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Chapter 6

The hepatocyte growth factor/Met pathway in development, tumorigenesis, and B-cell differentiation.

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Chapter 6

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I. INTRODUCTION

Hepatocyte growth factor/Scatter factor (HGF), originally described as a strong mitogen for hepatocytes (Michalopoulos et al., 1984; Nakamura et al., 1984; Russell et al., 1984a, 1984b), is a multifunctional cytokine with a domain structure and a proteolytic mechanism of activation similar to that of the blood serine protease plasminogen. Unlike plasminogen, however, HGF is devoid of protease activity but has pleiotropic effects on target cells, including stimulation of growth, motility, and morphogenesis. All known biological effects of HGF are transduced via a single receptor, i.e., Met, the product of the Met proto-oncogene. The Met protein is a receptor tyrosine kinase and is the prototype of a distinct subfamily, also encompassing Ron and Sea. Upon ligand binding, Met interacts with several cytoplasmic target proteins resulting in activation of a number of distinct signaling cascades including the Ras/MAP kinase and PI3-K/PKB pathways. In addition to binding Met, HGF has a high affinity for heparin and heparan sulfate. Heparan sulfate is present on the cell surface and in the ECM, in the form of heparan sulfate proteoglycans (HSPGs). By binding HGF, HSPGs function as co-regulators of Met signaling.

Genetic studies in mice have indicated that HGF is indispensable for mammalian development, as mutations of the HGF or Met genes cause abnormal development of the liver and placenta, and disrupt the migration of myogenic precursors into the limb bud. Other studies have provided evidence for important roles of HGF in angiogenesis, and in the three dimensional organization of kidney
tubular cells and various glandular structures, e.g. mammary glands. Apart from mediating these physiological functions, the HGF/Met pathway is also believed to play a key role in tumor growth, invasion, and metastasis. For example, Met was originally isolated as the product of a human oncogene Tpr-Met and Met and/or HGF overexpression have been reported in several human tumors. The tumorigenicity of HGF/Met signaling has been confirmed in transgenic mouse models, which develop tumors in many different tissues. In human hereditary papillary renal carcinomas, potentially activating Met mutations are found.

In this review, we discuss the structure, signal transduction, and physiological functions of the HGF/Met pathway, as well as its role in tumorigenesis. Furthermore, we highlight recent studies which indicate a role for the HGF/Met pathway in antigen-specific B cell differentiation and B cell neoplasia.

II. STRUCTURE AND FUNCTION OF HGF AND MET

A. Structure of HGF and Met

1. Structure of HGF

Hepatocyte growth factor/scatter factor (HGF) was independently identified by groups working in two different fields of research. In 1984, a factor present in serum of partially hepatectomized rats and in rat platelet lysates, was found to have a strong mitogenic effect on hepatocytes (Michalopoulos et al., 1984; Nakamura et al., 1984; Russel et al., 1984a, 1984b). Hence, this factor was designated hepatocyte growth factor (HGF). Almost simultaneously, Stoker and Perriman (1985) identified a molecule secreted by fibroblasts, which causes dissociation or "scattering" of epithelial cell colonies, and was thus named scatter factor (SF). Subsequent structural and functional studies showed HGF and SF to be identical (Gherardi and Stoker 1990; Weidner et al., 1990, 1991; Furlong et al., 1991; Konishi et al., 1991; Naldini et al., 1991c; Rubin et al., 1991).

The mature HGF protein has a relative molecular mass of 90 kDa under non-reducing conditions and is a heterodimer composed of an α-subunit of 60 kDa and a β-subunit of 30 kDa linked together by a disulfide interchain bridge (Nakamura et al., 1987, 1989; Weidner et al., 1990; Rubin et al., 1991). Due to differential glycosylation, two β-chains, of ~34 kDa and ~32 kDa, respectively, can generally be detected. HGF is secreted as a biologically inactive monomer which is activated through proteolytic cleavage (Naka et al., 1992). Several proteases have been shown to be able to activate HGF. These include urokinase-type (uPA) and
Figure 1. Schematic representation of the HGF protein. HGF is a secreted glycoprotein composed of a 60 kDa α-chain and a 30 kDa β-chain linked by a disulfide bridge. The α-chain contains an NH$_2$-terminal domain with a hairpin loop (HL), and 4 kringle domains (K1-4). The β-chain is homologous to the protease domain of plasminogen, but has no catalytic activity due to the lack of several essential amino acids.

tissue-type (tPA) plasminogen activator, proteases known to function in blood clotting and extracellular matrix (ECM) breakdown, blood-coagulating factor XIIa, and two new serine proteases, i.e. HGF activator and HGF converting enzyme (Naldini et al., 1992; Mars et al., 1993; Miyazawa et al., 1993; Mizuno et al., 1994; Shimomura et al., 1995). Recently, a negative regulator of HGF activation was identified, underscoring the complexity of this activation process (Shimomura et al., 1997).

The full length human HGF cDNA encodes a protein of 728 amino acids (Fig. 1) (Nakamura et al., 1989). Its amino acid sequence predicts translation as a precursor protein, which becomes activated by proteolytic cleavage at an Arg-Val cleavage site. This cleavage results in the above mentioned α and β-chains. Furthermore, the cDNA sequence contains 4 putative N-linked glycosylation sites. Interestingly, significant homology was found between HGF and plasminogen. Like this serine protease, the α-chain of HGF has 4 kringle domains, structures that play a role in protein–protein interaction. The β-chain shows high homology with the catalytic domain of plasminogen, but, due to the lack of 2 crucial amino acids from the active site, HGF has no proteolytic activity.
Several structurally different HGF transcripts were shown to exist. For instance, in cultured human fibroblasts, Northern blotting revealed 3 HGF mRNAs of 6, 3 and 1.5 kb, respectively (Chan et al., 1991; Miyazawa et al., 1991b; Rubin et al., 1991; Weidner et al., 1991). Molecular cloning and Northern blotting indicated that the 6 and 3 kb messages emanated from differential polyadenylation (Weidner et al., 1991). The 1.5 kb mRNA represents a splice variant encoding the N-terminal domain of HGF in combination with the first 2 kringle domains (Chan et al., 1991; Miyazawa et al., 1991b). This variant, NK2, behaves as an HGF antagonist (Chan et al., 1991). The subsequently described one kringle domain variant, NK1, functions as a partial HGF agonist (Cioce et al., 1996; Jakubczak et al., 1998). In addition to these two variants, a putative splice variant containing a deletion of 15 nucleotides in the first kringle domain has been described (Rubin et al., 1991; Weidner et al., 1991). This deletion results in a change of the biological activity of HGF, presumably caused by a change in its tertiary structure (Shima et al., 1994).

Genomic studies have revealed that human HGF is encoded by a single gene localized on the long arm of chromosome 7, band 21.1 (Weidner et al., 1991; Saccone et al., 1992). The gene spans about 70 kbp of DNA and contains 18 exons.
Figure 3. Schematic representation of the receptor tyrosine kinase Met. The receptor is composed of two disulfide-linked chains: a 50 kDa α-chain and a 145 kDa β-chain. The β-chain contains the tyrosine kinase domain (TK) and a ‘docking site’ (DS) which interacts with signaling molecules.

(Miyazawa et al., 1991a). The promoter region contains a number of regulatory sequences, including a TATA-like element, an IL-6 responsive element, and a potential binding site for nuclear factor-IL-6, a regulator of IL-6 expression. Also, wild-type, but not mutant, p53 was shown to activate the HGF promoter (Metcalf et al., 1997).

Han and colleagues (1991) identified a gene which shared about 50% sequence homology with HGF. The molecule was designated hepatocyte growth factor-like protein (HGFL), but was subsequently shown to be identical to macrophage stimulating protein (MSP) (Yoshimura et al., 1993), a molecule involved in macrophage chemotaxis and in phagocytosis (Skeel et al., 1991). Structural analysis suggests that HGF and MSP, together with plasminogen and apolipoprotein (a) have evolved from a common ancestral gene (Fig. 2) (Donate et al., 1994).
2. Structure of Met, the high affinity receptor for HGF

Met, the receptor for HGF, was originally identified as the product of an oncogene (Cooper et al., 1984). This oncogene, TPR-Met, results from a chromosomal translocation, fusing the sequence encoding the intracellular domain of Met to that of Tpr (Park et al., 1986), a protein with unknown function. Tpr-Met functions as a constitutively active homodimer with a strong transforming capacity (Cooper et al., 1984; Gonzatti-Haces et al., 1988). Molecular cloning of the 8 kb Met proto-oncogene indicated that this molecule is a cell-surface tyrosine kinase receptor for growth factors (Dean et al., 1985; Park et al., 1987; Rodrigues et al., 1991), whereas functional studies revealed that HGF is the ligand of Met (Bottaro et al., 1991; Naldini et al., 1991b; Rubin et al., 1991).

The Met protein is synthesized as a single-chain 170 kDa precursor. After synthesis, the molecule is cleaved and rearranged into a 190 kDa heterodimer linked by a disulfide bridge (Fig. 3) (Giordano et al., 1989a, 1989b). Komada et al. (1993) demonstrated that Met can be cleaved by furin, but that endoproteolytic processing is not essential for HGF-induced signal transduction. The Met heterodimer is composed of a 50 kDa α-subunit, and an 145 kDa β-subunit (Giordano et al., 1989a). The cytoplasmic tail of the β-chain contains the tyrosine kinase domain and a ‘docking site’, which interacts with multiple signaling molecules (Ponzetto et al., 1994). Both functional domains will be discussed in more detail in Section II.C.

The identification of Tpr-Met resulted in the assignment of the human Met gene to chromosome 7, band q31 (Cooper et al., 1984; Dean et al., 1985; Lin et al., 1996). The gene spans more than 110 kbp and contains 21 exons (Duh et al., 1997; Lin et al., 1998; Liu, 1998) The sequence of the Met promoter region revealed a number of binding sites for regulatory elements, including AP1, AP2, NF-κB, and, like the HGF gene, IL-6RE (Liu, 1998). Recently, wild-type, but not mutant p53, was shown to enhance the activity of the Met promoter (Seol et al., 1999), as had been shown before for the HGF gene.

Two receptor tyrosine kinases related to Met, i.e. Sea (Huff et al., 1993) and Ron (Ronsin et al., 1993), have been identified. MSP was shown to be the ligand for Ron (Gaudino et al., 1994; Wang et al., 1994), whereas Sea remains, as yet, an orphan receptor. In addition, Met shows homology with the putative receptor tyrosine kinase stem cell-derived tyrosine kinase (STK) and with the SEX family of transmembrane proteins (Iwama et al., 1994; Maestrini et al., 1996).
3. Low affinity receptors for HGF

Apart from binding to Met, HGF also binds to heparan sulfate proteoglycans (HSPGs). These interactions, which appear to play an important role in the regulation of HGF activity, will be discussed in Section II.D.

B. Expression and functions of HGF and Met

1. Introduction

The receptor tyrosine kinase Met is prominently expressed on a wide variety of epithelial cells, whereas its ligand, HGF, is expressed by stromal cells. This reciprocal expression pattern points to their important role in epithelial-mesenchymal interactions underlying branching morphogenesis and tubulogenesis during development of organs such as lungs, kidney and mammary glands. Over the past few years, it has become clear, however, that HGF and Met are also involved in a plethora of other biological processes. In the next paragraphs we will give an overview of the well established expression pattern and functions of HGF and MET, as well as of those attributed more recently. It places HGF and Met in the center of developmental processes, leading to a proper organization not only of epithelial tissues, but also of muscle, endothelium, and the nervous and haematopoietic systems.

