Small GTP-binding proteins and regulated secretion of von Willebrand factor by endothelial cells

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INTRODUCTION

Endothelial cells constitute a highly regulated barrier between tissue and blood, which can respond rapidly to changes in their microenvironment (Cines et al., 1998). Triggering of endothelium by inflammatory reagents results in altered expression of surface molecules allowing for modulation of their adhesive properties. In addition, endothelial cells contribute to a considerable extent to processes like vasodilatation and vasoconstriction. A further illustration of the active role of endothelium in maintaining vascular homeostasis is provided by the presence of intracellular storage pools for a number of bioactive components. Upon activation with an appropriate agonist the contents of these intracellular storage pools is released into the circulation. In 1964, a novel subcellular organelle was described that was exclusively present in endothelial cells (Weibel and Palade, 1964). Subsequent investigations revealed that the contents of these organelles, nowadays known as Weibel-Palade bodies, is secreted upon stimulation of endothelial cells with agonists such as thrombin. Several proteins have been localized to these subcellular organelles, which include von Willebrand factor (vWF) and P-selectin. In this thesis, the molecular mechanism of regulated exocytosis of Weibel-Palade bodies is studied. Recently, evidence has been obtained for the involvement of members of the superfamily of Ras-like small GTP-binding proteins in this process. This introduction summarizes the current knowledge on the biogenesis and exocytosis of Weibel-Palade bodies. In the last paragraph, a short introduction is given on small GTP-binding proteins that have been implicated in release of storage granules in cells of non-endothelial origin.

WEIBEL-PALADE BODIES

In 1964, Weibel and Palade described a rod-shaped cytoplasmic structure only present in endothelial cells which consisted of a bundle of fine tubules, enveloped by a tightly fitted membrane. They were found in endothelial cells of small arteries in various organs in rat and men. These organelles have a length of approximately 3 μm and a width of 0.15 μm (Weibel and Palade, 1964; Figure 1A). Vascular endothelial cells from a number of sources, including bovine aorta and human adipose tissue capillaries, do not contain Weibel-Palade bodies (Schwartz, 1978; Kern et al., 1983). Immunolocalization and cell fractionation studies revealed that vWF is a major component of Weibel-Palade bodies (Wagner et al., 1982; Reinders et al., 1984; Ewenstein et al., 1987). VWF is a multimeric protein involved in adhesion of blood platelets to a damaged vessel wall. In plasma, it serves as a carrier protein for factor VIII (Sadler, 1998). In addition to vWF, P-selectin, a transmembrane receptor which mediates adhesion of neutrophils, and CD63 (also called lysosome-associated membrane protein 3, LAMP-3) have been found in Weibel-Palade bodies (McEver et al., 1989; Bonfanti et al., 1989; Vischer and Wagner, 1993). In recent reports, also endothelin has been localised to these organelles and, after prolonged stimulation of endothelial cells,
interleukin-8 is stored in Weibel-Palade bodies as well (Russell et al., 1998; Wolff et al., 1998; Utgaard et al., 1998). A number of studies have shown that condensation of multimeric vWF plays a crucial role in the biogenesis of Weibel-Palade bodies (Wagner et al., 1991; Voorberg et al., 1993; Hop et al., 1997). In the next paragraph, intracellular processing steps, required for the generation of fully processed, high molecular weight multimers of vWF are described.

**Figure 1.** Weibel-Palade bodies are storage organelles for high molecular weight multimers of von Willebrand factor.

A. Electron microscopic image of endothelial cell stained with polyclonal antibody directed to von Willebrand factor. Labelling of a rod-shaped Weibel-Palade body is observed. B. Schematic representation of the domain organization of vWF. The arrow indicates the cleavage site at Arg763 that releases the propeptide from pro-vWF. C. Multimeric structure of vWF. Pro-vWF monomers dimerize through cysteine residues located at the carboxy terminus of vWF. Multimers are formed via cysteine residues in D1, D2, D', and D3 domain.

