. Levels of soluble CD26/DPPIV in plasma in relation with physiological or pathophysiological processes

<table>
<thead>
<tr>
<th>Condition</th>
<th>Serum DPPIV</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstinence in alcoholics</td>
<td></td>
<td>(Maes et al., 1999)</td>
</tr>
<tr>
<td>Ageing</td>
<td></td>
<td>(Durinx et al., 2001)</td>
</tr>
<tr>
<td>Allergic rejection, kidney transplantation</td>
<td></td>
<td>(Korom et al., 1997)</td>
</tr>
<tr>
<td>Anorexia nervosa, bulimia nervosa</td>
<td></td>
<td>(Hildebrandt et al., 1999)</td>
</tr>
<tr>
<td>Atopic dermatitis</td>
<td></td>
<td>(Katoh et al., 2000)</td>
</tr>
<tr>
<td>Autoimmune disease</td>
<td></td>
<td>(Kubota et al., 1994)</td>
</tr>
<tr>
<td>Biliary atresia</td>
<td></td>
<td>(Perner et al., 1999)</td>
</tr>
<tr>
<td>Cancer of bile duct or pancreas</td>
<td></td>
<td>(Haake et al., 1986)</td>
</tr>
<tr>
<td>Colorectal carcinoma</td>
<td></td>
<td>(Cordero et al., 2000)</td>
</tr>
<tr>
<td>Crohn's disease (CD26 higher on T-cells)</td>
<td></td>
<td>(Rose et al., 2002)</td>
</tr>
<tr>
<td>Depression/anxiety</td>
<td></td>
<td>(Polgar, 1992, Maes et al., 2001a, b)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td></td>
<td>(Korosi et al., 2001)</td>
</tr>
<tr>
<td>Encephalitis</td>
<td></td>
<td>(Steinbrecher et al., 2001)</td>
</tr>
<tr>
<td>Fibromyalgia</td>
<td></td>
<td>(Maes et al., 1998b)</td>
</tr>
<tr>
<td>Gastrointestinal cancer</td>
<td></td>
<td>(Cordero et al., 2000)</td>
</tr>
<tr>
<td>Graves' disease</td>
<td></td>
<td>(Nishikawa et al., 1995)</td>
</tr>
<tr>
<td>Hepatitis</td>
<td></td>
<td>(Friesz et al., 2001)</td>
</tr>
<tr>
<td>Hepatoma</td>
<td></td>
<td>(Hanski et al., 1986)</td>
</tr>
<tr>
<td>Hepatoma/melanoma cell line (more acidic isoforms)</td>
<td></td>
<td>(Ivanov et al., 1992, McCaughan et al., 1993)</td>
</tr>
<tr>
<td>HIV infection</td>
<td></td>
<td>(Subramanyam et al., 1993, Hosono et al., 1999)</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td>(Lefebvre et al., 2002)</td>
</tr>
<tr>
<td>Inflammatory bowel disease (CD26 higher on T-cells)</td>
<td></td>
<td>(Hildebrandt et al., 2001a)</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td></td>
<td>(Schmitz et al., 1996)</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td></td>
<td>(Lakatos et al., 2000)</td>
</tr>
<tr>
<td>Oral squamous cell carcinoma</td>
<td></td>
<td>(Fukasawa et al., 1982, Uematsu et al., 1998)</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td></td>
<td>(Gotoh et al., 1988)</td>
</tr>
<tr>
<td>Pregnancy</td>
<td></td>
<td>(Polgar, 1992, Hildebrandt et al., 2001b)</td>
</tr>
<tr>
<td>Psoriasis</td>
<td></td>
<td>(Katoh et al., 2000, Bock et al., 2001)</td>
</tr>
<tr>
<td>Rheumatoid arthritis (CD26 higher on T cells)</td>
<td></td>
<td>(Cordero et al., 2001, Cuchacovich et al., 2001)</td>
</tr>
<tr>
<td>Schizophrenia</td>
<td></td>
<td>(Maes et al., 1998a)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td>(Van Der Velden et al., 1999)</td>
</tr>
<tr>
<td>Systemic lupus erythematosus (hypersialylated isoform)</td>
<td></td>
<td>(Stanickova et al., 1992, Cuchacovich et al., 2001)</td>
</tr>
<tr>
<td>Women versus men</td>
<td></td>
<td>(Maes et al., 1998b, Durinx et al., 2001)</td>
</tr>
</tbody>
</table>

### Receptor function of CD26/DPPIV

The highly invasive human prostate tumor cell line LNCaP synthesizes and secretes large amounts of plasminogen activators and matrix metalloproteinases (MMPs). It was demonstrated in these cells that the receptor for plasminogen type 2 (Pg 2) is CD26/DPPIV. Pg 2 has six glycoforms that differ in their sialic acid content. Only the highly sialylated Pg 2γ, Pg 2β and Pg 2α glycoforms bind to CD26/DPPIV (Gonzalez-Gronow et al., 2001). In human rheumatoid synovial fibroblasts, Pg binding to the Pg 2 receptor and activation of Pg by urokinase-type plasminogen activator induce a significant rise in the cytosolic free Ca²⁺ concentration (Gonzalez-Gronow et al., 1993) via interactions of Pg with CD26/DPPIV associated with the integrin α(IIb)β3 on the cell surface (Gonzalez-Gronow et al., 1994, 1998). Lactose is a sugar which interferes with the binding of Neu5Ac(2→3) or (2→6) residues to sialic acid-binding proteins and inhibits the intracellular Ca²⁺ flux by blocking binding of Pg 2γ, Pg 2β and Pg 2α glycoforms to CD26/DPPIV on the surface of the fibroblasts (Gonzalez-Gronow et al., 1998), suggesting that binding occurs via carbohydrate chains. A similar inhibition of the intracellular Ca²⁺ flux was
observed when cells were preincubated with the anti-CD26/DPPIV mAb 236.3.

Triggering of CD26/DPPIV can induce MMP-9 expression, and when this occurs in cancer cells, it possibly facilitates metastasis (Gonzalez-Gronow et al., 2001). Pg 2e alone is also able to stimulate expression of proMMP-9 (Gonzalez-Gronow et al., 2001). On the other hand, CD26/DPPIV also plays a role in differentiation of cells, thereby blocking the cells from conversion into malignant phenotype. When a Ser→Ala mutation was inserted in the catalytic triad, CD26/DPPIV did not suppress tumorigenicity or anchorage-independent growth of cancer cells, nor did it reverse the block in differentiation, showing that DPPIV activity is required in suppressing the malignant phenotype (Wesley et al., 1999).

As a receptor, CD26/DPPIV is able to bind adenosine deaminase (ADA) (Kameoka et al., 1993). ADA is a 41-kDa enzyme expressed in all mammalian cells that catalyzes deamination of adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyxinosine, respectively. Although the location of this enzyme is mainly cytosolic, it is also expressed on the surface of B cells and T cells, and this expression is increased upon cell activation. CD26/DPPIV is directly associated with ADA and is identical with the so-called ADA-binding protein (Fig. 1). ADA plays a major role in the development and function of lymphoid tissues. ADA deficiency causes both defects in the development of the immune response and hematologic malignancies (Blackburn et al., 1998). In humans, ADA deficiency is inherited as an autosomal recessive condition and causes severe combined immunodeficiency due to the sensitivity of T cells and B cells to adenosine and deoxycadenosine, which downregulates T cell and B cell activation. Adenosine and deoxycadenosine are the substrates that are degraded by ADA. Thus, the ADA-CD26/DPPIV complex is able to reduce local concentrations of adenosine (Dong et al., 1996). Furthermore, binding of ADA to CD26/DPPIV on the surface of T cells induces cell proliferation (Martin et al., 1995), HIV gp120 protein inhibits binding between ADA and CD26/DPPIV, and thus inhibits adenosine breakdown. In this way, it may contribute to the pathogenesis of HIV-related diseases (Valenzuela et al., 1997), but the fact that adults with defective ADA-CD26/DPPIV binding are healthy suggests that binding is not essential (Richard et al., 2000). Gp120 interacts with CD26/DPPIV but gp120-mediated disruption of the ADA-CD26/DPPIV complex is a consequence of interactions between gp120 and a domain on the CD26/DPPIV protein different from the ADA-binding site (Franco et al., 1998).

CD26/DPPIV colocalizes with CXCR4 (Herrera et al., 2001), which is the receptor for one of the natural substrates of CD26/DPPIV, SDF-1α (Fig. 1). SDF-1α binding to CXCR4 induces chemotaxis and antiviral activity of Th2 cells, but not Th1 cells. CXCR4 and CD26/DPPIV on membranes of T (CD4+) and B (CD4−) cell lines co-immunoprecipitate. Treatment of cells with SDF-1α induces CD26/DPPIV to be cointernalized with CXCR4 (Fig. 2), SDF-1α-mediated down-regulation of plasma membrane-bound CD26/DPPIV by internalization is not blocked by pertussis toxin indicating that internalization is not mediated by Gi proteins. Internalization of CXCR4 receptors does not occur in cells that express mutant CXCR4 receptors that cannot be internalized, whereas CD26/DPPIV internalization is not hampered, indicating that internalization of CD26/DPPIV and CXCR4 follows different routes (Herrera et al., 2001). Longer periods of treatment with SDF-1α result in a homogeneous distribution of CXCR4 underneath the plasma membrane and a clustered localization of CD26/DPPIV in intracellular vesicles. This confirms that internalization of the two proteins follows different routes. Codistribution and cointernalization also occurs in peripheral blood lymphocytes. Since CD26/DPPIV is a cell surface ectopeptidase that has the capacity to cleave SDF-1α, the CXCR4-CD26/DPPIV complex is likely a functional unit in which CD26/DPPIV may directly modulate SDF-1α-induced chemotaxis and antiviral activity of lymphocytes (Fig. 3). The physical association of CXCR4 and CD26/DPPIV directly or as part of a supramolecular structure, suggests a role of CD26/DPPIV in the pathophysiology of viral infection. Furthermore, colocalization of CXCR4 and CD26/DPPIV by SDF-1α suggests that the ADA-CD26/DPPIV and the CXCR4-CD26/DPPIV-ADA complexes are important for the functioning of lymphocytes (Fig. 3) (Herrera et al., 2001).