2. Expression pattern during amphibian, avian and mammalian development

During embryogenesis of the tadpole Xenopus laevis, Met is present as early as in the gastrula stage and remains expressed at high levels throughout neurulation (Aoki et al., 1996). Sites of expression include the foregut region, tailbud mesenchyme, and, in neurulating embryos, neural tissues. HGF expression becomes apparent later, from the neurula stage onwards. The spatiotemporal expression pattern of both HGF and Met point to multifarious roles in amphibian organogenesis. This has been shown more specifically by use of dominant-negative Met constructs, introduced into fertilized Xenopus eggs. Embryos thus treated fail to develop a normal liver, whereas organogenesis of the gut and early kidney are greatly impaired (Aoki et al., 1997). Hence, in Xenopus embryos, a functional HGF-Met system is involved in early organogenesis, especially of organs derived from the primitive gut.

Interference with the HGF-Met system during early chick embryo development leads to abnormal axis formation, underscoring its determining role
during avian development, especially in neural induction and limb bud elevation (Stern et al., 1990). HGF, but not MET, is expressed in the mesoderm of the limb bud and in the central core region of mandibular arch and maxillary processes at stages 17 to 24 of development (Myokai et al., 1995; Théry et al., 1995). During limb bud extension, HGF is expressed in the mesenchyme and becomes later confined to the ventral and subapical mesenchyme of the limb bud, suggesting that HGF production in the limb bud is involved in the induction and maintenance of apical ectoderm during limb bud development (Myokai et al., 1995).

During embryonic development of rodents, HGF is prominently expressed in a multitude of tissues, mainly at sites where epithelial/mesenchymal interactions determine organogenesis (Iyer et al., 1990). In gastrulating mouse embryos, the expression of HGF and Met overlaps. Initially, the two genes are expressed in the endoderm and in the mesoderm along the rostro-intermediate part of the primitive streak and, later, in the node and in the notochord. Neither HGF nor Met is expressed in the ectodermal layer throughout gastrulation (Sonnenberg et al., 1993; Adermarcher et al., 1996). During early organogenesis, overlapping expression of HGF and Met is found in the heart, condensing somites, and neural crest cells. However, a second and distinct pattern of expression, characterized by the presence of the ligand in mesenchymal tissues and the receptor in the surrounding ectoderm, is seen in the bronchial arches and in the limb buds. At E13, only this second pattern of expression is observed in differentiated somites and several major organs, such as the lungs, the liver, and the gut (Adermarcher et al., 1996). The expression of the HGF and Met genes throughout embryogenesis suggests a shift from an autocrine to a paracrine signaling system. Halfway gestation, HGF is present in renal collecting tubes of the kidney, in the liver, in esophageal and skin squamous epithelium and in bronchial epithelium (Defrances et al., 1992; Lee et al., 1993). HGF is also detected in brain, somites, haematopoietic cells, and chondrocytes (Defrances et al., 1992).

Similar patterns of Met and HGF expression are found along human embryonic development. From the 5th week of gestation onwards, placental tissue highly expresses HGF and Met. HGF is secreted by amniotic epithelium, the placental villi and the villous core mesenchyme, whereas Met is present on the trophoblast and vascular endothelium (Kauma et al., 1997; Somerset et al., 1998; Wolf et al., 1991). A human pathological condition, known as intra-uterine growth restriction, is associated with an underdeveloped placenta and could be linked to a decreased secretion of HGF by the villous stromal cells (Somerset et al., 1998). The absolute dependence of placenta maturation on HGF has been unequivocally shown in HGF null mutant mouse embryos, whose placenta fail to develop properly and which die in utero (Schmidt et al., 1995; Uehara et al., 1995). From week 6-13 of gestation, when major organogenesis takes place, HGF and Met are co-expressed in liver, metanephric kidney, intestine, lung, gall bladder and spleen.
(Kolatsi-Joannou et al., 1997; Wang et al., 1994b). In the digestive tract of 7-8 week old embryos, Met is localized in epithelia of the liver, pancreas, esophagus, stomach, the small and large intestine, and in smooth muscle layers, whereas HGF becomes concentrated in mesenchymal tissue and smooth muscle (Kermorgant et al., 1997). Interestingly, HGF expression has also been shown in epithelial tissues in the interval from week 9-17 of gestation, particularly in the crypt region of the small intestine, keratinizing epithelium of the tongue, skin and esophagus (Wang et al., 1994b).

In conclusion, HGF and Met are highly conserved molecules in a wide range of species, not only structurally (see Section II.A), but also with respect to their particular role during embryogenesis. In the next paragraphs some specific

![Figure 4](image)

**Figure 4.** Scattering of MDCK cells induced by HGF. A, HGF treatment leads to dissociation of the islands and to migration of the cells. B, MDCK cells grown in the absence of HGF form islands.
functions of HGF and Met will be discussed. We will focus on branching morphogenesis, muscle development, angiogenesis and neuronal development.

3. Mesenchymal-epithelial interaction and branching morphogenesis

HGF induces scattering of epithelial cells in vitro (Stoker et al., 1987; Uehara and Kitamura, 1992) through activation of Met (Weidner et al., 1993) (Fig. 4). This effect can be mimicked by a constitutively active mutant of Met (Jeffers et al., 1998a), suggesting that activation of Met is sufficient in this process. Once activated, Met can in turn activate PI3K and the Ras-MAPK pathway (Boccaecio et al., 1998; Potempa and Ridley, 1998). Furthermore, enzymes involved in ECM proteolysis (e.g. uPA) are activated (Pepper et al., 1992). Partial ECM proteolysis may increase cell motility by diminishing adhesion properties of epithelial cells towards matrix components. Although scattering in vitro can hardly be considered as its physiological function, the phenomenon *per se* reflects the first phase of epithelial morphogenesis (by activation of MET) through mesenchymal induction (secretion of HGF), underlying the complex, but coordinated formation of branched organs, such as the lungs, the kidney and mammary gland (Sonnenberg et al., 1993).

Tubular differentiation can be induced under "ECM conditions", *i.e.* when epithelial cells are cultured in a 3-dimensional ECM-like environment. Thus, kidney epithelial cells, treated with HGF, form tubules resembling those emanating during kidney organogenesis in early embryonic development (Boccaecio et al., 1998; Cantley et al., 1994; Liu et al., 1998a; Sachs et al., 1996). Ezrin, a member of the ERM family of membrane to cytoskeleton linkers (reviewed in Tsukita and Yonemura, 1997), and a substrate of MET, is involved in the cytoskeletal reorganization associated with tubulogenesis (Crepaldi et al., 1997). Embryonic mesenchymal kidney cells undergo a mesenchymal to epithelial transition, which is accelerated by HGF (Karp et al., 1994). This conversion mimics developmental processes in the metanephros *in vivo*, where mesenchymal specialization is induced by the ingrowth of a branching ureteric bud and is in accordance with expression patterns of Met and HGF during development (Santos et al., 1994; Woolf et al., 1995).

Surprisingly, kidney epithelial cells derived from *Met* null mutant mouse embryos, and hence unresponsive to HGF, were able to form tubular structures *in vitro* and to express epithelial-specific markers after treatment with epidermal growth factor (EGF) (Kjelsberg et al., 1997). An intact HGF-Met pathway may thus not be necessary for kidney development, although it can play an auxiliary role. This opens the possibility that HGF may be involved in kidney epithelial regeneration, rather than embryonic kidney development. Indeed, following renal
injury. HGF expression is elevated (Horie et al., 1994; Igawa et al., 1993; Liu et al., 1999). Moreover, transgenic mice, overexpressing HGF in the kidney die of renal failure, associated with the stimulation of the HGF-Met autocrine pathway (Takayama et al., 1997a). In these mice, kidney pathology is not apparent at birth, but rather develops progressively.

In epithelial cells derived from another branched, lumen forming organ, the mammary gland, HGF treatment leads to the formation of branches and structures resembling mammary gland ducts when cultured in a 3-dimensional matrix (Berdichevsky et al., 1994; Brinkmann et al., 1995; Niemann et al., 1998; Soriano et al., 1995; Yang et al., 1995). In accordance with its role in mesenchymal-epithelial interaction in the mammary gland, Met expression is confined to the epithelial cells lining the mammary ducts, whereas HGF is produced by mammary gland fibroblasts (Niranjan et al., 1995; Tsarfaty et al., 1992; Wang et al., 1994a; Yang et al., 1995). During pregnancy, HGF and Met transcripts are progressively reduced to background levels during lactation, and increase during the phase of involution to pre-pregnancy levels. The reduction in HGF and Met expression corresponds to periods in which functions other than tubulogenesis predominate in the mammary gland: alveolar budding and milk protein synthesis (Pepper et al., 1995). Indeed, treating mammary gland cultures with the milk production inducing hormone prolactine, sharply reduces Met transcript levels (Pepper et al., 1995).

In the developing lung, HGF is expressed in the mesenchyme and Met in the pulmonary epithelium (Ohmichi et al., 1998). Alveolar type II cells, when cultured in the presence of HGF, are induced to proliferate (Mason et al., 1994; Shiratori et al., 1995), whereas tracheal epithelial cells are driven to differentiate into a polarized cell type (Shen et al., 1997). HGF also proved to be a mitogen for bronchial epithelial cells (Singh-Kaw et al., 1995) and furthermore to facilitate the organotypic rearrangement of cultured E15 mouse lung epithelial cells (Sato and Takahashi, 1997) and branching morphogenesis in organ cultures (Ohmichi et al., 1998). Apart from the function in pulmonary development, HGF can act as growth factor in vivo for alveolar type II cells after lung injury and can thus add to the restoration of epithelial integrity (Panos et al., 1996; Yanagita et al., 1993). Its tissue distribution in the developing lung, together with its proliferation and differentiation-stimulating effects, renders HGF a paracrine growth factor in lung development and regeneration.

Pancreatic epithelial cells, as well as pancreas carcinoma cells, are induced to proliferate and differentiate by HGF, forming tubular structures composed of a lumen, lined by polarized epithelial cells (Brinkmann et al., 1995). These cells have characteristics of pancreas ductal epithelia, including apical microvilli (Jeffers et al., 1996a) and the appearance of characteristic markers of normal ductal cells (Vila et al., 1995). HGF further influences pancreatic islet formation and β-cell differentiation, leading to the secretion of insulin (Otonkoski et al., 1994, 1996).
HGF has initially been described as a mitogenic factor for cultured hepatocytes (Michalopoulos et al., 1984; Nakamura et al., 1984) (see also Section II.A) and it has been implicated in embryonic hepatic development. In the liver, HGF is expressed in Ito cells, whereas Met transcripts are strongly expressed by hepatocytes (Hu et al., 1993). After chemical or mechanical liver injury HGF levels sharply increase, leading to a strong hepatocyte proliferation (Horimoto et al., 1995; Hu et al., 1993). Livers from transgenic mice with liver-specific overexpression of HGF are twice the size of livers of control animals and they regenerate much faster after partial hepatectomy (Sakata et al., 1996; Shiota et al., 1994). Apart from their placental phenotype described above, HGF null mutant mouse embryos fail to develop a fully functional liver (Schmidt et al., 1995), demonstrating that the presence of HGF is an absolute requirement during liver organogenesis. In rats with an experimental liver cirrhosis the administration of HGF through autologous gene transfer was shown to have a beneficial effect on overall survival (Ueki et al., 1999). Thus, HGF acts as a paracrine factor for hepatocyte proliferation and differentiation, both during embryonic liver development, as well as during post-trauma regeneration.