**Biosynthesis of von Willebrand factor**

During the last decade, significant insight has been obtained in the biosynthesis and processing of vWF (reviewed by Hop and Pannekoek, 1996; Sadler, 1998). VWF is
Introduction

Synthesised by megakaryocytes and endothelial cells as a precursor polypeptide of 2813 amino acids (pre-pro-vWF). The vWF gene is located on chromosome 12 and consists of 52 exons. A pseudogene representing partial duplication of exons 23-34 is located on chromosome 22 (Mancuso et al., 1991). The translation product includes a signal peptide of 22 amino acids, a propeptide of 741 amino acids and mature subunit of 2050 amino acids. VWF contains repetitive homologous domains D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2, where D1-D2 comprises the propeptide and D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2 the mature subunit (Figure 1B). The protein is remarkably rich in cysteines, which comprise 234 of the 2813 amino acids of pre-pro-vWF (Verweij et al., 1986; Bonthron et al., 1986). Following synthesis, the signal peptide is cleaved off upon translocation of the protein into the endoplasmic reticulum (ER). In the ER, disulphide bonding mediated by the cysteine residues located in the last 151 amino acids on the carboxy-terminal part results in the formation of pro-vWF dimers (Voorberg et al., 1991; Figure 1C). Interestingly, the last 90 amino acids at the carboxy terminal part constitute a conserved motif that also occurs in a number of growth factors and mucins (McDonald and Hendrickson, 1993; Meitinger et al., 1993). This so-called "cysteine knot motif" contributes to formation of dimers, that are often stabilized by disulphide bonds. The physiological importance of cysteine residues in this region is underscored by the presence of two point mutations (C2671Y and C2773R) in patients with the severe form of von Willebrand disease (Schneppenheim et al., 1996; Eikenboom et al., 1998). Von Willebrand disease is a bleeding disorder, characterized by a quantitative (type 1 and 3) or qualitative (type 2) defect in vWF (Sadler et al., 1998). Substitution of Cys2773 for an Arg interferes with dimerization of pro-vWF (Schneppenheim et al., 1996). Reduced levels of vWF in plasma of patients carrying this point mutation are most likely caused by the aberrant biosynthesis of vWF in endothelial cells and megakaryocytes. Correctly assembled pro-vWF dimers are transported to the Golgi apparatus where further processing takes place. Multimerization of pro-vWF dimers requires cysteine residues in domains D1, D2, D' and D3 of vWF (Voorberg et al., 1990; Mayadas and Wagner, 1992). Mutations in the D3 domain of vWF (C1159R and D879N), observed in type 1 vWD, result in aberrant assembly of multimers (Eikenboom et al., 1996; Jorieux et al., 1998). Proteolytic processing in the trans-Golgi network releases the propeptide (D1-D2) from the mature subunit (Wagner, 1990). Non-covalent interactions mediated by the vWF propeptide direct assembly of multimers. The presence of CXXC sequences, similar to that observed in thioredoxin, suggests that the propeptide catalyzes interchain disulfide bonding at the amino-terminus of mature vWF (Mayadas et al., 1992). These findings are supported by co-transfection experiments with propeptide (domains D1-D2) and vWFdelpro (lacking domains D1-D2) encoded on separate cDNAs. Under these conditions, formation of multimers proceeds as efficiently as observed upon transfection of full-length vWF cDNA (Wise et al., 1988; Voorberg et al., 1993). These results suggest that multimer assembly may continue following proteolytic processing of pro-vWF in endothelial cells.

Following transport through the Golgi apparatus, vWF partitions between two different
pathways in endothelial cells. Not fully processed, low molecular weight vWF multimers are secreted in a constitutive manner. In contrast, high molecular weight multimers are stored in Weibel-Palade bodies which release their contents upon stimulation of endothelial cells by agonists like thrombin (Wagner, 1990). The requirements for biogenesis of vWF containing granules have been studied extensively by heterologous expression of vWF cDNA variants. Dense granules containing vWF are observed following transfection with wild type vWF cDNA in AtT-20 mouse pituitary cells, RIN5F rat insulinoma cells, CV-1 monkey kidney cells and MDCK canine epithelial cells (Wagner et al., 1991; Mayadas and Wagner, 1992; Voorberg et al., 1993; Hop et al., 1997). The amino-acid sequences involved in directing vWF to storage compartments are only partially elucidated. Most likely, (non-) covalent interactions occurring at multiple sites on vWF direct the biogenesis of vWF-containing granules (Voorberg et al., 1993). Remarkably, secretion of vWF containing granules is not sensitive to extracellular stimuli in AtT-20, RIN5F and CV-1 cells (Wagner et al., 1991; Voorberg et al., 1993). In contrast, exocytosis of vWF storage organelles can be induced in MDCK cells upon stimulation with an agent that activates the protein kinase C pathway (Hop et al., 1997). This observation suggests that regulated exocytosis of vWF granules requires integration of exocytic machinery with appropriate signalling pathways.