Immunohistochemical analysis indicated that crosslinking of CD26/DPPIV with a monoconal antibody induces not only capping and internalization of the molecule, but also its colocalization with the mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGFIR), as this protein binds to CD26/DPPIV via M6P residues in the glycosylation domain of CD26/DPPIV (Ikushima et al., 2000). Colocalization was found in cytoplasmic vesicles close to the cell surface after crosslinking of CD26/DPPIV (Fig. 2). Addition of exogenous M6P inhibited not only the internalization of CD26/DPPIV induced by anti-CD26/DPPIV antibody crosslinking but also T cell proliferation induced by CD3 and CD26/DPPIV costimulation. Apparently, M6P/IGFIR binding to CD26/DPPIV is necessary for the costimulatory function of CD26/DPPIV. T cell proliferation induced by anti-CD3 and PHA is not inhibited by M6P (Ikushima et al., 2000).

CD26/DPPIV on the apical membrane of hepatocytes (Kreisel et al., 1993) and intestinal epithelial cells (Matter et al., 1990) is continuously internalized and reexpressed. Internalization of CD26/DPPIV is independent of DPPIV activity. Although internalization has been described for CD26/DPPIV to be modulated by M6P/IGFIR on T cells (Ikushima et al., 2000), it is not yet clear which carbohydrate moiety is responsible for this interaction. However, treatment of CD26/DPPIV with either a glycosidase or a phosphatase completely abolished this binding capacity, demonstrating that both glycosylation and phosphorylation of CD26/DPPIV are required for binding with M6P/IGFIR. It was found that only PHA-activated T cells express M6P-CD26/DPPIV, suggesting that activation induces mannose-6-phosphorylation of CD26/DPPIV (Ikushima et al., 2000). These data suggest that binding of CD26/DPPIV to M6P/IGFIR is mediated by M6P residues on the CD26/DPPIV carbohydrate moiety. The function of this recycling in hepatocytes and intestinal epithelial cells seems to be repair of the oligosaccharide chains attached to the glycoprotein, or in the regulation of terminal glycosylation, e.g. in response to physiological stimuli (Kreisel et al., 1993). Recycling of ectoenzymes has been demonstrated in only a few cases, such as for 5′-nucleotidase in a hepatoma cell line (Van den Bosch et al., 1988). Other membrane components such as the transferrin receptor (Snider and Rogers, 1985) and the M6P/IGFIR also exhibit recycling and resialylation of their oligosaccharides. In this setting, it is striking to see that all molecules associated with T cell activation, such as CD3/TCR complex (Moller et al., 1990; Morel et al., 1992; Herrera et al., 2001: Hwang and Sprent, 2001; Dietrich et al., 2002; Menne et al., 2002), CXCR4 (Herrera et al., 2001) and all the
Fig. 1. Schematic representation of molecules associated with CD26/DPPIV. From top to bottom: adenosine A1a receptor (A1aR) and its ligand adenosine, adenosine deaminase (ADA), CD45, the CD26/DPPIV dimer, its transporter the M6P/IGFRII, one of the natural substrates of CD26/DPPIV, SDF-1α, that is inactivated (in red frame) by CD26/DPPIV, the receptor of SDF-1α, CXCR4, RANTES (I) which is another natural substrate of CD26/DPPIV but is still bioactive (in green frame) after proteolysis, and the receptor of RANTES, CCR5, the TCR/CD3 complex and intracellular Fyn59, and ZAP70 which is associated with the TCR. The CD4 complex associated with intracellular Lck59 and phospholipase C (PLC) which liberates IP₃ and consequently induces an increase in intracellular Ca²⁺. Activation of T cells by CD26 crosslinking results in increased activity of phosphotyrosine kinases via CD45, ZAP70, Lck59 and Fyn59, phosphorylation of CD3 and Ca²⁺ influx via PLC which are similar effects as upon stimulation of the TCR/CD3 pathway. Modified after (Gorrell et al., 2000).
Fig. 2. Schematic representation of traffic of plasma membrane-bound proteins in cells. All proteins can be internalized and recycled. IL-4 stimulation elevates M6P/IGFRII expression and enhances mannose glycoconjugate uptake and endocytosis and downregulates CD26/DPPIV expression on the plasma membrane of Th2 cells. IFN-γ stimulation decreases M6P/IGFRII expression and thus diminishes internalization of CD26/DPPIV in Th1 cells and an elevated particle sorting towards the lysosomal compartment. Ligand binding by the M6P/IGFRII and subsequent internalization and accumulation in the endolysosomal compartment induces transcription activity. Early endosome (EE), sorting endosome (SE), late endosome (LE), endolysosome (EL) and lysosome (L). Membrane-bound proteins are represented by the same symbols as in Figure 1.

molecules associated with CD26/DPPIV are internalized and recycled in T lymphocytes as is shown in the schematic representation in Figure 2.

Further studies on the exact cellular distribution patterns of CD26/DPPIV and all the molecules that it is associated with in living cells during T cell activation will provide further insight in functions of CD26/DPPIV, and possibly novel mechanisms in which costimulatory molecules are involved in the T cell signaling process.
Fig. 3. Schematic representation of Th1 and Th2 actions towards sites of inflammation and preferential attraction of Th1 cells by chemokines that are bioactive after CD26/DPPIV proteolysis, IP-10 and RANTES (in green frames). Chemokines that are inactivated by CD26/DPPIV, SDF-1α, eotaxin and MDC (in red frames) attract Th2 cells. The chemokines are released by dendritic cells, epithelial cells, stromal cells and endothelial cells at the site of inflammation. Neutrophils and macrophages are preferentially recruited to the site of inflammation by Th1 cells, whereas basophils and eosinophils are preferentially recruited by Th2 cells.
Costimulatory function of CD26/DPPIV

Crosslinking of CD26/DPPIV and CD3 with immobilized mAbs induces T cell activation and IL-2 production (Morimoto and Schlossman, 1998; Boonacker et al., 2002). Treatment of T cells with anti-CD26/DPPIV antibodies leads to a decreased surface expression of CD26/DPPIV due to internalization (Fig. 2), and this modulation of CD26/DPPIV results in an elevated proliferative response to anti-CD3 or anti-CD2 stimulation (Dang et al., 1990a). T cell activation leads to redistribution of proteins in lipid rafts (Montixi et al., 1998; Xavier et al., 1998; Horejsi et al., 1999; Janes et al., 1999). This redistribution in rafts results in association of the T cell receptor (TCR/CD3) with signal-transduction molecules, indicating a specific role of rafts in T cell activation. Costimulatory molecules such as CD2, CD3, CD4, and CD44 are also raft associated (Yashiro-Ohtani et al., 2000), and strengthen TCR-mediated signaling when coligated with CD3 by inducing aggregation of rafts as well as enhancing association of TCR/CD3 and rafts. Crosslinking of CD26/DPPIV induces tyrosine phosphorylation of a panel of cellular proteins, which are similar to those that are phosphorylated after TCR/CD3 stimulation (Hegen et al., 1997). Moreover, inhibition by M6P was demonstrated for anti-CD3 and anti-CD26 costimulation, but not by G6P or M1P. The inhibitory effect was not observed in cells stimulated by anti-CD3 and PHA (Kushima et al., 2000). Stimulation with a combination of anti-CD3 and anti-CD26/DPPIV antibodies induces substantially more IL-2 production by CD26(DPPIV-) transfected Jurkat cells (Jurkat cells are T cells that lack CD26(DPPIV)) than when the mutant lacking enzyme activity – CD26(DPPIV-) transfected Jurkat cells – is stimulated. However, mutant CD26(DPPIV-) transfected cells produce significantly more IL-2 than control Jurkat cells lacking CD26(DPPIV) expression (Tanaka et al., 1993).

IL-2 has to be processed by CD26(DPPIV) to become fully activated. As a costimulatory molecule, CD26(DPPIV) is also able to induce cell proliferation via interactions with CD45 (Fig. 1). CD45 is a tyrosine phosphatase, which in its inactive state forms a dimer (Desai et al., 1993). It has been demonstrated that CD26(DPPIV) is present in lipid rafts, and that crosslinking with anti-CD26(DPPIV) antibodies increases the recruitment of CD26(DPPIV) and CD45 molecules to these rafts (Ishii et al., 2001). This indicates that CD26(DPPIV) and CD45 are associated. Recruitment results in increased tyrosine phosphorylation of receptor signaling molecules such as c-Cbl, p56Lck, ZAP-70 Erk1/2, and CD3ζ (Hegen et al., 1997; Ishii et al., 2001), suggesting that CD26(DPPIV) induces monomerisation of CD45 and stimulates TCR signaling (Braun et al., 1998). However, the costimulatory function of CD26(DPPIV) remains incompletely understood, because proteolytic activity of CD26(DPPIV) seems to play an important but not an essential role in the costimulatory function of CD26(DPPIV) (Tanaka et al., 1993).

Adhesion of CD26/DPPIV to collagen and fibronectin

Indications of interactions of CD26/DPPIV as an adhesion molecule with proteins of the extracellular matrix were obtained by experiments that show that the small peptide Gly-Pro-Ala which is recognized by CD26(DPPIV) as substrate interferes with spreading of rat hepatocytes on a matrix containing fibronectin and collagen (Hanski et al., 1985). Similar results were obtained with substrates and inhibitors of CD26(DPPIV), but not with tripesides that were not substrates for CD26(DPPIV). In agreement with this observation, it was found that peripheral T cells can migrate through a monolayer of endothelial cells on a collagen gel when high levels of CD26(DPPIV) are expressed (Masayama et al., 1992). Moreover, an antibody against CD26(DPPIV) delayed fibronectin-mediated adhesion of rat hepatocytes to denaturated collagen (Hanski et al., 1988). Nitrocellulose binding assays using 125I-labelled DPPIV that was purified to homogeneity from rat hepatocytes revealed a direct interaction of DPPIV and fibronectin. Binding to fibronectin occurred at a site in the CD26(DPPIV) molecule that is distinct from its exopeptidase substrate recognition site because competitive peptide inhibitors and phenylmethylsulphonyl fluoride enhanced fibronectin binding, possibly as a result of an altered conformation of DPPIV (Piazza et al., 1989). CD26(DPPIV) acts as collagen receptor on murine fibroblasts (Bauvois, 1988), and on human T cells as a collagen receptor that induces cell activation (Dang et al., 1990b). The binding site for collagen was found to be present in the cysteine-rich region of the molecule (Loster et al., 1995).