HGF and Met are furthermore involved in the proliferation and migration of a wide variety of epithelial cells, and in the morphogenesis of epithelial tissue. In colon epithelial cells, a complete epithelial developmental program is enrolled upon treatment with HGF, including apical/basal polarization and the formation of crypt-like structures (Brinkmann et al., 1995). Prostate stromal cells produce HGF in vivo (Kasai et al., 1996) and prostate epithelial cells, grown in the presence of HGF proliferate and develop tubular structures reminiscent of those found in the prostate (Brinkmann et al., 1995). Other implications of HGF-Met include the development of bone (especially of cartilage) (Blanquaert et al., 1999; Grumbles et al., 1996; Takebayashi et al., 1995), teeth (Tabata et al., 1996), the (male and female) reproductive tract (Depuydt et al., 1996; Naz et al., 1994; Parrott and Skinner, 1998), thyroid (Schulte et al., 1998; Trovato et al., 1998), and the regulation of hair growth (Jindo et al., 1994, 1998; Shimaoka et al., 1995).

During epithelial wound healing, many cellular programs that play a role during embryonic development are re-activated. The HGF-Met axis has been implicated in epithelial wound healing of various epithelia, including gastric, intestinal and corneal epithelia (Nusrat et al., 1994; Takahashi et al., 1995a, b; Wilson et al., 1999).

In conclusion, HGF and Met are involved in tissue-specific programs of differentiation in a wide variety of lumen-forming organs, leading to the formation of contiguous, polarized epithelial cell layers and, depending on the type of tissue, tubulogenesis and branching.
As described above, HGF and Met are already expressed in the developing central and peripheral nervous system (CNS and PNS), but they remain present during adulthood. Embryonic prospective chick neural plate explants, when treated with HGF, differentiate into cells with a neuronal morphology, and start to express neuronal markers (Streit et al., 1995), whereas in transgenic mice that ectopically express HGF, cells of the neural crest lineage become inappropriately targeted (Takayama et al., 1996). Thus, HGF is involved in neural induction, as well as in later stages of neuronal development, when neural cells adopt a migratory phenotype. In the mammalian CNS, Met is abundantly expressed in the neurons of the hippocampus, cerebral cortex, septum, amygdala, pons, olfactory bulb, medulla and spinal cord (Achim et al., 1997; Honda et al., 1995; Jung et al., 1994; Thewke and Seeds, 1999; Wong et al., 1997). During embryogenesis, HGF expression seems to be confined to prospective target cells for the outgrowing neurites. HGF is therefore considered as a chemotactic, e.g. for spinal motoneurons. Furthermore, HGF has been identified as a survival factor for these neurons, and is secreted by their target tissue, muscle, during later stages of development (Ebends et al., 1996; Yamamoto et al., 1997). HGF has been shown to act synergistically with another neurotrophic factor, ciliary neurotrophic factor (CNTF), in motoneuron survival (Wong et al., 1997). During adulthood, HGF remains expressed in the CNS, where it is found, apart from its localization in neurons, in astrocentral glial, ependymal cells, and cells lining the choroid plexus (Honda et al., 1995; Jung et al., 1994). In cultured sympathetic neurons, which express both HGF and Met throughout development, HGF acts as an autocrine axonal outgrowth-stimulating factor, and not as a survival factor (Maina et al., 1998; Yang et al., 1998). However, in the precursor cells of the sympathetic neurons, the sympathetic neuroblasts, HGF does have a stimulating effect on cellular survival, pointing to a shift in the dependence upon HGF from a survival factor to an outgrowth-stimulating factor (Maina et al., 1998). Transgenic mice expressing dominant negative Met fail to develop a complete set of sensory innervating connections (Maina et al., 1997). In cultured dorsal root ganglia of these mice, which contain predominantly sensory neurons, HGF acts synergistically with nerve growth factor (NGF) in axonal outgrowth (Maina et al., 1997). Neurotrophic effects of HGF have also been reported in mesencephalic dopaminergic neurons from neocortical explants in vitro (Hamanoue et al., 1996). In these explants, HGF is mainly expressed in microglia, suggesting a role in CNS development.

Thus, depending on the spatiotemporal distribution pattern and the type of neurons involved, HGF may act as a neural inducer, a neuronal survival factor, or an axonal guidance factor.
5. Angiogenesis

HGF is a potent in vitro motility-stimulating factor for endothelial cells under 2-dimensional culture conditions (Rosen et al., 1990), whereas in 3-dimensional collagen matrices, endothelial cells can be induced by HGF to adapt an elongated phenotype, or to even form tubular, vessel-like structures (Bussolino et al., 1992; Grant et al., 1993). Abundant and genuine angiogenesis in vivo is observed when rabbit cornea is treated with HGF (Bussolino et al., 1992). Accordingly, after implantation into mice, tumor cells that express both Met and HGF, expand much faster than cells that do not secrete HGF and they constitute larger tumors. This coincides with increased and abundant microvascularization of the HGF-secreting tumors (Lamszus et al., 1997; Laterra et al., 1997). Blood vessel endothelial cells express Met on their plasma membrane (Bussolino et al., 1992), but it is not clear whether HGF-induced angiogenesis is a direct consequence of increased endothelial cell motility and proliferation. HGF can also enlarge the expression of vascular endothelial growth factor (VEGF) in gastric epithelial cells and could thus be responsible for neovascularization in gastric tumors (Takahashi et al., 1997). On the other hand, VEGF induction has been described in endothelial smooth muscle cells after HGF treatment, where it may act synergistically with HGF in angiogenesis (Van Belle et al., 1998). Finally, HGF has been described to induce platelet-activating factor in macrophages that are in the vicinity of the site of neovascularization (Camussi et al., 1997). HGF thus increases proliferation and migration of endothelial cells and may engender angiogenesis directly, or indirectly via VEGF and platelet-activating factor.

6. Muscle development

During embryonic muscle development, HGF secreted by limb bud mesenchymal cells induces migration of Met expressing myogenic precursor cells from the somites (Bladt et al., 1995; Yang et al., 1996). Met signaling is essential for the detachment of the myogenic precursor cells and the subsequent migration into the limb bud and diaphragm (Brand-Saberi et al., 1996). In Met null mutant mouse embryos, myogenic precursor cells remain in the dermomyotome and consequently, the limb bud and diaphragm are not colonized, leading to the absence of skeletal muscles in the limb and diaphragm (Bladt et al., 1995; Dietrich et al., 1999). In contrast, development of the axial skeletal muscles proceeds in the absence of Met signaling. Ectopic HGF expression leads to aberrant muscle development as shown in chick embryos, where additional limb buds had been induced by the ectopic application of fibroblast growth factor (FGF). Here, myogenic precursor cells colonize this newly formed limb bud, through chemotactic response.
attraction towards HGF (Heymann et al., 1996), whereas in transgenic mice that inappropriately express HGF, ectopic muscle formation occurs (Takayama et al., 1996).

Met and HGF mRNA are present in immature neonatal rat skeletal muscle, but in adult skeletal muscle their levels are below detection limits. After muscle damage, both HGF and Met expression is upregulated in the regenerating muscle (Jennische et al., 1993; Anastasi et al., 1997; Tatsumi et al., 1998). In a cultured undifferentiated myoblast cell line both genes are also co-expressed, pointing to the existence of an autocrine pathway in the regulation of cell proliferation (Anastasi et al., 1997). It appears that HGF expression is developmentally regulated in skeletal muscle and transiently re-expressed during muscle regeneration. The latter process may involve the concerted activation of quiescent satellite cells to proliferate (Allen et al., 1995), while at the same time their differentiation is inhibited (Gal-Levi et al., 1998).

HGF and Met are also expressed in progenitor cells of the cardiomyocytes and may play a role in cardiomyogenic differentiation and heart organogenesis (Rappolee et al., 1996; Song et al., 1999).

HGF is hence an inducer of myogenic migration during embryonic development and of satellite cell proliferation during muscle regeneration. Contrarily to the mutually exclusive expression pattern generally found in mesenchymal/epithelial tissues, myoblast proliferation may be regulated by HGF-Met in an autocrine fashion.

7. Haematopoiesis

The HGF/Met pathway has also been implicated in haematopoiesis. Both HGF and Met are expressed in the yolk sac of the chicken embryo (Thery et al., 1995), and in the human and rodent fetal liver, primordial sites of haematopoiesis (Seiden et al., 1990; Hu et al., 1993). Within the adult haematopoietic microenvironment, the bone marrow, Met is expressed by a subset of haematopoietic precursor cells (HPC), whereas HGF is expressed by stromal cells, suggesting that HGF functions as a paracrine growth factor (Kmiecik et al., 1992; Galimi et al., 1994; Takai et al., 1997; Weimar et al., 1998). Indeed, it was shown that HGF promotes differentiation and proliferation of HPC induced by other haematopoietic growth factors. In the presence of IL-3, HGF stimulates the formation from CD34+ progenitors of burst forming units erythroid, as well as colony forming units granulocyte erythroid macrophage, but not of colony forming units granulocyte monocyte (Galimi et al., 1994; Takai et al., 1997). In the presence of stem cell factor, an even stronger synergistic effect is obtained (Galimi et al., 1994; Weimar
Figure 5. A schematic representation of the most relevant signaling pathways activated by HGF. For reasons of clarity both relevant autophosphorylation sites of Met, i.e. Y1349 and Y1356, are depicted twice. Furthermore, the Met-associating proteins, except for Grb2, are able to interact with either autophosphorylation site. Crossed shapes represent adaptor or docking proteins without catalytic activity, squares represent kinases, circles represent GTPases, octagons represent guanine nucleotide dissociation stimulators, and pentagons represent transcription factors. The solid arrows indicate a direct activation, whereas the dotted arrows indicate activation via known or unknown intermediate proteins or phospholipid metabolites, and the blunted arrows indicate a direct inhibition. Although only Met is depicted, most signaling pathways also apply to the oncoprotein Tpr-Met. See text for further details.
et al., 1998). Apart from effects on growth and differentiation, HGF stimulation of CD34⁺ cells leads to integrin activation and adhesion to fibronectin. This adhesive interaction prolonged survival of haematopoietic cells in culture (Weimar et al., 1998). Taken together, these data indicate that the HGF/Met pathway is involved in the regulation of the proliferation, differentiation and survival of haematopoietic progenitors.