Involvement of CD26/DPPIV in apoptosis

Jurkat T cells transfected with CD26(DPPIV-) appeared to be less apoptotic than CD26 transfected with CD26(DPPIV+). The higher rates of apoptosis of the CD26(DPPIV+) transfectants was explained by the finding that CD95 (Fas/Apo-1) was upregulated in mutants without DPPIV activity in comparison with transflectants with DPPIV activity (Morimoto et al., 1994). CD95 is a member of the nerve growth factor/tumor necrosis factor receptor family that mediates apoptosis (Trauth et al., 1989). In a human hepatoma cell line, CD26(DPPIV) functions in the absence of most receptors expressed on T cells. Surprisingly, these hepatoma cells underwent apoptosis after stimulation with an immobilized anti-CD26(DPPIV) mAb. This effect was not limited to this cell line, because CD26(DPPIV) stimulation of another hepatoma cell line, HepG2, also induced apoptosis. This effect was transduced by a tyrosine kinase, because phosphatase inhibition enhanced apoptosis (Gaetaniello et al., 1998). This is in line with the fact that tyrosine phosphorylation can be demonstrated after crosslinking of CD26(DPPIV), but in contrast with the finding that CD26(DPPIV-) transfected Jurkat cells are protected from apoptosis after HIV infection, apparently because CD95 was not upregulated in the hepatoma cells (Morimoto et al., 1994).

A possible explanation is that the opposite functions of CD26(DPPIV) depend on its moonlighting properties and thus the molecule either exerts its different functions in different cell types, or different isoforms exert unique functions in different cellular contexts. The remarkable difference between the role of CD26(DPPIV) in lymphocytes and hepatoma cells in relation to apoptosis is an example that the protein exerts its specific functions depending on the context of multifunctional mole-
ular machineries and thus that CD26/DPPIV is a moonlighting
protein.

The role of CD26/DPPIV in immune responses

CD26/DPPIV is expressed on T lymphocytes, B lymphocytes and NK cells (Ansorte et al., 1995; Mattern et al., 1995). CD26-positive T helper cells are for 76% CD4+ and for 16% CD8+ (Mattern et al., 1995). Expression of CD26/DPPIV on T helper cells is tightly regulated upon activation (Boonacker et al., 2002). Detailed analysis of subsets of human CD4+ T helper cells indicates that CD26/DPPIV expression is more restricted than that of most other accessory proteins since it is expressed only on CD4+ memory T helper (CD45RO+/CD29+) cells. This unique population is the only cell type that can recall antigens, can induce immunoglobulin synthesis in B cells and activate MHC-restricted cytotoxic T cells (Morimoto et al., 1989; Dang et al., 1990a). CD4+ cells lacking CD26/DPPIV cannot be triggered to elicit helper functions but can respond to mitogens and alloantigens. CD26/DPPIV is not a stable surface marker; T cell activation increases expression of CD26/DPPIV on the T cell surface in the initial stages of proliferation and it reaches a maximum after three days. Then, expression decreases again and cells stop to proliferate after 11 days of culture (Ansorte et al., 1995; Mattern et al., 1995). The increase in CD26/DPPIV expression upon stimulation is more profound on Th1 cells than on Th2 cells, so that CD26/DPPIV expression is many-fold higher on Th1 cells during activation and proliferation, but Th1 and Th2 cells show similar DPPIV activity due to posttranslational alterations in $K_m$ and $V_{max}$ values of DPPIV (Boonacker et al., 2002).

Kahne et al. (1996) demonstrated differences in intracellular localization patterns of isoforms of DPPIV. These patterns were found to change significantly upon mitogenic stimulation. Some isoforms were found only in soluble fractions of resting T cells, whereas after PHA-stimulation they were present in membrane fractions. These isoforms on the plasma membrane either originate from the cytosol and are translocated or represent a newly synthesized form of the enzyme.

CD26/DPPIV isoforms of haematogenous sources are strongly heterogeneous. Mitogenic stimulation of human mononuclear cells leads to changes in both isoelectric points and cellular localization patterns of distinct isoforms of CD26/DPPIV. When comparing staining patterns of DPPIV activity and immunolocalization patterns of CD26/DPPIV isoforms, strong differences were found. Lysates from healthy individuals also showed strong differences in activity patterns after iso-electric focussing (Kahne et al., 1996). Noteworthy is that these isoforms of CD26/DPPIV are recognized by all known anti-CD26/DPPIV mAbs. A direct quantitative relationship between the amount of antibody binding and DPPIV activity was not found. It can be concluded on the basis of these phenomena that the CD26 family consists of isoforms exhibiting DPPIV activity as well as isoforms without detectable activity (Klobučka and Babusíková, 1999a). However, it is not completely clear whether CD26/DPPIV was responsible for all bands that showed substrate conversion, because substrate conversion could also be performed by homologous proteases, such as attractin, DPPIII, DPPIV-β, or DPPVIII (Table V).

Since CD26/DPPIV participates in various functions of the immune system, variations in expression of this molecule and its DPPIV activity may be the basis of the roles of CD26/DPPIV in regulating T cell development in particular and the immune response in general. Because IFN-γ, CD26/DPPIV, CCR5, CXC4, and CXCR3 (the latter 3 are the receptors of IP-10, Mig and I-TAC which are all processed by CD26/DPPIV: Table I) are Th1 hallmarks (Bonecchi et al., 1998; Loetscher et al., 1998), processing of chemokines may constitute an important mechanism to reduce attraction of leukocytes in a Th1 response (Fig. 3). It is crucial to dampen the immune response after influx of sufficient immune cells in an inflamed site. At the moment that CD26/DPPIV-mediated cleavage of chemokines has reduced the bioactivity of chemokines, expression of proteolytically less active isoforms of CD26/DPPIV makes sense. This is in accordance with the finding that CD26/DPPIV expression on Th2 cells is lower than on Th1 cells, whereas DPPIV activity on Th1 cells and Th2 cells is similar (Boonacker et al., 2002). Moreover, adult and neonate T cell populations differ in their DPPIV activity with higher activity on neonatal cells. The shifting of the T cell repertoire is more dynamic and faster during the neonatal period and has been linked with a difference in CD26/DPPIV expression (Vissinga et al., 1987).

Consequences of abnormal expression of CD26/DPPIV

Many diseases are related with altered plasma levels of sCD26/DPPIV (Table VI).

CD26/DPPIV and cancer

Malignant cells often show altered CD26/DPPIV expression or even do not express CD26/DPPIV (Wesley et al., 1999). For example, loss of CD26/DPPIV occurs during malignant transformation of melanocytes when the cells become independent of exogenous growth factors for survival (Albino et al., 1992; Morrison et al., 1993). Tetracycline-inducible expression of DPPIV/CD26 in malignant human melanoma cells that were transfected with the CD26/DPPIV gene induced a profound switch of phenotype of the cells to nonmalignancy. CD26/DPPIV re-expression led to inhibition of tumorgenicity and anchorage-independent growth, reversal of the block in differentiation, and a re-acquired dependence on exogenous growth factors for survival. Suppression of tumorigenicity and reversal of the block in differentiation were dependent on DPPIV activity (Wesley et al., 1999). However, other studies using melanoma cells expressing mutant CD26/DPPIV lacking either extracellular serine protease activity or the six amino acid long cytoplasmic tail demonstrated that DPPIV activity and the cytoplasmic tail are not required for decreased metastatic potential (Pethiyagoda et al., 2000). Surprisingly, dependence on exogenous growth factors was not related with DPPIV activity. Re-expression of either wild-type CD26/DPPIV or mutant CD26/DPPIV rescued expression of a second cell surface serine protease, FAP-α, which can form a heterodimer with CD26/DPPIV. This observation suggests that FAP-α also plays a role in regulating growth of melanocytes (Wesley et al., 1999) and demonstrates the complex roles that these multifunctional proteases play. Downregulation of CD26/DPPIV seems to play an important role in the early development of melanomas. Because CD26/DPPIV can inactivate
circulating growth hormone-releasing factor (GHRF) (Frohman et al., 1989), downregulation of CD26/DPPIV may facilitate tumor growth by prolonging the presence of GHRF in the circulation, resulting in higher levels of the growth hormone.

Differentiation of gliomas is related with CD26/DPPIV activity and its subcellular distribution pattern. Low activity is found in poorly differentiated gliomas and vice versa. These differences may be related with changes in pl-isoforms of CD26/DPPIV. When comparing differentiated slowly proliferating glioma cells with poorly differentiated rapidly proliferating glioma cells, DPPIV activity of a number of pl-isoforms was distinctly higher in differentiated glioma cells. The higher DPPIV activity in differentiated glioma cells was particularly related with membrane-associated isoforms (Sedo et al., 1998).

Stromal cells in benign prostate tumors express different cell-surface peptidases than normal prostatic stromal cells and epithelial cells. For example, expression of aminopeptidase A (APA) is increased in tumor stroma, whereas aminopeptidase N (APN) is absent in most cancer cells (Bogenrieder et al., 1997). CD26/DPPIV levels are also decreased in metastatic prostatic tumors. Binding of CD26/DPPIV to fibronectin and collagen may alter an aggressive or metastatic phenotype to a more quiescent phenotype.

Along this line, Cheng et al. (1998) have demonstrated that adhesion of CD26/DPPIV to fibronectin is involved in metastasis of breast cancer in lung. Remarkably, CD26/DPPIV was expressed on lung endothelial cells whereas fibronectin was present on the surface of the metastatic cancer cells.