C. Signal transduction by Met

1. Introduction

Like in most other receptor tyrosine kinases, the activation of the kinase domain of Met is believed to depend upon receptor dimerization or oligomerization, resulting in intermolecular transphosphorylation. This process of di- or oligomerization may be facilitated by the action of HSPGs, as discussed in Section II.D. Upon stimulation by HGF, the C-terminus of the β-chain of Met is strongly tyrosine phosphorylated (Bottaro et al., 1991; Naldini et al., 1991a,b). The autophosphorylation of the tyrosine residues Y1349 and Y1356 of Met, as well as the equivalent residues Y482 and Y489 of the oncoprotein Tpr-Met, are critical for most biological responses (Ponzetto et al., 1994, 1996; Zhu et al., 1994; Fixman et al., 1995). These tyrosine residues serve as a multisubstrate docking site for several proteins, including Gab1, Grb2, phosphatidylinositol 3-kinase (PI3-K), phospholipase C (PLCγ), Src, She, SHP-2 and STAT3 (Fig. 5). Except for Gab1, which has a unique Met-binding domain (Weidner et al., 1996; see, however, discussion below), these proteins interact with Met via their SH2 domains: Grb2 specifically to Y1356, the other proteins to both Y1349 and Y1356 (Ponzetto et al., 1993, 1994, 1996; Pelicci et al., 1995; Nguyen et al., 1997). Here we will discuss the nature and function of the different Met-associating signaling molecules. Furthermore, the signaling pathways activating Met, and their biological function, will be discussed.

2. The role of Grb2 and signal transduction via Ras

One of the signaling molecules that associates directly with Met upon HGF stimulation is Grb2 (Ponzetto et al., 1994). Grb2 is an adapter protein consisting of one SH2 and two SH3 domains. SH2 domains are involved in binding to phosphorylated tyrosine residues, whereas SH3 domains bind to proline-rich regions. By means of its SH3 domain, Grb2 is constitutively associated with Sos, an exchange factor for Ras. The Grb2-Sos complex is recruited by receptor tyrosine
HGF/Met signaling and function

kinases, via their autophosphorylated tyrosine residues, to the plasma membrane where Ras is localized. As a consequence, Ras becomes activated (Fig. 5). After HGF-induced autophosphorylation, Met also associates with the She adapter protein (Pelici et al., 1995). Interestingly, upon phosphorylation, She is also able to associate with Grb2 (Pelici et al., 1995). Thus, HGF stimulation can trigger the Ras-pathway by both direct and She-mediated association of the Grb2-Sos complex to Met (Fig. 5).

Activation of Ras has been implicated in a wide variety of cellular responses including cytoskeletal reorganization, adhesion, proliferation, differentiation and apoptosis. The first identified and best characterized effector molecule for Ras is the serine/threonine kinase Raf1, which phosphorylates and activates MEK, resulting in the phosphorylation and activation of MAP kinase (Campbell et al., 1998; Vojtek and Der, 1998) (Fig. 5). Among the substrates for MAP kinase are the transcription factors Elk-1 and Ets-2 involved in ternary complex formation at serum response elements. Upon activation, these transcription factors regulate expression of immediate early genes, such as c-fos, eventually leading to cell proliferation (Wasylyk et al., 1998). Besides Raf, several additional effector molecules for Ras have been identified. These include PI3-K (Rodriguez-Viciana et al., 1994), which will be discussed below, and RalGDS (Spaargaren and Bischoff, 1994), an exchange factor for Ral (Albright et al., 1993; Feig et al., 1996) (Fig. 5). Ral has been implicated in Ras-dependent proliferation, gene expression, phospholipase D activation and transformation (Wolthuis et al., 1999), however, no studies have been conducted yet to investigate its involvement in Met signal transduction and functional responses.

In initial studies using mutants of Met, it was shown that Y1356, the Grb2 binding site, is required for scattering and branching tubulogenesis of MDCK cells, whereas the equivalent residue Y489 of Tpr-Met is required for cell proliferation and transformation (Zhu et al., 1994; Fixman et al., 1995). However, these mutations also reduced binding of other Met associating proteins (Ponzetto et al., 1993, 1994; Pelici et al., 1995). Using a more sophisticated mutant that selectively fails to bind Grb2 only, it was shown that Grb2 association by Met is required for HGF-induced branching tubulogenesis of MDCK cells, but not for scattering (Fournier et al., 1996; Ponzetto et al., 1996; Royal et al., 1997). Similarly, whereas Grb2 binding by Tpr-Met is not required for motility, it is required for induction of transformation and invasion, in vitro, as well as metastasis and tumorigenicity, in vivo (Fixman et al., 1996; Ponzetto et al., 1996; Giordano et al., 1997; Jeffers et al., 1998b; Bardelli et al., 1999). Intriguingly, however, Grb2 binding to Met is dispensable for transformation, metastasis and tumorigenicity, when Met is activated by either a point mutation or by autoerine HGF stimulation (Jeffers et al., 1998b).
Interestingly, it has been shown that expression of N17-Ras, a dominant negative mutant of Ras, abolishes HGF-induced cell scattering (Hartmann et al., 1994; Ridley et al., 1995). Since scattering does not require a Grb2 binding site, this suggests that HGF/Met activates Ras by a Grb2-independent mechanism. Indeed, a recent study by Tulasne et al. (1999), shows that a mutant of Met, which lacks four major autophosphorylation tyrosine residues (including Y1349 and 1356 which constitute the multisubstrate docking site), despite its loss of Grb2 binding ability, is still able to induce Ras activation. Moreover, the scattering response, which was clearly not affected by these mutations, was abolished upon treatment with the specific pharmacological inhibitor PD98059 of the Ras-MAP kinase pathway intermediate MEK (Tulasne et al., 1999). Using either this MEK-inhibitor or constitutively active or dominant negative mutants of Ras or MAP kinase, it was concluded that the activation of the Ras-MAPK pathway is required but not sufficient for HGF-induced scattering (Ridley et al., 1995; Potempa and Ridley 1998; Herrera 1998; Khwaja et al., 1998; Tanimura et al., 1998; Tulasne et al., 1999), and for tubulogenesis of MDCK cells (Khwaja et al., 1998). In conclusion, Grb2 and the Ras-MAP kinase pathway appear to play an important regulatory role in a variety of responses elicited by HGF/Met, including mitogenesis, motogenesis and morphogenesis, as well as in Tpr-Met-induced transformation, invasion, metastasis and tumorigenicity.

3. The role of Gab1 and signal transduction via PI3-K

Another important substrate for Met is the docking protein Gab1 (Weidner et al., 1996). In vitro, Gab1 interacts directly with Met via a proline rich binding domain (Weidner et al., 1996), but it has been concluded that the interaction of Gab1 with Met and Tpr-Met in vivo is mediated by Grb2 (Nguyen et al., 1997; Bardelli et al., 1997; Fixman et al., 1997). Gab1, which contains a PH-domain as well as a proline-rich region, and can become heavily tyrosine phosphorylated, has the ability to directly associate with several signaling molecules such as Grb2, PI3-K, PLCγ and SHP2 (Holgado-Madruga et al., 1996).

Overexpression of Gab1 partially mimics the action of HGF, as it results in tubulogenesis of mammary epithelial cells (Niemann et al., 1998), as well as in enhanced MAP kinase activity, cell scattering and tubulogenesis of MDCK cells (Weidner et al., 1996). The HGF responses in MDCK cells could be abrogated by overexpressing the Met binding domain of Gab1 (Weidner et al., 1996). In NIH3T3 fibroblasts, however, Holgado-Madruga et al. (1996) did not observe enhanced MAP kinase activity or activation upon overexpression of Gab1. Moreover, a recent study shows that Met with mutations of the multisubstrate docking site, which abolish recruitment of Gab1, as well as Grb2, She and PI3-K, although
indeed impaired in the induction of morphogenesis, is still able to activate the Ras-MAP kinase pathway and to induce MEK-dependent scattering (Tulasne et al., 1999). Finally, the transforming potential of Tpr-Met mutants correlates with their ability to induce tyrosine phosphorylation of Gab1 (Bardelli et al., 1997; Fixman et al., 1997). Taken together, these findings convincingly demonstrate the involvement of Gab1 in Met-induced morphogenesis (Weidner et al., 1996; Nguyen et al., 1997; Niemann et al., 1998; Maroun et al., 1999; Tulasne et al., 1999), and suggest a role for Gab1 in transformation by Tpr-Met.

One of the first molecules that was shown to become associated with Met upon HGF stimulation was PI3-K (Graziani et al., 1991; Ponzetto et al., 1993). This interaction of PI3-K with Met may enhance PI3-K activity and/or localize PI3-K in the proximity of its substrate (Ponzetto et al., 1993). PI3-K is composed of a p85 adapter subunit, which contains the Met interacting SH2 domain, and a p110 catalytic subunit. PI3-K is able to phosphorylatePIP2 in order to produce PIP3. PIP3 in its turn can bind to the PH domain of target proteins, resulting in their translocation, membrane localization and, indirectly, in their activation. Among the PH-domain-containing effector molecules of PI3-K is the kinase Akt/PKB (Burgering and Coffer, 1995), which, upon membrane localization, is phosphorylated and activated by PDK1 (Stokoe et al., 1997; Stephens et al., 1998) (Fig. 5). Downstream effector molecules for the PI3-K-regulated kinase PKB include the Bel-2 family member Bad, which can exert pro-apoptotic activity by interacting with Bel-2 (Datta et al., 1997; del Peso et al., 1997); glycogen synthase kinase 3 (GSK3), involved in regulation of glycogen synthesis and, as discussed below, in phosphorylation of β-catenin (Cross et al., 1995); p70S6K, involved in regulation of protein synthesis and gene expression (Proud, 1996); and the forkhead transcription factor AFX (Kops et al., 1999). The function of these effector molecules in Met signal transduction has not yet been investigated.

Besides the ability of PI3-K to directly interact with Met, two additional mechanisms may account for Met-induced PI3-K activation (Fig. 5). Firstly, the p85 subunit of PI3-K was also found to associate with Gab1 (Holgado-Madruga et al., 1996), and, at least in cells overexpressing both Met and Gab1, more PI3-K activity is associated with Gab1 than with Met (Maroun et al., 1999). Interestingly, besides being able to associate with PI3-K, Gab1 requires PI3-K activity and an intact PH domain for proper localization and induction of morphogenesis (Maroun et al., 1999). Secondly, PI3-K has been identified as an effector molecule for Ras, as Ras has the ability to directly interact with the p110 catalytic subunit of PI3-K (Rodriguez-Viciana et al., 1994). To what extent these three different mechanisms contribute to HGF-induced PI3-K activation remains to be established. However, Ras-mediated PI3-K activation has been implicated in HGF-induced adherens junction disassembly in MDCK cells (Potempa and Ridley, 1998).
Chapter 6

By means of either specific pharmacological inhibitors such as Wortmannin and LY294002, or by expression of dominant negative or constitutively active mutants of PI3-K, its function in Met signaling has been extensively studied. These studies revealed a prominent regulatory role for PI3-K in Met-induced mitogenesis, motility and morphogenesis. (Royal and Park, 1995; Rahimi et al., 1996; Royal et al., 1997; Potempa and Ridley, 1998; Khwaja et al., 1998). Activation of PI3-K has been reported to be required and sufficient for tubulogenesis, and required for scattering (Royal and Park, 1995; Khwaja et al., 1998; Potempa and Ridley, 1998). Interestingly, however, mutation of the multissubstrate docking site of Met, which results in the loss of PI3-K and Gab1 association with Met upon HGF stimulation, does not abrogate HGF-induced scattering or Ras activation (Tulasne et al., 1999). This indicates that Ras-mediated, rather than direct Met-induced or Gab1-mediated, activation of PI3-K is required for HGF-induced scattering.