Attachment of circulating cancer cells to endothelial cells via adhesion molecules is considered to be responsible for organ-specific metastasis. Rat R3230AC-MET cancer cells and RPC-2 prostate cancer cells that metastasize to the lung bind membrane vesicles isolated from the lung vasculature. In contrast, vesicles that are derived from the vasculature of organs to which these cancer cells do not metastasize, do not bind. Purification of the endothelial cell adhesion molecule from rat lung extracts and the use of a mAb that inhibited selective adhesion revealed that the protein was CD26/DPPIV (Johnson et al., 1993).

CD26/DPPIV has been studied in T-acute lymphoblastic leukemia (T-ALL). Noteworthy is the discrepancy between the high expression of CD26/DPPIV but moderate DPPIV activity on Tlymphoblasts in the majority of T-ALL cases (Klobusicka and Babusikova, 1999a, b).

Degradation of extracellular matrix by proteinases has been implicated during invasion and metastasis (Werb, 1997; Van Noorden et al., 1998). Interactions between plasminogen and CD26/DPPIV have been reported to initiate signal transduction that regulates expression of MMP-9 in prostate cancer cells (Gonzalez-Gronow et al., 2001). Therefore, the role of CD26/DPPIV does not seem to be unequivocal in carcinogenesis. On the one hand, CD26/DPPIV expression in cancer cells seems to downregulate cancer progression because it induces differentiation. On the other hand, CD26/DPPIV activity seems to play a direct role in invasion and metastasis as well.

**CD26/DPPIV and autoimmune diseases**

In T-cell-mediated experimental autoimmune encephalomyelitis (EAE), a critical role for CD26/DPPIV in the modulation of effector functions of CD4+ T lymphocytes has been demonstrated (Natarajan and Bright, 2002). Signs of EAE were partially suppressed by in vivo administration of the specific CD26/DPPIV inhibitor I40 both in a preventive way and in a therapeutic way (Steinbrecher et al., 2001). The protective mechanism of CD26/DPPIV inhibition can be explained by its modulation of T cell effector function. The CD26/DPPIV inhibitors I40 and I49 also suppressed secretion of TNF-α and to a lesser extent that of IFN-γ, cytokines that upregulate the immune response. Earlier reports show that T cell proliferation and secretion of IL-2, IL-6, and IL-10 in mouse spleen and thymocytes is suppressed by in vivo administration of CD26/DPPIV inhibitors (Reinhold et al., 1997a). Likewise, proliferation and secretion of various cytokines including TNF-α and IFN-γ were suppressed in human T cells (Reinhold et al., 1997b, 1998). These findings are in line with the fact that EAE is mediated by CD4+ Th1 cells which typically secrete TNF-α, IFN-γ and lymphotxin. Moreover, it was found that the immunosuppressive TGF-β1 cytokine was upregulated in spinal cord tissue and plasma of mice that were treated with the DPPIV inhibitor I40 (Reinhold et al., 1997a, b; Steinbrecher et al., 2001). Expression of CD26 molecules on the surface of T cells from peripheral blood and cerebrospinal fluid of patients with progressive multiple sclerosis (Hafler et al., 1985) and Graves' disease is elevated (Eguchi et al., 1989).

In conclusion, it can be stated that autoimmune diseases are related with elevated CD26/DPPIV expression on T cells.

**CD26/DPPIV and AIDS**

CD26/DPPIV on human T cells consists of a set of isoforms as has been demonstrated by isoelectric focussing studies (Tomimoto et al., 1992; Ansorge et al., 1995; Mattern et al., 1995; Kahne et al., 1996; Smith et al., 1998). DPPIV activity is found only in basic isoforms of CD26/DPPIV, but not acidic isoforms. A shift to acidic isoforms has been observed during HIV infection (Smith et al., 1998). CD4+ cells of patients infected with HIV have an intrinsic defect in their ability to recognize and respond to antigens before a reduction of the total number of CD4+ cells occurs in these patients (Lane et al., 1985; Van Noesel et al., 1990; Schnittman et al., 1990). The memory function to recall antigens is a property of CD4+ T cells expressing CD26/DPPIV. It is the only type of T helper cell that is known to proliferate in response to soluble antigens. They also activate both MHC-restricted cytotoxic T cells to kill virally-infected target cells and B cells to secrete immunoglobulins (Morimoto et al., 1989). In this respect, it is striking that there is a selective decrease in CD26/DPPIV-positive T cells in HIV-1-infected individuals prior to a general reduction in the number of CD4+ cells (Blazquez et al., 1992). These findings indicate the importance of the immuno-modulating role of CD26/DPPIV which is supported by the finding that antigen response in HIV-1-infected individuals can be restored by the addition of sCD26/DPPIV in vitro (Schmitz et al., 1996). Furthermore, it has been shown that DPPIV protease activity of plasma sCD26/DPPIV was decreased in HIV-1-infected individuals and inversely correlated with HIV-1 RNA levels in cells.

In contrast, cleavage of SDF-1 by CD26/DPPIV reduces its anti-HIV activity and thus the presence of CD26/DPPIV may facilitate entry of the virus into cells (Ohnatsuki et al., 1998; Poot et al., 1998). Antigen-specific memory CD4+ T cells infected with HIV-1 showed significantly higher CXCR4 and HIV-1 expression in Th12-oriented responses (with low CD26/DPPIV expression) in comparison with Th1-oriented responses (with high CD26/DPPIV expression). Similarly, in naive CD4+ T cells activated in the presence of IL-4 or IL-12 and infected with the same HIV-1 virus, IL-4 upregulated CXCR4 and HIV-1 expression whereas IL-12 downregulated both (Fig. 3). The
Thus, a lower degree of sialylation is found in children as type Jurkat cells. Moreover, the costimulatory effect of CD26/DPPIV is reduced in CD26(DPPIV) transfectants as compared to wild-type. Whereas replication of HIV-1 virus is inhibited when CD26/DPPIV activity is blocked, Jurkat cells with costimulatory function of CD26/DPPIV. Inhibition of CD26/DPPIV protease activity by sialylation fits in this concept, since IL-12 processing that is necessary for proliferation of T cells is downregulated by sialylation because interaction of CD26/DPPIV with other proteins (e.g., ADA and CD45) are reduced.

Possible pathological effects of hypersialylation of CD26/DPPIV are the following: (1) modification of the affinity of CD26/DPPIV as an adhesion molecule, (2) impairment of CD26/DPPIV as receptor to respond to signals for cell activation, (3) alterations in the affinity of DPPIV for its natural substrates, and (4) conformational changes of CD26/DPPIV that may directly or indirectly lead to premature apoptosis. Concentrated domains of sialic acids linked to CD26/DPPIV by extended N-linked oligosaccharides as occurs for example during aging and HIV-1 infection (Smith et al., 1998) are more effective as a negative charge than limited domains of negatively-charged amino acids in the protein moiety for binding viral proteins or peptides. Similarly, it was shown that sialylation plays a key role in the apical targeting of a secreted CD26/DPPIV isoform. So, hypersialylation of CD26/DPPIV is both a natural and a pathological phenomenon. Increased sialylation occurs in principle with all proteins on the plasma membrane of cells during aging (Abdul-Salam et al., 2000). Thus, a lower degree of sialylation is found in children as compared with adults. Therefore, it is striking that older HIV-infected individuals develop HIV-related diseases more rapidly than younger HIV-infected persons, and die more rapidly after infection (Fletcher et al., 1992; Adler et al., 1997). HIV-1-infected cells from older individuals do not appear to be more susceptible to immune-mediated destruction, but the more rapid progression of HIV-related diseases appears to be due to the inability of older persons to replace functional T cells that have been destroyed. This is related to the pro tease activity and costimulatory function of CD26/DPPIV. Inhibition of CD26/DPPIV protease activity by sialylation fits in this concept, since IL-2 processing that is necessary for proliferation of T cells is inhibited when CD26/DPPIV activity is blocked. Jurkat cells transfected with CD26/DPPIV (Morimoto et al., 1994), whereas replication of HIV-1 virus is reduced in CD26/DPPIV-transfectants as compared to wild-type Jurkat cells. Moreover, the costimulatory effect of CD26/DPPIV can be downregulated by sialylation because interactions of CD26/DPPIV with other proteins (e.g., ADA and CD45) are reduced.

Therapeutic potentials of CD26/DPPIV targeting

The exact picture of all biological functions of CD26/DPPIV is difficult to compose due to its moonlighting properties. However, evidence exists for therapeutic potentials of DPPIV inhibitors, especially because processing of biologically active peptides by DPPIV activity modulates their metabolism. Tables I, II and III list biologically active peptides that can be processed by CD26/DPPIV.

GHFR is degraded by DPPIV and administration of a DPPIV inhibitor together with GHFR may be useful to treat children with growth hormone deficiency to prolong the availability of GHFR (Bongers et al., 1992).

DPPIV is involved in the degradation of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) in vitro and in vivo. GIP and GLP-1 are considered to be the most important insulin-releasing hormones that are part of the enteroinsular axis. The term enteroinsular axis refers to the signaling pathway between gut and pancreatic islets that amplify the insulin response to induce absorption of glucose.

N-terminal truncation by DPPIV abolishes insulinotropic activity of GIP and GLP-1 and this hydrolysis is the primary mechanism of their inactivation in vivo. Inhibition of DPPIV with orally administered Ille-thiazolidine enhances insulin secretion and improves glucose tolerance in response to an oral challenge in lean and obese Zucker rats. These rats exhibit characteristics of non-insulin-dependent diabetes mellitus (Pederson et al., 1998). This phenomenon was attributed to a block in the inactivation of GIP and GLP-1 by DPPIV, resulting in signal amplification of the enteroinsular axis. Therefore, it is believed that inhibitors of DPPIV alone or in combination with GLP-1 can be used to lower glucose levels in diabetes (Drucker, 1998).

Likewise, GLP-2 can also be inactivated by DPPIV. GLP-2 displays intestinal growth factor activity in rodents. Administration of a modified form of GLP-2, [Gly]-GLP-2, which is resistant to DPPIV hydrolysis in mice, increases their small bowel weight, predominantly due to a significant increase in villous height (Brubaker et al., 1997; Drucker et al., 1997a, b). Therefore, DPPIV inhibitors may be useful to increase the intestinalotopic properties of GLP-2 on mucosal regeneration in patients with intestinal disease (Drucker, 1998).

Binding of plasminogen to CD26/DPPIV initiates a signal transduction mechanism which regulates expression of MMP-9 by prostate cancer cells (Gonzalez-Gronow et al., 2001). Therefore, inhibitors of DPPIV may block metastasis of cancer.

Alkylamine-induced arthritis in rats, a model that shares several pathological features with rheumatoid arthritis, was suppressed by a series of DPPIV inhibitors in a dose-dependent manner (Tanaka et al., 1997). T cells with high levels of CD26/DPPIV preferentially migrate into the rheumatoid synovium to induce inflammation and tissue destruction (Mizokami et al., 1996). Again, DPPIV inhibitors may be useful as immunosuppressants for the treatment of autoimmune diseases such as rheumatoid arthritis and for the prevention of rejection of transplants, as DPPIV can alter the balance of chemokine activity towards stimulation and attraction of Th1 cells. CD26/DPPIV is preferentially expressed on Th1 cells and cleaves RANTES, eotaxin, MDC, SDF-1α, and SDF-1β. The cleavage products of these chemokines trigger Th1-specific but not Th2-specific immune responses.
Therefore, DPPIV inhibitors can be used to prolong the life of Japanese Fischer 334 rats that are displaying only pathologies of aging. This is because the digestive apparatus, despite a complete lack of CD26/DPPIV expression (Tiruppathi et al., 1993), bears important dynamically titrated features of the cell phenotype. From a functional point of view, an individual DASH molecule itself does not need to be highly specific, it is sufficient to change light properties and thus on the site and context of their expression in living cells.

In conclusion, there is growing evidence suggesting that the contextual repertoire of DASH proteins, with partially overlapping functional and molecular characteristics, seems to be an important dynamically titrated feature of the cell phenotype. Eventually, from a functional point of view, an individual DASH molecule itself does not need to be highly specific, it depends on the microenvironment of the molecule what function and what specificity are exerted. In other words, specificity of the proteins can be provided by cells and substrates in the immediate environment (Monsky et al., 1994; Duke-Cohan et al., 1996; Jacotot et al., 1996; Chiravuri et al., 1999; Mentlein, 1999). Most of the molecules belonging to this group fulfill multiple functions relying on their moonlighting properties and thus on the site and context of their expression in living cells.

**Conclusions**

The role of CD26/DPPIV in many processes seems logical. Expression of CD26/DPPIV in renal, intestinal and liver epithelial cells is mainly involved in the degradation/catabolism of proteins, but its functional role in the immune response is still largely unknown. To elucidate the function of CD26/DPPIV in the immune system, it is necessary to find a good model in which it is possible to monitor this extraordinary protein. Especially, a model is desired in which it is possible to study redistribution and oligosaccharide-reprocessing of CD26/DPPIV during T cell activation and subsequent interactions with other molecules to help to elucidate the functions of CD26/DPPIV in the immune response. High numbers of CD26/DPPIV-positive cells in inflamed tissues are related to autoimmune diseases (Hafler et al., 1985; Nakao et al., 1989) whereas low numbers of CD26/DPPIV-positive cells indicate immunodeficiency. However, the main function(s) of CD26/DPPIV on T cells still remain(s) to be elucidated.

Therefore, a better understanding of the functions of the various isoforms of the multifunctional CD26/DPPIV protein and other proline-specific peptidases in the specific cellular compartments and in extracellular fluids as scCD26/DPPIV is necessary.

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CHAPTER 9

Conclusions
Conclusions

Enzymes are the hardware of cells and play a crucial role in health and disease. Pathological alterations in enzymatic activity may have many causes. An enzyme can be either absent, inactive, overexpressed or located in a wrong cellular subcompartment. When the activity is normal but its location is wrong, deviations are hard to detect when using a biochemical approach for the detection of enzyme activity. On the other hand, when localizing the presence of an enzyme protein by immunohistochemistry, it does not give any information on whether the enzyme is active or not. Moreover, activity of enzymes is often regulated by the microenvironment of the enzyme. Therefore, detection of both enzyme protein and its activity in living cells provides a better understanding of cellular functioning. Many techniques are available to demonstrate activity of enzymes, but most of these techniques are not compatible with fragile living cells, because they are based on the use of compounds or methods that are toxic or damaging, and are thus not suited to study enzymatic activity and its regulation in living cells. Furthermore, membranes may be a limiting factor when detecting activity of intracellular enzymes in living cells. Usually, cells are permeabilized to enable compounds of the incubation medium to reach the site of the enzyme but this is not allowed on living cells. Micro-injection of (caged) substrates is a possibility to overcome the problem but it remains a cumbersome technique. Selectivity of substrates may be a limiting factor as well. Homologue enzymes may convert the substrate used for visualization of the activity of a particular enzyme, as is demonstrated in the present thesis for CD26/DPPIV. [Ala-Pro]²-cresyl violet appeared to be specific for DPPIV activity whereas Ala-Pro-rhodamine 110 was cleaved by DPPIV homologues as well.

Enzyme reactions are often performed at high substrate concentrations to be able to detect generation of product, although measurements should reflect the reaction as occurs in vivo, but that is often difficult to realize because physiological substrate concentrations are usually (very) low. Finally, the final reaction product should not interfere with the activity of the enzyme and it should accumulate at the site of conversion.

The present thesis describes the development of a technique to detect and quantify CD26/DPPIV activity in living cells. The approach that was selected for the visualization of enzyme activity in living cells is based on the use of synthetic fluorogenic enzyme substrates and 4D microscopy. Because many isoforms of enzymes exist that may show different localization patterns in cells and different activity, specificity, and affinity on the one hand and many enzymes have overlapping activities on the other hand, synthetic substrates should allow discrimination between different isoforms of enzymes or enzymes with overlapping activities, thus enabling detection and quantification of specific enzyme activity. It is demonstrated in this thesis that specific substrates can be applied that do not react with homologous enzymes and thus the technique can be used to visualize enzymatic properties of a specific enzyme. In this case, activity was demonstrated of the multifunctional or moonlighting protein CD26/DPPIV and posttranslational regulation of CD26/DPPIV activity as it occurs in living cells and tissues.

We have used a novel substrate, [Ala-Pro]²-cresyl violet, which shows a shift in fluorescence
upon hydrolysis, thus enabling visualization of both fluorescence of synthetic substrate and product which have different colors. The advantage of a fluorescent substrate that shifts its fluorescence upon enzymatic processing over a quenched non-fluorescent substrate is that substrate uptake and also which cell compartments are reached by the substrate can be visualized in living cells. Moreover, local substrate concentrations can be determined which is important to establish local kinetic parameters of the enzyme under study. With this technique, it is demonstrated that most cells take up the substrate and hydrolyze it whereas others take up the substrate but do not hydrolyze it because the enzyme is inactive, demonstrating again the complex regulation of enzyme activity in living cells.

Visualization of CD26/DPPIV activity in living cells was used to elucidate how two of its functions, signal transduction and proteolysis, are regulated. It appeared that on T helper cells, signal transduction was directly related with the number of CD26/DPPIV molecules on cells whereas the proteolytic DPPIV activity per cell was similar at physiological substrate concentrations, irrespective the number of molecules present on cells. This DPPIV activity is kept constant irrespective of the number of CD26/DPPIV molecules on cells by variation of kinetic parameters. Finally, a method was developed to rapidly screen whether a peptide or a protein is a natural substrate for a protease with the example of β-casomorphin₁₅ as substrate for CD26/DPPIV.
CHAPTER 10

Summary
Summary

Chapter 1 introduces the general issue that is addressed in the present thesis. The way measurements of enzyme activity are usually performed is not ideal and far from reality in vivo. Enzyme activities are usually studied in homogenates or dilute solutions in which the complex interactions as exist in living cells are lost. This approach eliminates many factors which affect enzyme activity. Most proteins and thus enzymes as well function in complexes, in which interactions with proteins and other types of macromolecules involved play an important role in the regulation of enzyme activity. Furthermore, enzymes can have multiple functions, which must be regulated separately in time and space. This phenomenon is called moonlighting and adds another dimension to cellular complexity. The functioning of moonlighting enzymes is therefore not easy to understand. Visualization of the various functions of enzymes in living cells can be a powerful tool to understand the complex regulation and functioning of moonlighting enzymes.

Chapter 2 is an overview of methods for the detection of enzyme activity in living cells and tissues. It shows that molecular interactions that take place during incubation and recording of the formation of a colored or fluorescent reaction product are not well understood. Because specific enzymes play key roles in many pathophysiological processes, they are targets for therapeutic strategies. Therefore, it is essential to study the activity of specific enzymes in living cells and tissues in a quantitative manner in relation to pathophysiological processes to understand their relevance and the potential impact of their targeting by drugs. Proteases, in particular, are crucial in every aspect of life and death of an organism and as a consequence are important for drug development. Enzyme activity in living cells can be studied with various tools. These can be endogenous fluorescent metabolites or synthetic chromogenic or fluorogenic substrates. The use of endogenous metabolites is rather limited and nonspecific because they are involved in many biological processes, but novel chromogenic and fluorogenic substrates have been developed to monitor activity of enzymes, and particularly proteases, in living cells and tissues. This chapter discusses these substrates and the methods in which they are applied, as well as their advantages and disadvantages for metabolic mapping in living cells (J. Histochem. Cytochem. 49:1473-1486, 2001).