Whether PI3-K activation alone is also sufficient for HGF-induced scattering of MDCK cells is still a matter of debate. On the one hand Potempa and Ridley (1998) reported that neither expression of an active mutant of PI3-K, nor the combined expression of PI3-K with active Raf or MEK, was sufficient for adherens junction disassembly, a prerequisite for scattering. On the other hand, Khwaja et al. (1998) reported that expression of an active mutant of PI3-K is sufficient to induce scattering, provided a basal level of MAP kinase activity is present, however, expression of active Rac or PKB is not sufficient to induce scattering. Based upon these observation, both studies suggested the requirement of an additional (novel) mitogenic pathway for HGF-induced scattering, either downstream of Ras, other than PI3-K or Raf (Potempa and Ridley, 1998), or downstream of PI3-K, other than PKB or Rac (Khwaja et al., 1998). Noteworthy, a recent study also suggested the existence of an additional mitogenic signaling pathway, as NK2, the truncated HGF isoform as described in Section II.A, despite its ability to induce both PI3-K and MAP kinase activation, as well as a mitogenic response, is unable to induce a mitogenic response in breast epithelial cells (Day et al., 1999). In agreement with the data from Khwaja et al. (1998), it has been reported that expression of active PI3-K disrupts the polarized tubular growth of well-differentiated mammary epithelial cells, resulting in enhanced motility and invasion (Keely et al., 1997). Taken together, PI3-K activation is required, and may also be sufficient, for HGF-induced scattering.

PI3-K has also been implicated in the responses elicited by Tpr-Met. A mutant of Tpr-Met, which preferentially binds PI3-K over Grb2, although still able to elicit cell motility, is unable to induce transformation, invasion and metastasis (Bardelli et al., 1999). However, a mutant of Tpr-Met which selectively binds Grb2 only, is also impaired in its ability to induce invasion and metastasis (Giordano et al., 1997). This could, however, be overcome by expression of constitutively active
PI3-K (Bardelli et al., 1999). Thus, these data indicate that simultaneous activation of the Ras and PI3-K pathway is required and sufficient for full invasive and metastatic activity of Tpr-Met. In conclusion, the PI3-K pathway is an important regulatory pathway in HGF/Met-induced mitogenesis, motogenesis and morphogenesis, as well as in Tpr-Met-induced motility, invasion and metastasis.

4. The role of signal transduction via Rho-family GTPases and β-catenin

Members of the Rho subfamily of Ras-related GTPases, as well as Ras itself, have been implicated in HGF-induced cytoskeletal reorganization, cell scattering and tubulogenesis. Initially, mainly based upon studies in fibroblasts, cdc42, Rac and Rho were shown to regulate the formation of filopodia, lamellipodia, and stress fibers, respectively (Hall, 1998; van Aelst and D’Souza-Schorey, 1997). Evidence was presented indicating that cdc42 may function upstream from Rac, which in turn may function upstream from Rho (Zigmond, 1996). Furthermore, it was shown that Ras can induce PI3-K-dependent activation of Rac (Rodriguez-Viciana et al., 1997), which may be mediated by the PH domain-containing exchange factor for Rac, Tiam (Michiels et al., 1995). More recently, these Rho-family GTPases were shown to be involved in the regulation of a wide variety of cellular functions, including regulation of membrane trafficking, transcriptional activation and cell growth control (Hall, 1998; van Aelst and D’Souza-Schorey, 1997). Several effector molecules for Rho-family GTPases have been identified, including the Rac effectors ROK and ROCK, involved in stress fiber formation, and the Rac effector PAK, involved in JNK activation and cytoskeletal organization (van Aelst and D’Souza-Schorey, 1997).

With respect to HGF/Met signaling, it has been reported that the activation of Rho is required for HGF-induced membrane ruffling and cell motility in keratinocytes (Takaishi et al., 1994; Nishiyama et al., 1994). Furthermore, HGF-induced actin reorganization, membrane ruffling, spreading and scattering of MDCK cells was reported to require activation of Ras and Rac (Hartmann et al., 1994; Ridley et al., 1995; Potempa and Ridley, 1998), but not of Rho (Ridley et al., 1995). Interestingly, recent studies revealed that Rac and Rho are involved in intercellular E-cadherin-mediated adhesions in epithelial cells. In MDCK cells and keratinocytes the basal cadherin-mediated cell-cell adhesion was inhibited by the dominant negative mutant N17-Rac and by inhibition of Rho (Braga et al., 1997; Takaishi et al., 1997). Furthermore, expression of constitutively active V12-Rac enhanced cadherin-mediated cell-cell adhesion in MDCK cells (Takaishi et al., 1997).

In agreement with the stimulatory effect of V12-Rac on cell-cell adhesion, overexpression of either V12-Rac or Tiam1 inhibits HGF-induced scattering of
MDCK cells (Hordijk et al., 1997). Furthermore, V12-Rac and Haml suppressed the scattered appearance and invasion of fibroblast-like Ras-transformed MDCK13 cells. This was shown to be due to restoration of E-cadherin-mediated cell-cell adhesion, as a consequence of enhanced levels of β-catenin and E-cadherin at intercellular junctions (Hordijk et al., 1997) (Fig. 5). In contrast, however, in well- differentiated mammary epithelial cells expression of active mutants of Rac, cdc42 or PI3-K disrupts their polarized tubular growth, and rather promotes their motility and invasion (Keely et al., 1997). Most likely, these apparent contradictory results reflect the delicate balance between the Rac-dependent regulation of cell-cell adhesion, cell-matrix adhesion, and matrix-dependent cell migration (Sander et al., 1998). Finally, expression of N17-Rac in MDCK cells prevents HGF-induced and Ras-mediated dispersal of β-catenin and E-cadherin, adherens junction disassembly and, as mentioned before, scattering (Potempa and Ridley, 1998).

Since tyrosine phosphorylation of β-catenin has been implicated in the dissociation of the cadherin/β-catenin complex from the actin cytoskeleton, it is noteworthy that, although this was not observed in MDCK cells (Potempa and Ridley, 1998), β-catenin as well as plakoglobin (γ-catenin) become phosphorylated on tyrosine residues upon HGF stimulation of HT29 colon adenocarcinoma cells (Shibamoto et al., 1994). In conclusion, Rac, Rho and β-catenin play an important regulatory role in cell-cell adhesion and HGF-induced cytoskeletal organization and motogenesis.

Besides its involvement in cell-cell adhesion, β-catenin has also been implicated in the regulation of gene transcription. β-Catenin can form mutually exclusive complexes with either cadherins or with APC, the tumor suppressor gene product that is mutated in colon carcinoma (Rubinfeld et al., 1993). GSK3, which can be phosphorylated and inactivated by the PI3-K-dependent PKB, can in turn phosphorylate APC and β-catenin on serine residues (Rubinfeld et al., 1996; Cross et al., 1995) (Fig. 5). As a consequence of this phosphorylation, β-catenin is degraded (Morin et al., 1997). However, upon phosphorylation and thus inhibition of GSK3, free β-catenin will accumulate, translocate to the nucleus and interact with the transcriptional regulator T cell factor/lymphocyte enhancer-binding factor (TCF/LEF-1), thereby inducing expression of TCF/LEF-1 target genes (Behrens et al., 1996; Molenaar et al., 1996) (Fig. 5). Interestingly, HGF stimulation of mouse mammary cells was reported to result in a decrease in GSK3 activity, the nuclear accumulation of β-catenin, and the activation of TCF/LEF-1 (Papkoff and Aikawa, 1998). Thus, via direct or indirect phosphorylation of β-catenin on either tyrosine or serine residues. Met appears to be able to regulate cell-cell adhesion as well as gene expression.

Expression of N17-Rac has also been shown to inhibit both JNK activation and transformation by Tpr-Met (Rodrigues et al., 1997). Based upon this observation it was suggested that activation of the JNK pathway, which is mediated by the sequential activation of PI3-K and Rac (Coso et al., 1995; Minden et al.,
1995) (Fig. 5), is essential for transformation by the Tpr-Met oncoprotein (Rodrigues et al., 1997). JNK in turn is able to phosphorylate a number of transcription factors, including the immediate early gene c-jun. Noteworthy, both GTPases Rac and Rho have also been implicated in regulation of the transcriptional activity of the Serum response factor (SRF) (Hill et al., 1995). In conclusion, Rac, Rho and β-catenin play an important regulatory role in HGF/Met-induced mitogenesis and motogenesis, whereas Rac is also involved in Tpr-Met-induced transformation.

4. The role of additional signaling molecules

An additional Met associating protein is PLCγ (Ponzetto et al., 1994). PLCγ mediates the production of IP3, which results in enhanced calcium release from intracellular stores, and diacylglycerol, which activates of PKC. Indeed, PKC has been implicated in Met signaling in a variety of cell types (Santos et al., 1993; Adachi et al., 1996; Dunsmore et al., 1996; Laping et al., 1998; Machide et al., 1998). Furthermore, both PKC and calcium have also been implicated in the negative regulation of Met signaling, by phosphorylation of residue S985 of Met, resulting in decreased kinase activity (Gandin o et al., 1990, 1991, 1994). Using a pharmacological inhibitor, PLCγ has been implicated in the chemotactic response elicited by stimulation of a chimeric PDGF-Met receptor molecule expressed in renal epithelial cells (Derman et al., 1996).

HGF also activates STAT3 (Schaper et al., 1997), and stimulates recruitment of STAT3 to the autophosphorylated Y1356 of Met (Boccaccio et al., 1998). Upon phosphorylation, the STAT proteins can dimerize and translocate to the nucleus, where they act as transcription factors controlling the promoter activity of target genes. Inhibition of STAT-mediated transcription prevents HGF-induced tubulogenesis, whereas scattering and proliferation were unaffected (Boccaccio et al., 1998).

Furthermore, the Src tyrosine kinase was shown to directly associate with Met (Ponzetto et al., 1994). This association with Met and activation of Src was shown to play a critical role in carcinoma cell motility (Rahimi et al., 1998), and in HGF-induced phosphorylation of FAK (Chen et al., 1998). With respect to another Met associating protein, the tyrosine phosphatase SHP-2 (Nguyen et al., 1997), no functional data are available yet. Recent data indicate that SHP-2 can also be indirectly recruited to Met via Gab1 (Maroun et al., 1999).

In addition, BAG-1, a cell death suppressor gene product that binds the anti-apoptotic proto-oncogene product Bel-2 in a cooperative fashion (Takayama et al., 1995), interacts with Met. This interaction was independent of phosphorylation of either Y1349 or Y1356 of Met (Bardelli et al., 1996), and may very well be
mediated by the molecular chaperone Hsp70 (Takayama et al., 1997c). Overexpression of BAG-1 enhances the anti-apoptotic effect of HGF on liver progenitor cells (Bardelli et al., 1996).

Finally, it is noteworthy that ezrin, a member of the ERM protein family involved in membrane-cytoskeleton interactions, is a substrate for Met in vitro and also becomes phosphorylated on tyrosine residues in vivo. Both a truncated and a tyrosine mutated variant of ezrin impair the motogenic and morphogenic response of epithelial kidney cells to HGF (Crepaldi et al., 1997). Furthermore, as will be discussed in more detail in the next Section, the observations that the HSPG CD44 can directly interact with ezrin (Tsukita et al., 1994), and can bind and present HGF to Met (van der Voort et al., 1999; Taher et al. 1999), adds an extra dimension to the role of ezrin in Met-signaling.