Chapter 3 describes the reactivity of two fluorogenic substrates, [Ala-Pro]²-cresyl violet and Ala-Pro-rhodamine 110 for microscopical detection of CD26/dipeptidyl peptidase IV (DPPIV) proteinase activity in living cells to establish to what extent the Ala-Pro-containing substrates are specific for CD26/DPPIV. DPPIV activity is one of the many functions of the moonlighting protein CD26/DPPIV. As a model, we used Jurkat cells, which are T cells that lack CD26/DPPIV expression, and CD26/DPPIV-transfected Jurkat cells. Ala-Pro-rhodamine 110 is not fluorescent but after proteolytic cleavage rhodamine 110 fluoresces. [Ala-Pro]²-cresyl violet is fluorescent by itself but proteolytic cleavage into cresyl violet induces a shift to longer wavelengths. This phenomenon enables the simultaneous determination of local (intracellular) substrate and product concentrations which is important for the analysis of kinetics of cleavage reactions. [Ala-Pro]²-
cresyl violet but not Ala-Pro-rhodamine 110 appeared to be specific for CD26/DPPIV. When microscopical analysis is performed on living cells during the first minutes of the enzyme reaction, DPPIV activity can be precisely localized in cells with the use of [Ala-Pro]²-cresyl violet. Fluorescent product is rapidly internalized in submembrane granules in transfected Jurkat cells and redistributed intracellularly via internalization pathways that have been described for CD26/DPPIV. Ala-Pro-rhodamine 110 appeared to be cleaved by homologues of CD26/DPPIV as well. It is concluded that [Ala-Pro]²-cresyl violet is a good fluorogenic substrate to detect DPPIV activity in living cells when the correct wavelengths are used for excitation and emission and images are captured in the early stages of the enzyme reaction. Furthermore, we conclude that the nature of the fluorophore can significantly affect interactions of synthetic protease substrates with its active site (J. Histochem. Cytochem. 2003, in press).

Chapter 4 describes methods to analyze enzyme activity in living cells by flow cytometry for rapid measurements of enzyme reactions in individual cells. Since enzyme activities often display a heterogeneous character in cell populations, flow cytometry is an ideal tool to study quantitatively enzyme activity in individual living cells. Furthermore, activity can be related to other relevant parameters such as the amount of DNA in the case of polyploid hepatocytes and the amount of enzyme molecules present as detected immunocytochemically, for example to determine posttranslational control or switching of functions as occurs with moonlighting proteins. In contrast to fluorescence microscopy in which time lapse series of digital images can be made of living cells during incubation to determine activity of a specific enzyme, each cell is measured only once by flow cytometry. In the latter case, fading of fluorescence is of negligible influence on the measurements, but on the other hand, enzyme reactions cannot be determined in time per individual cell. We solved this problem by analysis of enzyme reactions in time in large numbers of living cells while the cells are incubated. (Cytometry: Cytomics, proteomics, genomics. Cytometry CD vol 6. Multimedia Knowledge, Inc. www.mmke.com; in conjunction with Purdue University Cytometry Labs, New York, pp 1-8).

Chapter 5 describes properties of [Ala-Pro]²-cresyl violet as substrate for CD26/DPPIV for localization and quantification of its activity in individual freshly isolated living rat hepatocytes using confocal microscopy, image analysis, and flow cytometry. DPPIV activity was localized exclusively in patches at plasma membranes likely being bile canalicular domains. Activity was analyzed quantitatively in individual cells by capturing series of images in time. Production of fluorescence was analyzed on the basis of the series of digital images and it appeared to be nonlinear with time. By calculation of the initial velocity at time zero, activity of DPPIV per individual hepatocyte was calculated. Cresyl violet-dependent fluorescence appeared in a similar way when cells were analyzed by flow cytometry. A dipeptide phosphonate inhibitor inhibited production of fluorescence competitively with a $K_i$ of 7 μM. $K_m$ values in individual hepatocytes varied in the range of 6-22 μM depending on the individual rat from which the hepatocytes were obtained, whereas the $V_{max}$ varied in the range of 4-16 nU. $K_m$ and $V_{max}$ values per individual rat were inversely correlated indicating posttranslational regulation of the kinetic parameters of DPPIV. This relationship was lost when membrane fractions of the same hepatocyte suspensions
were analyzed. It is concluded that cresyl violet-based protease substrates are favourable compounds of choice to localize and quantify protease activity in living cells and tissues (Anal. Biochem. 252:71-77, 1997).

Chapter 6 describes the independent regulation of two of the functions of CD26/DPPIV, signal transduction and proteolysis. To investigate how two separate functions of one molecule are regulated, we analyzed CD26 protein expression and DPPIV activity on living human T-helper 1 (Th1) and T-helper 2 (Th2) cells that express different levels of CD26/DPPIV. DPPIV activity was specifically determined with the synthetic fluorogenic substrate [Ala-Pro]₂-cresyl violet and CD26 protein expression was demonstrated with an FITC-conjugated CD26-specific antibody. Fluorescence of liberated cresyl violet (red) and FITC (green) was detected simultaneously in living T cells using flow cytometry and spectrofluorometry. Th1 cells expressed three- to six-fold more CD26 protein than Th2 cells. The signal transduction function of the CD26/DPPIV complex, tested by measuring its co-stimulatory potential for proliferation, was directly related to the amount of CD26 protein at the cell surface. However, DPPIV activity was similar in both cell populations at physiological substrate concentrations because of differences in Vₘₐₓ and Kₘ values of DPPIV activity measured on Th1 and Th2 cells. Western blotting and zymography of Th1 and Th2 membrane fractions demonstrated similar patterns. This study shows that two functions of one molecule can be controlled differentially (J. Histochem. Cytochem. 50:1169-1177, 2002).

Chapter 7 describes a method for the detection and identification of natural substrates of proteases. Proteolysis of bioactive peptides is a regulating step in many physiological processes, but which proteases in what cellular sites are involved in activation and/or degradation of specific peptides is not well known. We developed a rapid microwell plate assay consisting of living cells and fluorogenic protease substrates to determine which bioactive peptides are natural substrates of a specific protease. The multifunctional or moonlighting protein CD26/DPPIV specifically catalyzes the cleavage of peptides from the amino terminus of peptides with proline as the penultimate amino acid. Many biologically active peptides, such as β-casomorphin₁₋₅, contain proline in that penultimate position. We incubated Jurkat cells and CD26/DPPIV-transfected Jurkat cells in the presence of the fluorogenic substrates [Ala-Pro]₂-cresyl violet and Ala-Pro-rhodamine 110 and β-casomorphin₁₋₅. Fluorescent cresyl violet was generated by CD26/DPPIV-transfected Jurkat cells but not by wild-type Jurkat cells with a Kₘ of 3.7 μM. β-Casomorphin₁₋₅ appeared to be a natural substrate of CD26/DPPIV, because it inhibited production of fluorescence competitively (Kᵢ = 60 μM). The microwell plate assay appeared to be an efficient system to determine whether specific peptides are natural substrates of a specific protease or not and vice versa whether fluorescent synthetic substrates are good substrates to compete with established natural substrates (BioTechniques, submitted).

Chapter 8 gives an overview of the different functions of the multifunctional or moonlighting protein CD26/DPPIV. CD26/DPPIV exerts its different functions depending on cell type and intracellular or extracellular conditions in which it is expressed. All its known functions are discussed in relation to physiological and pathophysiological conditions. The protein is a
proteolytic enzyme, receptor, costimulatory protein, and is involved in apoptosis. The CD26/DPPIV protein plays a major role in the immune response. Abnormal expression is found in the case of autoimmune diseases, HIV-related diseases and cancer. Natural substrates for CD26/DPPIV are involved in immunomodulation, psycho/neuronal modulation and physiological processes in general. Therefore, targeting of CD26/DPPIV and especially its proteolytic activity has many therapeutic potentials. On the other hand, there are homologous proteins with overlapping proteolytic activity, which thus may prevent specific modulation of CD26/DPPIV. It is concluded that CD26/DPPIV is a protein present in various cellular compartments and extracellularly where it exerts different functions and thus is a true moonlighting protein (Eur. J. Cell Biol. 82:53-73, 2003).

Finally, chapter 9 is a general discussion to put the findings presented in this thesis on visualization of functions of the moonlighting protein CD26/DPPIV using digital microscopy into perspective of biocomplexity and the dynamics of living cells and tissues.
Hoofdstuk 1 geeft een overzicht van de probleemstellingen die in dit proefschrift worden behandeld. De manier waarop enzymactiviteit wordt gemeten, staat ver van de werkelijkheid zoals die zich in vivo voordoet, wanneer activiteit gemeten wordt in homogenaten of sterk verdunnde oplossingen waardoor de complexe interacties, die zich voordoen in levende cellen, verloren gaan. Bij deze benadering kan de de rol van belangrijke factoren, die uiteindelijk activiteit van enzymen in intacte cellen bepalen, verloren gaan. Vele eiwitten zoals enzymen functioneren in complexen, waarin interacties met eiwitten en andere soorten macromoleculen een grote rol spelen in de regulatie van hun activiteit. Verder kunnen eiwitten meerdere functies hebben die separaat in ruimte en tijd gereguleerd moeten worden. Dit fenomeen wordt moonlighting genoemd, en voegt een extra dimensie toe aan de complexiteit van de levende cel. Hoe moonlighting eiwitten functioneren is echter nauwelijks bekend. Visualisatie van de verschillende functies van eiwitten in levende cellen kan daarom een goede manier zijn om de complexe regulatie en functionering van moonlighting eiwitten te begrijpen.


Hoofdstuk 3 beschrijft de reactiviteit van twee fluorogene substraten [Ala-Pro]-cresyl violet en Ala-Pro-rhodamine 110 voor microscopische detectie van protease activiteit van CD26/dipeptidyl peptidase IV (DPPIV) in levende cellen met het doel de specificiteit van de Ala-Pro bevattende substraten voor de detectie van CD26/DPPIV activiteit te bepalen. DPPIV activiteit is een van de vele functies van het moonlighting eiwit CD26/DPPIV. Als model hebben we Jurkat cellen gebruikt (T cellen die niet CD26/DPPIV tot expressie brengen) en Jurkat cellen die met CD26/DPPIV zijn getransfecteerd. Ala-Pro-rhodamine 110 substraat is niet fluorescent, maar na
proteolytische afsplitsing van Ala-Pro fluoresceert rhodamine 110 wel. [Ala-Pro]-cresyl violet is wel fluorescent, maar proteolytische afsplitsing van Ala-Pro induceert een shift in fluorescentie van cresyl violet naar langere golflengte. Dit fenomeen maakt het mogelijk om zowel locale (intracellulaire) concentraties van substraat [Ala-Pro]-cresyl violet als product (cresyl violet) te bepalen wat belangrijk is voor de analyse van de kinetiek van enzymatische reacties. [Ala-Pro]-cresyl violet maar niet Ala-Pro-rhodamine 110 bleek specifiek te zijn voor CD26/DPPIV. Ala-Pro-rhodamine 110 werd ook door andere proteases gesplitst. Aangetoond werd dat, als de microscopische analyse wordt uitgevoerd gedurende de eerste minuten van de enzymreactie, DPPIV aktiviteit nauwkeurig kan worden bepaald in cellen met [Ala-Pro]-cresyl violet substraat. Fluorescerend product bleek snel geïnternaliseerd te worden in granula in getransfecteerde Jurkat cellen direct onder de celmembran en werd dan intracellulair geredistribueerd zoals ook beschreven is voor CD26/DPPIV. Geconcludeerd is dat [Ala-Pro]-cresyl violet een goed fluorogeen substraat is voor de localisatie van DPPIV activiteit in levende cellen, mits de juiste golflengten worden gebruikt voor excitatie en emissie en microscopische beelden in de vroege stadia van de enzymreactie worden gemaakt. Vorder concluderen we dat de eigenschappen van een fluorofoor een grote invloed kunnen hebben op de interacties tussen een synthetisch substraat en de enzymatische groeve van een protease (J. Histochem. Cytochem. 2003, in press).

Hoofdstuk 4 beschrijft een nieuw ontwikkelde methode om enzymactiviteit in individuele levende cellen te bestuderen m.b.v. flowcytometrie. Omdat enzymactiviteit vaak een heterogeen karakter ten toon spreekt in celpopulaties, is flowcytometrie een ideaal gereedschap om enzymactiviteit kwantitatief te analyseren in individuele levende cellen. Voorts kan enzymactiviteit per individuele cel gekoppeld worden aan andere cellulaire parameters, zoals de hoeveelheid DNA in het geval van polyploidie, of de hoeveelheid aanwezig enzymewit zoals kan worden gedetecteerd met immunocytochemie. Op deze wijze kan bijvoorbeeld posttranslationale regulatie van enzymactiviteit worden bestudeerd. In tegenstelling tot fluorescentie microscopy waarbij reeksen van beelden in de tijd worden gemaakt van cellen om daarin enzymactiviteit te detecteren, wordt bij flowcytometrie elke cel slechts op één tijdstip tijdens de enzymincubatie gemeten, namelijk op het moment dat de cel door de laserstraal gaat. Dit heeft als voordeel dat er geen artefacten, samenhangend met fluorescentie metingen zoals fading optreden (Cytometry: Cytomics, proteomics, genomics. Cytometry CD vol. 6. Multimedia Knowledge, Inc. www.mmke.com in conjuction with Purdue University Cytometry Labs, New York).

Hoofdstuk 5 beschrijft de eigenschappen van [Ala-Pro]-cresyl violet als substraat voor CD26/DPPIV voor de localisatie en kwantificering van DPPIV protease activiteit in vers geïsoleerde hepatocyten van ratten, gebruikmakend van confocale microscopy, image analysis en flowcytometrie. DPPIV activiteit werd exclusief gelocaliseerd in structuren op de plasmamembraan die oorspronkelijk het apicale deel van de plasmamembraan, ofwel de galcanaliculaire membraan, vormden. Series van microscopische beelden werden kwantitatief geanalyseerd voor DPPIV activiteit per individuele cel m.b.v. image analysis. De enzymreactie bleek niet lineair in de tijd te zijn. Door de initiële reactiesnelheid te berekenen op basis van de productie van fluorescentie in de tijd, was het mogelijk om de DPPIV activiteit per hepatocyt te bepalen.
Productie van cresyl violet-afhankelijke fluorescentie per individuele hepatocyt gaf eenzelfde beeld te zien wanneer de cellen met behulp van flowcytomietrie werden geanalyseerd. Een dipeptide fosfonaatremmer remde de productie van fluorescentie op een competitieve manier met een $K_i$ van 7 μM. $K_m$ waarden van individuele hepatocyten varieerden tussen 6-22 μM, afhankelijk van de rat waarvan ze afkomstig waren. $V_{max}$ waarden varieerden tussen 4-16 nU. $K_m$ en $V_{max}$ waarden per individuele rat waren omgekeerd evenredig aan elkaar wat duidt op een posttranslationele regulatie van de kinetische parameters van DPPIV. Deze relatie ging verloren als membraanfracties van dezelfde hepatocyt suspenesies werden geanalyseerd (Anal. Biochem. 252:71-77, 1997).

Hoofdstuk 6 beschrijft dat de regulatie van twee van de functies van CD26/DPPIV, de signaaltransductie functie en de proteolytische functie, onafhankelijk is. Om te onderzoeken hoe deze twee functies van een en hetzelfde molecuul worden gereguleerd, hebben we CD26/DPPIV eiwitexpressie en DPPIV activiteit simultaan gelocaliseerd op levende humane T-helper 1 (Th1) en T-helper 2 (Th2) cellen die verschillende expressieniveaus van CD26/DPPIV hebben. DPPIV activiteit werd specifiek gemeten met het synthetische substraat [Ala-Pro]$_2$-cresyl violet en CD26/DPPIV eiwitexpressie met een FITC-geconjugueerd antilichaam dat specifiek is voor CD26/DPPIV. Fluorescentie van vrijgekomen cresyl violet (rood) en gebonden FITC (groen) werd tegelijkertijd gemeten in levende T-cellen met behulp van flowcytomietrie, spectrofluorometrie en confocale microscopic. Th1 cellen brengen drie tot zes keer zo veel CD26/DPPIV eiwit tot expressie als Th2 cellen. De signaaltransductie functie van het CD26/DPPIV complex, getest als co-stimulatie voor proliferatie, was direct gecorreleerd aan de hoeveelheid CD26/DPPIV eiwit op het celoppervlak. Echter, DPPIV activiteit bleek ongeveer dezelfde te zijn in beide typen cellen als deze werden geïncubeerd met fysiologische substraatconcentraties als gevolg van variatie in $V_{max}$ en $K_m$ waarden van DPPIV op Th1 en Th2 cellen. Western blotting en zymografie van Th1 en Th2 membraanfracties vertoonden eenzelfde beeld. Deze studie laat zien dat twee functies van een en hetzelfde (moonlighting) eiwit onafhankelijk gereguleerd kunnen worden (J. Histochem. Cytochem. 50:1169-1177, 2002).

Hoofdstuk 7 beschrijft een nieuw ontwikkelde assay voor de identificatie van natuurlijke substraten van enzymen. Proteolyse van bio-actieve peptiden is een regulerende stap in vele fysiologische processen, maar welk protease op welke (intra-)cellulaire locatie betrokken is bij de activatie of afsplitsing van een specifiek peptide is nauwelijks bekend. Wij ontwikkelden een snelle microwell plate assay bestaande uit levende cellen en een fluorogeen protease substraat om te bepalen welke bio-actieve peptiden natuurlijke substraten zijn voor een specifiek protease met het multifunctionele moonlighting eiwit CD26/DPPIV als model. CD26/DPPIV katalyseert specifiek de afsplitsing van een dipeptide van de aminoterminus van een eiwit met als voorlaatste aminozuur proline. Vele biologisch actieve peptiden, zoals β-casomorphin$_{1-5}$, bevatten proline in de voorlaatste positie. We inicubeerden Jurkat cellen, T-cellen die CD26/DPPIV expressie missen, en T-cellen getransfecteerd met CD26/DPPIV in de aanwezigheid van Ala-Pro-rhodamine 110 en β-casomorphin$_{1-5}$ in 96-wells platen in een standaard plaatreader en in aanwezigheid van [Ala-Pro]$_2$-cresyl violet en β-casomorphin$_{1-5}$ in een spectrofluorometer. Fluorescerend cresyl violet
werd gegenereerd door CD26/DPPIV getransfecteerde cellen, maar niet door wild type Jurkat cellen, met een $K_m$ van 3.7 µM. β-Casomorphin$_{5-9}$ bleek een natuurlijk substrate voor CD26/DPPIV, omdat het de vorming van fluorescentie competitief remde ($K_i = 60$ µM). De microwell plate assay, gebruikmakend van levende cellen en fluorescerende substraten, bleek een efficiënt systeem te zijn om te bepalen of een specifiek peptide een natuurlijk substrate is voor een specifiek protease en omgekeerd, of een fluorogeen synthetisch substrate een goed substrate is om met natuurlijke substraten te competeren (BioTechniques, submitted).


Als laatste hoofdstuk is hoofdstuk 9 een algemene discussie van dit proefschrift betreffende de visualisatie van functies van het moonlighting eiwit CD26/DPPIV met behulp van digitale microscopie om deze functies in het perspectief te plaatsen van de biocomplexiteit en dynamiek van levende cellen.
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Lieve Trees, ik moet er niet aan denken dat ik alles zelf had moeten schrijven en corrigeren. Jouw geduld en precisie voor de kleinste details heeft mij veel werk uit handen genomen, zeker toen ik er maar één ter beschikking had. Als ik iets aan je overliet dan behoefde ik mij er absoluut geen zorgen over te maken, want een kanjer zoals jij kom je maar zelden tegen, laat staan dat ze er zo precies een puntje aan zuigen.

Beste schoonheid (Jan Peeterse), vaak heb ik je het moeilijk gemaakt, maar wat wil je, 4D imaging op een plat A4’tje, en dan ook nog gisteren klaar, want toen was ik natuurlijk weer eens te laat voor een presentatie. Je bent werkelijk de meester van cut, cut, cut, en...... paste, en wist mijn afgrijzelijke figuren en beelden om te toveren tot het niveau van Karel Appel (ik bedoel dus niet van Oven, en ook niet zo’n apparaat met vage programma’s die OOOOOOH zo handig zijn maar niet te vinden op je desktop, laat staan dat ze een verbinding hebben met een printer en bovendien kleurenblind zijn). Dat overkwam Olaf tenminste, en was de enige keer dat hij uit z’n dak ging en die ik helaas heb gemist. Ik hoop dat je nog een paar cp’tjes over hebt voor Olaf, want ook hij moet nog een boekie publiceren in kleur.