D. Heparan sulfate proteoglycans and HGF/Met function

Besides Met, heparan sulfate proteoglycans (HSPGs) have been identified as a second class of HGF-binding sites on various cell types. These binding have a much lower affinity than the Met receptor, but they are considerably more numerous (10-1000 fold) (Higuchi et al., 1991; Tajima et al., 1992).

HSPGs are proteins that carry one or more covalently linked heparan sulfate chains. They are widespread throughout mammalian tissues both as cell surface molecules, e.g. the syndecans, glypicans, and CD44-HS, and as ECM components, e.g. perlecan. HSPGs have been implicated in several important biological processes including cell adhesion and migration, angiogenesis, tissue morphogenesis, and regulation of blood coagulation. In these processes, they are believed to function as scaffold structures, designed to accommodate proteins through non covalent binding to their heparan sulfate (HS)-chains (reviewed in Schlessinger et al., 1995; Lindahl et al., 1998). The ligand-binding sites reside within discrete sulfated domains formed by complex, cell-specific modifications to the HS disaccharide repeat. Binding of proteins, including many growth factors and cytokines, e.g. FGFs, VEGF, HB-EGF, IL-3, IL-7, GM-CSF, and certain chemokines, to HS-chains may serve a variety of functions ranging from immobilization and concentration, to distinct modulation of their biological function. This functional importance is illustrated by fibroblast growth factor 2 (FGF-2), whose binding to its signal-transducing receptor and consequent biological effects is critically dependent on its interaction with cell-surface HSPGs (Rapraeger et al., 1991; Yayon et al., 1991; Schlessinger et al., 1995). Furthermore, a number of cell biological and genetic studies have recently provided compelling evidence for an in vivo role of cell-surface HSPGs in growth
control and morphogenesis in Drosophila, mice and humans (reviewed by Selleck, 1998).

The modular structure of HGF has facilitated the identification of the domains responsible for binding to Met and heparin/HS. By using deletion mutants of HGF and examining their binding ability to immobilized heparin, Mizuno et al. (1994) identified the hairpin loop of the amino-terminal domain and the second kringle domain as sites essential for heparin binding. The same domains, are also critical for Met binding and signaling (Matsumoto et al., 1991; Hartmann et al., 1992; Lokker et al., 1992; Okigaki et al., 1992). In order to dissect the binding sites for Met and heparin/HS in HGF, the groups of Gherardi and Blundell generated three-dimensional models of the individual HGF domains to help to design specific mutants (Donate et al., 1994). Based on the X-ray structures of antithrombin-(Carrell et al., 1994) and FGF-heparin complexes (Faham et al., 1996), they predicted the heparin-binding sites to contain clusters of positively charged residues which make electrostatic contact with negatively charged groups in HS-chains. Indeed, three such clusters were identified on the surface of HGF, two in the hairpin loop and one in the kringle 2 domain (Donate et al., 1994). By introducing specific mutations at these sites, it was confirmed that these residues play a key role in heparin-binding (Hartmann et al., 1998). A study by Chirgadze et al. (1999) has recently reported the crystal structure of NK1, a natural splice variant of HGF with agonistic activity, consisting of the N- and first kringle-domains (see Section II.A). It was shown that NK1 assembles as an asymmetric homodimer in which the N-domain of one partner interacts with the kringle domain of the other. Short heparin fragments (14-mer) effectively dimerized NK1 in solution, suggesting that heparan sulfate chains expressed on cells or in the ECM may stabilize the NK1 dimers in vivo.

Although HS-chains are composed of a linear array of disaccharide units consisting of alternating hexuronic acid (L-iduronic acid (IdoA) or D-glucuronic acid) and D-glucosamine, there is evidence that they are capable of highly specific protein binding. Variations in O-sulfation pattern, hexuronate composition, and length of the sulfated segments determine this specificity (Lindahl et al., 1998). The first specific binding domain identified, was a pentasaccharide which binds with high affinity to antithrombin III (AT III), a serine protease inhibitor. This sequence induces a conformational change in AT III and accelerates its binding to factor Xa and thrombin, and in this way promotes the anticoagulant action of AT III (Lindahl et al., 1984). Lyon et al. (1994) and Ashikari et al. (1995) have analyzed the structural basis of the interaction between HGF and HSPGs. Both studies indicate that high affinity HGF-binding requires oligosaccharides with a minimum length of 8-12 units containing 6-O-sulfated GlcNSO residues, which may be flanked by IdoA(SO₃) units (Ashikari et al., 1995). Interestingly, this
Figure 6. Different isoforms of CD44 can promote Met signaling by distinct mechanisms. CD44v3-10 promotes Met signaling by heparan sulfate-mediated presentation of HGF to Met, whereas CD44 can also promote Met signaling as a consequence of adhesion to HA. See text for further details.

Structural specificity for binding to HS differs radically from that of FGF-2 (Maccarana et al., 1993), illustrating the importance of structural diversity of the HS-chain in selective growth factor binding.

Whereas HSPGs have been shown to be crucial for FGF interaction with its receptor, and thus for FGF functioning, their role in HGF/Met interaction is, as yet, less well defined. Binding to heparin/HS does not appear to affect the affinity of full-length HGF for the Met receptor, but it increases receptor phosphorylation and mitogenicity on rat hepatocytes (Zioncheck et al., 1995; Schwall et al., 1996). In contrast, Met binding and mitogenicity of NK1 has been reported to require HSPGs (Schwall et al., 1996; Sakata et al., 1997). Recently, we have shown that HGF binds to a HSPG splice variant of CD44 (CD44-HS) expressed on B cells (van der Voort et al., 1999). This binding strongly promotes the HGF-induced tyrosine
phosphorylation of Met as well as phosphorylation of several substrates (Fig. 6) (see also Section III.C). Taken together, these *in vitro* studies indicate that HSPGs may play an important regulatory role in HGF/Met signaling.

Interaction of HGF with HSPG could modulate Met-signaling via several mechanisms (Fig. 6). Firstly, as already mentioned, HSPGs may promote dimerization of HGF, thereby promoting receptor cross-linking and tyrosine kinase activity (Chirgadze *et al.*, 1999). Secondly, by inducing a conformational change HSPGs may influence the affinity of HGF for Met, as has been demonstrated for the NK1 splice variant (Sakata *et al.*, 1997). Thirdly, HGF may mediate colocalization of HSPGs and Met, which may bring relevant intracellular signaling molecules in the proximity of each other. For example, we have shown that the cytoplasmic tail of CD44 interacts physically and functionally with Src-family protein tyrosine kinases (Taher *et al.*, 1996), which have also been implicated in Met signaling (Ponzetto *et al.*, 1994). The above-mentioned processes may involve the formation of a ternary complex between HGF, Met, and a HSPG. In case of ternary-complex-formation between CD44-HS, HGF, and Met, Src kinases associated with the cytoplasmic tail of CD44, might be recruited into the complex (Taher *et al.*, 1999). This may facilitate their activation by Met. Similarly, the ERM family member ezrin, which is also associated with CD44 and a downstream target for Met, may also be assembled into the complex. Ezrin acts as a linker between the intracellular domain of CD44 and the actin-based cytoskeleton (Tsukita *et al.*, 1994), and has been shown to be involved in HGF-induced cell migration (Crepaldi *et al.*, 1997). By recruiting ezrin, CD44-HS might thus contribute to the regulation of cell adhesion and migration.

The preceding data suggest an important physiological role for HSPG in the regulation of HGF function. To directly address this issue, Hartmann *et al.* (1998) performed *in vivo* studies comparing wild-type HGF with HGF mutants with a strongly (50 fold) reduced affinity for heparin. Mutant HGF showed a delayed clearance from the blood, and interestingly, induced a higher DNA synthesis in normal mouse liver. Based on these findings, the authors suggest a role for HSPGs in promoting the internalization and degradation of HGF *in vivo*. Although no further *in vivo* data are as yet available, it is tempting speculate on other physiological roles for HSPGs. HSPGs may help to localize HGF to specific cells or ECM components within the microenvironment and may be required for the establishment of a chemotactic gradient. Examples are the migration of myogenic precursor cells to the limb bud during embryogenesis, which is critically dependent on HGF and Met, and possibly, the migration B cells within the germinal center. Furthermore, membrane and matrix HSPG may also protect HGF from proteolytic degradation (Saksela *et al.*, 1988). Further studies are needed to explore these possible roles of HSPG.
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E. The HGF/Met pathway in tumor growth, invasion and metastasis

In Section II.A.2, we briefly discussed the oncogenic potential of the Tpr-Met chimera. In this chimera, the intracellular domain of Met is fused to Tpr, resulting in a constitutively active homodimer with transforming capacity (Cooper et al., 1984; Park et al., 1986; Gonzatti-Haces et al., 1988). A vast body of clinical and experimental data shows that, apart from Tpr-Met, also the Met proto-oncogene and HGF play a pivotal role in tumorigenesis. For instance, overexpression and high levels of autophosphorylation of Met have been found in human tumor cell lines (Fig. 7) (Tempest et al., 1988; Giordano et al., 1989a; Ponzetto et al., 1991; Kuniyasu et al., 1992). Often this overexpression is caused by gene amplification (Giordano et al., 1989a; Ponzetto et al., 1991; Kuniyasu et al., 1992). Interestingly, however, transfection of tumor cells with activated ras and ret oncogenes also causes Met overexpression and enhance HGF-dependent invasion (Ivan et al., 1997; Webb et al., 1998). Furthermore, Met overexpression can be induced by HGF itself, as well as by a number of other cytokines, including EGF, IL-1, and IL-6 (Chen et al., 1997). These data indicate that both oncogenes and cytokines present in the tumor microenvironment can induce Met expression and thereby promote tumorigenesis.

In addition to Met overexpression, overproduction of HGF can also occur in tumors. For instance, it was demonstrated that tumor cells can release factors, e.g., IL-1, FGF-2 or PDGF, which stimulate neighboring fibroblasts to secrete HGF (Fig. 7) (Rosen et al., 1994a, 1994b; Nakamura et al., 1997). Alternatively, some tumor cell lines were shown to express both HGF and Met, suggesting the presence of an autocrine loop (Fig. 7) (Naidu et al., 1994; Moriyama et al., 1995; Tuck et al., 1996; Trusolino et al., 1998). Aberrant expression and activation of the HGF/Met pathway are not only present in tumor cell lines, but also in many native human tumors. Overexpression of Met was detected in carcinomas of the stomach, liver, colon, pancreas, lung, and thyroid gland (Di Renzo et al., 1991, 1992, 1995a, 1995b; Prat et al., 1991; Kuniyasu et al., 1992; Liu et al., 1992; Boix et al., 1994; Kurukawa et al., 1995; Ueki et al., 1997). For colorectal, liver, thyroid, and brain cancer, it was shown to be correlated with disease progression (Di Renzo et al., 1995b; Belfiore et al., 1997; Koochekpour et al., 1997; Ueki et al., 1997). Similarly, overexpression of HGF was also reported in human cancer, e.g., in tumors of the pancreas and in gliomas (Furukawa et al., 1995; Koochekpour et al., 1997; Lamszu et al., 1998). Importantly, in patients with breast or non-small cell lung cancer, expression of HGF was shown to be a strong and independent predictor of recurrence and tumor-related death (Yamashita et al., 1994; Siegfried et al., 1997).
Figure 7. The HGF/MET pathway in tumor growth, invasion and metastasis. Uncontrolled MET-signaling can be caused by receptor overexpression, illegitimate autocrine or paracrine stimulation, activating mutations, and translocation and fusion with Tpr. In addition, uncontrolled MET-signaling may also result from overexpression of HSPG. As a consequence of enhanced MET activity, tumor cells may increase their growth rate and become resistant to apoptosis, resulting in a growth and/or survival advantage. Furthermore, MET activation may lead to cytoskeletal reorganization, and integrin activation, as well as to activation of proteolytic systems involved in ECM degradation, resulting in an increased invasive and metastatic capacity. HGF production, either by fibroblasts in the tumor stroma or by the tumor cells, may stimulate angiogenesis.