Beste Ron, ik heb je leren kennen op de cursus die ik destijds volgde. Het practicum enzymatische technieken was mij wel bevallen door de geringe hoeveelheid tijd v/d incubaties, hooguit een uur. Aangezien ik liever lui dan moe ben, dacht ik, dat is een goede keuze voor een stage. Bovendien waren we vanaf het begin zeer goed bevriend, waarschijnlijk door onze gemeenschappelijke interesses (vrouwen, drinken, vertier en de flair en aanzien van het wetenschappelijke onderzoek). Jouw motivatie heeft dan ook het beste in mij doen ontspruiten. Ik denk dat het een van jouw grootste kwaliteiten is om mensen te stimuleren en te interesseren in de wetenschap. Gedurende de periode die op de stage volgde, mijn promotie onderzoek, heb ik je het echter niet makkelijk gemaakt. Ik denk dat de wanhoop die ik je vaak bezorgde wel tot enige slapeloze nachten heeft geleid. (Raar eigenlijk dat je me hebt aangenomen nadat ik je in de kou liet staan in Groesbeek, tijdens een van onze onvergetelijke congressen). Gelukkig ben je vergevingsgezind en hebben we het tot een goed en vruchtbare einde weten te brengen, al dan niet postranslationeel (ik bedoel dat mijn spelling en omschrijvingen vaak naderhand door jou moesten worden vertaald opdat datgene wat ik uitkramde voor een ieder begrijpelijk werd). Je hebt me geleerd om door te zetten en niet na een paar pogingen op te geven. Een eigenschap die zeer nuttig is voor een losbandige jonge man, en mij hopelijk lang zal heugen.
Lieve Maril, ouwe spek trekkerd. ik hoop dat ook jij het licht zult zien, al dan niet gepolariseerd door gestrekt varkenscollageen. Het is lastig keuzes maken in het leven. De wetenschap zei je eerst vaarwel om een carrière te beginnen bij het ministerie. Maar na enige tijd kwam je toch weer sporadisch bij ons op de afdeling klussen. Hopelijk vind je tijd voor beide zodat je uitgebalanceerd door het leven heen kan wedelen.

Beste Wilma, G6PDH heeft en speelt een grote rol in je leven, het is werkelijk een grote verdienste dat je na al die tijd dat je naar dit enzym kijkt elke keer weer met frisse moed en inspiratie dit wetenschappelijke fenomeen te lijf gaat. Petje af, zeker nadat je zelf O₂ ongevoelig werd verklaard. Je wist onze chef altijd af te remmen als hij weer eens te kort door de bocht ging (vandaar dat hij geen rijbewijs heeft) en staat garant voor een degelijk stuk wetenschap waar geen speld tussen te krijgen is.

Lieve Klazien, ik geloof dat we samen alleen de cursus hebben gedaan, en wat buffers gemaakt, maar zoals je al langer weet, ben ik een vreemde eend in de bijt en heb daarom helaas nooit (voor jou waarschijnlijk gelukkig) samen mogen werken met jou. Ik hoop dat je je met de cellen ploffert aan je zijde echter wel in de richting van de bijna nog net levende cleytochimie zult verplaatsen, opdat je de cacodylaat en andere troep niet meer hoeft te hanteren, en de fluorescentiemicroscopie op het gebied van de enzymkinetiek zal begeren.

Ex roomies, Heleen, Wikky, Kees en Cars, harde werkers van het eerste uur, jullie gezelschap en ervaring heeft me altijd leren beseffen dat er vele wegen zijn die naar Rome leiden, helaas hebben jullie zelf ook niet altijd de snelste routebeschrijving gekregen, maar zijn toch altijd weer in staat geweest om in de oude stad te arriveren. Petje af voor al dat werk, al dan niet in een kamer zonder licht, of met plakband in de weer om toch maar het beoogde resultaat te mogen boeken.

P.S. Cars, voor de decoupeerzaag zijn ook ijzerzaagjes te verkrijgen tegenwoordig!

Beste Ard, je bent een ware kunstenaar, vandaar dat jouw voorkeur uitgaat naar een appel. Voor ieder biologisch probleem ben je in staat om een digitale analoog op het scherm van de apple te toveren. Ik heb samen met jou met veel genoegen aan onze eerste digitale microscoop gesleuteld opdat we alles via de computer aan konden sturen, zoals belichtingstijd, interval en minimale incubatietijd voor levende cellen, het was een waar genoegen om jou de elektronica te zien implementeren waar ik geen snars van begrijp.

Carel, Jan (met de korte achternaam), Ron Hoebe en Jacob Aten, jullie zijn echte techneuten, altijd op zoek naar de sublieme oplossing. Jan, ik weet dat ik je een stukje biologie heb laten zien waar je nog steeds nachtmerries van krijgt. Maar zo werkt het nu eenmaal met cellen die je van oude lui aftapt. De ene doet het beter dan de andere, en dat kun je nou eenmaal niet standaardiseren. Beste Carel, flow-jo, ik weet niet wat er gebeurd is, maar waar is in godsnaam dat filter gebleven? Is ie soms ‘go with the flow’? Want daarna is het nooit meer gelukt om een mooie enzymreactie te visualiseren. Lag het aan de gevoeligheid van de nieuwe apparatuur, de modificatie van mijn substrate, voor mij is het een groot raadsel. Lang leve de techniek, een van jullie grootste passies, maar let wel, jullie moeten publiceren om je bestaansrecht te verifiëren!

Beste Ton, je bent een echt lab beest. Werken met jou is altijd een feest. Resultaat-belust en altijd van de moeilijkheden bewust heb je me veel geholpen en geleerd. Jouw nuchtere kijk (van 9 tot 5 tenminste) heeft mij veel geholpen en zaken gesimplificeerd, absoluut Ton, niet verkeerd. Zou Vincent het je zo hebben geleerd, of ben je gewoonweg zo door je ouders geïncubeerd? In ieder geval, koop die nieuwe Skoda toch maar niet, ik heb gezien dat ze snel hun waarde verliezen, dus als je er per se één wilt, koop dan een gebruikte, bijv. een V6 TDI’tje kost een vermogen, maar na 5 jaar minder dan je voor charisma hebt betaald. PS ook leverbaar met zwart leer.
(Schelte-like). Denk je maar eens in, elke dag in het leer naar je werk, naar huis, naar je beste vriend, je fluit op de leren achterbank, etc, een groot feest.

Beste Eddy, ook al hebben T-lymfocyten volgens jou geen lysosomen, ze zitten wel bomvol met kleine organel len die daar verdomd veel op lijken. Ik denk dat de naamgeving echter enige verwarring heeft veroorzaakt in de immunologie, een wetenschap die kijkt naar het sturen van de immuun respons, en dus niet naar het basisprincipe van iedere levende cel. (Vraag Ron nog maar eens om het je uit te leggen) Ik hoop dat je nog veel vliegtuigen mag spotten in verre oorden, maar peil wel effe of de olie voordat je weer vertrekt, want na 250.000 km zitten de zuigers wat losser in hun jas! P.S. Welk nummer staat er eigenlijk op de bezemsteel van je vriendin?

Beste Jan (Wormmeester), trouwe vriend en drinkmaat. (Bepaal jij dat!) Je bent zeker een goede vriend geworden in al die jaren dat ik je ken (1991-nu). Ook met jou heb ik altijd veel kunnen lachen, zelfs toen we onderste boven lagen met mijn bootje in de gracht in het midden van de nacht. Arme Marco, zijn proefschrift hebben we moeten drogen en ook de floppies deden het gelukkig weer dank zij jou! Als er iemand op de afdeling is die altijd paraat is om voor wat voor een probleem dan ook een oplossing te zoeken, dan ben jij het wel. Zelfs nu dat ik in Italië zit help je me nog! Het enige probleem waar je geen raad me wist was de dierenarts in spe, ik hoop dat ook zij weer een beetje kan lachen om al de fratsen die ik heb uitgehaald.

Beste Joost, Robert, Hermelijn en alle andere immuno’s, ik heb jullie vaak in de maling genomen, maar altijd met een knipoog. Oh wat heb ik lachen bij het isoleren van de proteosomen, maar kennelijk is er niks mis met de motilitiet van mijn cellen. Zelfs na 500 G gingen ze gewoon weer aan de haal. Je moet nu eenmaal dat overhebben voor de wetenschap. Ook denk ik het van veel genoegen terug aan het dagje uit, alwaar onze sjef het voor je (Robert) opnam. Ik hoop dat jullie nog veel interleukines zullen ontdekken en hun RNA’s mogen sequencen.

Beste Dave, Fred en de dames van de afdeling Biochemie, dank jullie wel voor de ondersteuning. Jammer genoeg hebben we nooit een van onze hilarische experimenten mogen publiceren, maar dank zij vallen en opstaan worden we nu eenmaal wijzer. Ik hoop dat jullie na het lezen van dit proefschrift eindelijk begrijpen wat ik al die jaren op jullie afdeling heb gedaan. Dank jullie wel voor de bereidheid om mij te helpen, ik bezocht jullie afdeling altijd met veel genoegen en voelde me altijd gesteund door jullie professionaliteit.

Leica Microscopical Systems, Dr. Werner Knebel, Filipppe Clauws en Jan worden hartelijk bedankt voor hun medewerking. Zonder uw medewerking was het nooit mogelijk geweest om zeer specifiek zowel het substraat als het product te detecteren in de confocaal microcoop. Ook al was onze samenwerking van zeer korte duur, het mag zeker een zeer succesvolle samenwerking worden genoemd.

De boys van de afdeling voor microscopisch onderzoek, Jan van Marle en Henk van Veen wil ik ook hartelijk bedanken. Zonder hun technische ondersteuning zou ik nooit wijs zijn geworden in het aansturen van de confocaal. Zeker in het begin, bij al die rare commando’s zoals “prompt off” en “hash on” had ik hele andere ideeën.

Als laatste wil ik graag de dames en heren van de Epstein bar bedanken, alwaar de harde kern van de bovengenoemde wetenschappers hun dorst lessen op de donderdagmiddag, inclusief mezelf.

Ik weet echter niet of het barpersoneel het erg vindt dat ik niet meer langs kom.
Curriculum Vitae