Weidner and colleagues (1990) demonstrated that HGF induces invasion of carcinoma cell lines into collagen gels. Similar effects of HGF were subsequently reported for many other tumor cell lines, including mammary, colon and squamous cell carcinoma, and melanoma lines (Jiang et al., 1993; Matsumoto et al., 1994;
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Rosen et al., 1994b; Hendrix et al., 1998). In accordance with these findings, Date et al. (1998) showed that a 4-kringle-containing HGF antagonist (NK4) inhibits HGF-induced tumor invasion. Interestingly, Jiang et al. (1993) reported that, while colon carcinoma cells became more motile in the presence of HGF, their growth was inhibited. Similar observations were made by Giordano et al. (1993) in NIH 3T3 fibroblasts. A recent report suggests that HGF-induced growth suppression can be caused by the induction of oxidative stress (Arakaki et al., 1999).

At least two mechanisms may be involved in the promotion of invasiveness by the HGF/Met pathway. First, HGF may induce invasion and metastasis by causing cytoskeletal rearrangement and by activating adhesion molecules (Fig. 7). For example, HGF was shown to induce tyrosine phosphorylation of molecules involved in the assembly of focal adhesions, resulting in cell spreading and migration (Matsumoto et al., 1994) (see also Sections II.B and C). In addition, we and others have shown that HGF induces activation of integrins and consequent adhesion and migration (van der Voort et al., 1997; Weimar et al., 1997; Trusolino et al., 1998). Secondly, activation of Met may lead to an enhanced degradation of the ECM by invasive cells (Fig. 7). Met activation by HGF increases the expression of urokinase-type plasminogen (uPA) and its receptor (Pepper et al., 1992; Rosen et al., 1994b; Jeffers et al., 1996b), molecules known to play a role in ECM proteolysis.

Studies using in vivo models confirm the involvement of HGF and Met in tumorigenesis. Autocrine stimulation of Met transfected NIH 3T3 cells with HGF enhanced the tumorigenic and metastatic capacity of these cells in nude mice (Rong et al., 1992, 1994). Similar findings were reported for SK-LMS-1 human leiomyosarcoma cells and for mouse mammary tumor cells (Rosen et al., 1994b; Jeffers et al., 1996b; Lamszus et al., 1997). In the leiomyosarcoma model, HGF/Met signaling was shown to increase the expression of both uPA and its receptor uPAR, suggesting a role for this proteolysis network in promoting invasiveness and metastasis. In the mouse mammary tumor model as well as in a glioma model, stimulation of angiogenesis by HGF appeared to play a key role (Fig. 7) (Lamszus et al., 1997; Laterra et al., 1997).

Studies in transgenic mice corroborate the role of HGF and Met in tumorigenesis. HGF transgenic mice were shown to develop a broad array of primary tumors and metastases of mesenchymal as well as epithelial origin, including malignant melanoma, fibrosarcoma, and mammary carcinoma (Takayama et al., 1997b; Otsuka et al., 1998). Many of these tumors arose from abnormally developed tissues, suggesting a functional link between HGF-dependent morphogenesis and tumorigenesis. Since most neoplasms, melanomas in particular, demonstrated overexpression and enhanced activation of Met, autocrine signaling via Met was thought to be a major cause of tumorigenesis.
Recently, Met has been implicated in the genesis of hereditary papillary renal carcinomas (HPRC). Schmidt et al. (1997) showed that missense mutations in the Met gene are present in the germline of affected members of HPRC families. Remarkably, affected individuals often have a duplication of the chromosome bearing the mutated Met allele (Fischer et al., 1998; Zhuang et al., 1998). Similar mutations were also found in a subset of sporadic papillary renal carcinomas and of childhood hepatocellular carcinomas (Zhuang et al., 1998; Park et al., 1999). Importantly, NIH 3T3 fibroblasts transfected with Met constructs containing the mutations detected in HPRC, are transforming in vitro and tumorigenic in vivo (Jeffers et al., 1997). Taken together, these data strongly suggest that the Met mutants expressed in HPRC initiate tumorigenesis.

III. HGF/MET IN B CELL DEVELOPMENT AND NEOPLASIA

A. HGF/Met in antigen-specific B cell differentiation

Interestingly, recent studies from our laboratory have provided evidence for a role of the HGF/Met pathway in the immune system, i.e. in the regulation of antigen-specific B cell differentiation (van der Voort et al., 1997, 1999; Pals et al., 1998; Taher et al., 1999). During this process, naive B cells develop into memory cells or plasma cells. This requires multiple interactions of B cells with other cells, such as T cells and follicular dendritic cells (FDC), and with the ECM, which take place within distinct microenvironmental compartments of the lymphoid tissues (Fig. 8) (MacLennan, 1994; Nossal, 1994; Thorbecke et al., 1994; Liu et al., 1996a; Rajewsky, 1996; Lindhout et al., 1997). After their initial activation in the extrafollicular T cell (paracortical) area, germinal center (GC) founder cells migrate into B cell follicles where they initiate the formation of GCs (Liu et al., 1991; Jacob et al., 1991a). Once in the GC, the B cells first pass the dark zone where they undergo rapid clonal expansion and somatic hypermutation in their IgV genes (Berek et al., 1991; Jacob et al., 1991b; Küppers et al., 1993; McHeyzer-Williams et al., 1993; Pascual et al., 1994). Mutated B cells then progress to centrocytes and move to the basal light zone of the GC. Here they reencounter antigen, presented as low levels of immune complexes on FDC, and undergo affinity selection (Tew et al., 1990; Hardie et al., 1993; MacLennan, 1994). Whereas low affinity mutants and autoreactive mutants die by apoptosis, high affinity mutants internalize antigen and process it on their migration pathway to the apical light and outer-zones of the GC. In these areas, the affinity selected B cells present antigen to antigen-specific GC T cells (Fuller et al., 1993; Casamayor-Palleja et al., 1995; Zheng et al., 1996). Cognate T-B interaction results in expansion and Ig-isotype switching of high affinity B cells (Kraal et al., 1982; Liu
et al., 1996b), that mature into memory B cells or plasma cells and receive signals mediating their export from the lymphoid organ (MacLennan, 1994).

We observed that stimulation of human tonsillar B cells by phorbol ester and, more importantly, by concurrent CD40 and B cell receptor (BCR) ligation, leads to a rapid transient Met induction (van der Voort et al., 1997). Presumably, BCR- and CD40-mediated signals are also instrumental in the physiological induction of Met, as Met is expressed in vivo on tonsillar centroblasts (CD38+CD77+), which are the offspring of B cells that have recently been activated at extrafollicular sites by antigen plus T cells signals (MacLennan, 1994). These activating signals critically involve CD40/CD40L interactions: Patients with the X-linked hyper-IgM syndrome (due to mutated and consequently defective CD40L) do not develop GC and blocking of the CD40/CD40L pathway in mice leads to complete inhibition of GC reactions (Banchereau et al., 1994; Foy et al., 1994; Han et al., 1995; Kawabe et al., 1994; Facchetti et al., 1995). Our findings thus link Met induction to the initiation of the B cell immune response.

**Figure 8.** Schematic representation of the T cell-dependent B cell differentiation in secondary lymphoid organs. See text for further details. B, B cell; FDC, follicular dendritic cell; T, T cell.
Adhesion regulation, particularly regulation of lymphocyte integrin function, is believed to be fundamental to the control of cell migration and microenvironmental homing during B cell differentiation (Koopman and Pals, 1992; Butcher and Picker, 1996). In functional studies, we observed that HGF augments adhesion of Met positive B cell lines to VCAM-1 and fibronectin by activating the integrin α4β1 (van der Voort et al., 1997). Similar effects of HGF on integrin-mediated adhesion of B cells to fibronectin were also reported by Weimar et al. (1997). The physiological relevance of these findings is strongly supported by our observation that HGF is produced by stromal cells and FDC (van der Voort et al., 1997; and our unpublished observations). During B cell differentiation the integrin α4β1 mediates B cell adhesion to FDC (Freedman et al., 1990; Koopman et al., 1991, 1994), an interaction that regulates the formation of the microenvironment required for the affinity selection of GC B cells. Apart from establishing physical contact between B cells and FDC, α4β1 presumably contributes directly to the B cell selection process itself, as signaling through the α4β1/VCAM-1 pathway costimulates rescue of GC B cell from apoptosis (Koopman et al., 1994, 1997). Furthermore, α4β1 also regulates cell adhesion to fibronectin (Wayner et al., 1989), an important substrate for cell migration. Interestingly, Weimar et al. (1997) reported that HGF indeed stimulates B cell migration on fibronectin.

In view of the pleiotropic effects of HGF on many cell types, it is possible that, HGF may have other, as yet unknown, roles in antigen-specific B cell differentiation in addition to adhesion regulation. For example, as will be discussed in Section III.B, Met signaling might promote B cell proliferation and survival.

Preliminary results from in vivo studies support the involvement of the HGF/Met pathway in antigen-specific B cell differentiation. We explored this role by a molecular genetic approach using Met knock-out mice. Since homozygous Met knock-out mice die in utero at around day E15.5 (Bladt et al., 1995), the immune function of these mice can not be studied directly. To circumvent this problem, we reconstituted RAG-2−/IL-2γR− mice with fetal liver cells from MET−/− mice or control littermates. After i.v. injection, the haematopoietic stem cells present in the fetal liver migrate to the bone marrow, and regenerate B- and T-cell populations. Thus far, analysis of the B and T-cell compartments, the organization of the lymphoid organs, and of the baseline levels of immunoglobulin, demonstrated no significant difference between MET−/− and control mice. Interestingly, however, the immune response against the T cell-dependent antigen TNP-KLH was reduced in the MET−/− mice. Furthermore, in the spleens of immunized MET−/− mice, we observed a reduction in the number of plasma cells, the Ig-secreting population of B cells.
B. Met signaling in B cells

Although most data on Met-signaling have been obtained in epithelial cells, and hence do not necessarily apply to B cells, we have recently been able to demonstrate HGF/Met-induced phosphorylation and/or activation in B cells of at least several key signaling molecules such as Ras, MAP kinase, PI3-K, PKB and Gab1 (Fig. 5) (our unpublished observations). Here we will outline the putative roles of these molecules in B cell development.

As discussed above, the HGF/Met pathway is implicated in integrin regulation in B cells (van der Voort et al., 1997; Weimar et al., 1997). Several different signaling pathways have been implicated in inside-out signaling to integrins. Key regulatory proteins in these pathways appear to be PI3-K and different Ras-like GTPases (Howe et al., 1998; Kolanus and Seed, 1997; Hughes and Paff, 1998). PI3-K is involved in the activation of integrins in leukocytes (Shimizu et al., 1995), T cells (Zell et al., 1998) and platelets (Zhang et al., 1996a), whereas activated R-ras increases integrin activity in myeloid cells (Zhang et al., 1996b) and epithelial cells (Keely et al., 1999). Since R-ras is able to bind the same effector molecules as Ras, including PI3-K (Spaargaren et al., 1994; Spaargaren and Bischoff, 1994; Marte et al., 1997), integrin activation by R-ras may involve the activation of PI3-K.

In contrast to the stimulatory effect of R-ras, activated H-ras and Raf were found to inhibit activity of platelet integrins expressed in CHO cells (Hughes et al., 1997). Interestingly, R-ras appears to function as an antagonist of Ras-suppressed integrin activity in this cell system (Sethi et al., 1999). However, unlike its inhibitory effects on the activation of platelet-integrins, active Ras promotes TCR-triggered integrin-mediated T cell adhesion to ICAM-1 (O’Rourke et al., 1998).

These data imply that, dependent on the specific cell system studied, Ras may either inhibit or promote integrin activity. In addition to Ras and R-ras, the GTPase Rho has also been implicated in integrin activation, specifically in chemoattractant-induced integrin activation in lymphocytes and neutrophils (Laudanna et al., 1996). Since HGF stimulation of Met in B cells results in activation of PI3-K as well as the Ras-MAP kinase pathway (van der Voort et al., 1999) (our unpublished observations), and Rho has been implicated in Met signaling in epithelial cells (Takaishi et al., 1994, Nishiyama et al., 1994), it is likely that these proteins also play an important role in the regulation of integrin activity by HGF in B cells.

Recent studies have revealed several points of convergence between B-cell antigen receptor (BCR) and HGF/Met signaling. Firstly, the prominent Met substrate Gab1 was recently shown to become phosphorylated, and associated with Grb2, PI3-K, Shc and SHP-2, upon BCR triggering (Ingham et al., 1998; Nishida et al., 1999) (our unpublished observations). Secondly, PI3-K, which is activated upon stimulation of B cells by HGF, has a prominent role in BCR-signaling and B

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cell development: targeted disruption of the gene encoding the p85 subunit of PI3-K arrests B cell development at the pro-B cell stage, resulting in decreased immunoglobulin production (Fruman et al., 1999; Suzuki et al., 1999), similar to that in X-linked hypoglobulinemia. Thirdly, STAT3, which becomes phosphorylated and translocates to the nucleus upon stimulation by HGF (Boccaccio et al., 1998), also plays a role in BCR-mediated signaling (Karras et al., 1997). Finally, we observed that HGF activates the Ras-MAPK pathway in B cells. This presumably constitutes an important transcription regulatory and proliferative signal for Met expressing GC B cells, which are in the process of undergoing rapid clonal expansion and selection (Lindhout et al., 1997; Tarlinton, 1998b). Ras is also involved in BCR-signaling and B cell development. Expression of dominant negative Ras arrests development at a very early stage, prior to formation of the pre-B cell receptor (Iritani et al., 1997). Furthermore, activated Ras causes progression of RAG1-deficient pro-B cells to pre-B cells and to cells with characteristics of the more mature GC B cells (Shaw et al., 1999).

Successful B cell selection in the GC requires tight regulation of cell survival. Interestingly, several studies indicate that the HGF/Met pathway may generate survival signals. HGF can rescue MDCK cells from apoptosis (Frisch and Francis, 1994), and inhibits apoptosis induced by staurosporin or DNA damaging agents of liver progenitor and carcinoma cells (Bardelli et al., 1996; Fan et al., 1998; Liu et al., 1998b). In addition, overexpression of an active Met mutant renders hepatocytes resistant to anoikis and staurosporin-induced apoptosis (Amicone et al., 1997). Given the ability of Met to interact with the anti-apoptotic BAG-1 upon HGF stimulation (Bardelli et al., 1996), it is interesting to note that BAG-1 has been reported to play a role in survival and proliferation of the IL-3-dependent B cell line Ba/F3 (Cleveenger et al., 1997). Furthermore, we have found that Met can activate PKB, in a PI3-K-dependent fashion, in B cells (our unpublished observation). PKB is able to phosphorylate Bad, a Bcl-2 antagonist expressed in GC B cells (Ghia et al., 1998), and thereby may suppresses the pro-apoptotic activity of Bad (Datta et al., 1997; Mok et al., 1999) (Fig. 5). Taken together, these data suggest that Met may play an important role in the regulation of apoptosis in the GC B cells, and thus in the process of affinity selection, which is critical for antigen-specific B cell differentiation.

Finally, regulation of GC B cell migration is important for the antigen-specific B cell differentiation (Tarlinton, 1998a; Pals et al., 1998). Several chemokines have been shown to be involved in this process, including SDF-1, which is produced by reticulum cells surrounding the GC and acts via the G protein-coupled receptor CXCR4 (Bleul et al., 1996, 1998). A recent study has reported the ability of the BCR to arrest SDF-1α-induced migration (Bleul et al., 1998). Interestingly, it was shown that this arrest was caused by PKC-mediated CXCR4 downregulation (Guinamard et al., 1999). Since HGF stimulation also
results in activation of PKC, this mechanism may provide a means to arrest migration of Met expressing B cells in the GC.

C. Heparan sulfate proteoglycans on B cells promote Met signaling

We have recently obtained evidence that HSPGs expressed on the cell-surface of specific B cell subsets may play an important role in regulating Met signaling. Several human B cell subpopulations, including plasma cells and memory B cells, express HSPGs (van der Voort et al., 2000). Interestingly, this HSPG expression was greatly enhanced by activation of B cells with the phorbol ester PMA, and more importantly, by ligating the co-stimulatory molecule CD40. An even stronger induction of HSPG was obtained after simultaneous ligation of CD40 and the BCR, signals which also induce expression of Met (van der Voort et al., 1997). Since CD40 and the BCR play key roles during the T cell-dependent B cell differentiation (see Section III.A), these data suggest that Met and HSPGs act in concert during this biological process. Indeed, we observed that, upon activation, B cells acquire the capacity to bind large amounts of HGF via HS-moieties. CD44 isoforms carrying HS-chains (CD44-HS) are the major proteoglycan core proteins on these activated B cells, which did not express the core proteins of syndecan-1, 2, 4, or glypican. However, others have shown that human as well as murine plasma cells express syndecan-1 (Sanderson et al., 1989), whereas syndecan-4 was recently shown to be expressed by murine immature and mature B cells (Yamashita et al., 1999).

By using Burkitt’s lymphoma cells transfected with either CD44-HS or a CD44 isoform lacking HS (CD44s), we demonstrated that CD44-HS strongly promotes signal transduction via Met, including Met phosphorylation, phosphorylation of Gab-1, activation of the MAP kinases ERK1/2, and phosphorylation of PKB. Taken together, our results identify HSPGs, specifically CD44-HS, as functional co-receptors for HGF promoting signal transduction through Met (Fig. 6). We hypothesize that, via concentration and presentation of HGF, HSPGs regulate the biological activity of the HGF/Met pathway in B cells.

D. The HGF/Met pathway in B cell neoplasia

The HGF/Met pathway presumably is not only involved in normal B cell differentiation, but also in the development and progression of B cell neoplasia. Met is constitutively expressed by several Burkitt’s lymphoma cell lines, including Raji, BJAB, and EB4B (Jücker et al., 1994; van der Voort et al., 1997, Weimar et al., 1997), as well as by a subset of native Burkitt’s lymphomas (Weimar et al., 1997).
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In these tumor cells, which represent the malignant counterparts of GC centroblasts, HGF induces Met phosphorylation, as well as activation of downstream signaling molecules including MAP kinases and PKB (van der Voort et al., 1997, 1999; Taher et al., 1999; our unpublished observations) (see also Section III.B). Furthermore, HGF stimulation of Met positive Burkitt's lymphoma cells enhances α4β1 and α5β1-mediated adhesion to fibronectin, collagen and VCAM-1, and promotes their invasion into fibroblast monolayers (van der Voort et al., 1997; Weimar et al., 1997). Since HGF is produced by follicular dendritic cells and lymphoid stromal cells (van der Voort et al., 1997) (our unpublished observation), paracrine stimulation of Burkitt's lymphoma cells by HGF most likely takes place within the lymphoid microenvironment, promoting tumor growth and/or survival. HGF/Met signaling may stimulate survival via at least two distinct routes. As a direct consequence of HGF stimulation, Met may become associated with the anti-apoptotic protein BAG-1 (Bardelli et al., 1996) (see also Section III.B). At the same time, HGF stimulation may down-modulate the activity of the Bcl-2 antagonist Bad (Datta et al., 1997). Alternatively, activation of integrins by HGF/Met signaling may prevent tumor cell death by anoikis. This scenario is supported by our previous observation that integrin mediated adhesion to FDC presents a strong anti-apoptotic signal for GC B cells (Koopman et al., 1994, 1997).

Although precise data concerning the expression of Met and HGF in different subtypes of malignant lymphoma are at present not available, a study by Weimar and colleagues (1997) indicates that Met expression is not confined to Burkitt's lymphoma. In 8 out of 11 follicle center cell lymphomas, and in some cases of large B cell lymphoma, Met expression was observed. Furthermore, in approximately half of the cases of Hodgkin's disease Met expression was found in Hodgkin's/ Reed-Sternberg (RS) cells, which presumably represent "crippled" GC B cells (Braeuninger et al., 1997). Interestingly, Met expression in RS cells was strongly correlated with the presence of Epstein-Barr virus (EBV), suggesting a role for EBV in Met regulation (Weimar et al., 1997). Although recent studies from our laboratory do not confirm the high percentage of Met positive cases among follicle center cell lymphomas, we did find Met expression in large B cell lymphomas (our unpublished observations).

HGF has also been identified as a potential growth factor for multiple myeloma (MM), a neoplasm of terminally differentiated B cell (i.e. plasma cells). By screening myeloma supernatants for their ability to inhibit the activity of transforming growth factor-β (TGF-β), Borset and colleagues (1996c) isolated an antagonist. This protein was identified as HGF, and was produced by all five myeloma cell lines tested. Interestingly, in four of these cell lines Met was also expressed, suggesting the existence of an autocrine HGF/Met loop. Indeed, in the human myeloma cell line JJN-3, Met was found to be constitutively
phosphorylated and could be dephosphorylated by anti-HGF antibodies (Borset et al., 1996b). These findings were further extended by analyzing MM cells freshly isolated from patients. In all seven cases studied, co-expression of HGF and Met was observed on MM cells isolated from the bone marrow (Borset et al., 1996a). Recently, the Nordic Myeloma Study Group reported the HGF serum levels of over 400 MM patients. In approximately half of these patients elevated HGF levels were present; these patients had an unfavorable prognosis and poor response to melphalan/prednisone treatment (Seidel et al., 1998).

In conclusion, while its precise role needs to be elucidated, the above data suggests that deregulated HGF/Met signaling may contribute to the development and progression of specific subtypes of B cell neoplasia, including Burkitt’s lymphoma, large B cell lymphoma, and multiple myeloma.

IV. SUMMARY

This review summarizes the structure, signal transduction and physiological functions of the HGF/Met pathway, as well as its role in tumor growth, invasion, and metastasis. Moreover, it highlights recent studies indicating a role for the HGF/Met pathway in antigen-specific B cell development and B cell neoplasia.

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