**Exocytosis of Weibel-Palade bodies in endothelial cells**

In endothelial cells, the contents of Weibel-Palade bodies is released upon stimulation of the cells with various agents such as thrombin, epinephrine, reactive oxygen intermediates, adenosine nucleotides, peptido-leukotrienes, cytokines and under conditions of hypoxia (Levine et al., 1982; Vischer et al., 1995, 1997; Palmer et al., 1994; Datta et al., 1995; Paleolog et al., 1990; Pinsky et al., 1996). In plasma, the level of vWF can be increased by infusion of the vasopressin analog 1-desamino-8-D-arginine vasopressin (DDAVP) (Mannucci et al., 1977; Borchiellini et al., 1996). The action of DDAVP in vivo seems to be indirect since cultured endothelial cells do not respond to DDAVP (Booth et al., 1987). Monocytes treated with DDAVP secrete platelet-activating factor that mediates vWF release from endothelial cells (Hashemi et al., 1993).

Exocytosis of Weibel-Palade bodies induced by thrombin is coupled to phospholipid methylation and can be blocked by 3-deazaadenosine, a methyltransferase inhibitor (de Groot et al., 1984). Evidence for the involvement of calcium in the regulated exocytosis of vWF has been provided by the observation that BAPTAM, a calcium chelator, in combination with the extracellular calcium chelator EGTA, inhibits thrombin-induced secretion (Van den Eijnden-Schrauwen et al., 1997). In permeabilized cells, thrombin-induced exocytosis of Weibel-Palade bodies can be blocked by a calmodulin-binding inhibitory peptide suggesting that this process is mediated by calcium/calmodulin (Birch et al., 1992). Activation of protein kinase C is not necessary for thrombin-induced release of vWF while staurosporin, an inhibitor of PKC, did not affect exocytosis of Weibel-Palade bodies (Birch et al., 1992).

Limited information is available concerning the cellular components that are involved in
exocytosis of Weibel-Palade bodies. In many cell types, regulated secretion is mediated by small GTP-binding proteins of the Ras superfamily. Therefore, it seems likely that also in endothelial cells small GTP-binding proteins are involved in release of vWF through the regulated pathway. Initially, no evidence could be obtained for the involvement of GTP-binding proteins in the exocytosis of Weibel-Palade bodies using electroporpermeabilized endothelial cells (Frearson et al., 1995). Only recently, a role for GTP-binding proteins has been suggested in this process (Van den Eijnden-Schrauwen, 1997; Fayos and Wattenberg, 1997). For instance, thrombin-induced release of vWF could be blocked by GDPβS, an inhibitor of GTP-binding proteins. To distinguish between heterotrimeric G proteins and small GTP-binding proteins, human umbilical vein endothelial cells (intact or saponin-permeabilized) were incubated with aluminium fluoride, an activator of heterotrimeric G proteins. A stimulatory effect was observed on vWF secretion suggesting that at least heterotrimeric G proteins are involved in this process (Van den Eijnden-Schrauwen, 1997). Another study using digitonin-permeabilized cells suggested the involvement of a small GTP-binding protein of the Ras superfamily while incubation of the cells with aluminium fluoride did not affect secretion of vWF (Fayos and Wattenberg, 1997). Taken together, these studies do not provide evidence that this process is indeed mediated by small GTP-binding proteins. However, in view of their pivotal role in vesicular transport and intracellular signalling we postulated that small GTP-binding proteins serve an essential role in regulated exocytosis of Weibel-Palade bodies in endothelial cells.

SMALL GTP-BINDING PROTEINS OF THE RAS SUPERFAMILY INVOLVED IN REGULATED EXOCYTOSIS

Small GTP-binding proteins function as a molecular switch alternating between an active ‘GTP’- and inactive ‘GDP’- bound conformation. The superfamily of Ras-like GTPases consists of more than 50 proteins that can be divided in five subfamilies: Ras, Rho, Rab, Ran and Arf. Ras proteins are involved in intracellular signalling pathways that determine growth and differentiation of cells (Scheffzek et al., 1998). Single point mutations in Ras result in oncogenic transformation and have been detected in a variety of human tumours (Bos, 1989). Members of the Rho subfamily play a role in organization of the cytoskeleton (Nobes and Hall, 1995; Allen et al., 1997). In endothelial cells, Cdc42 and Rho have been shown to regulate cytoskeleton reorganization in response to shear stress (Li et al., 1999). Ran functions in transport through the nuclear pore complex (Moore, 1998). The Rab and Arf subfamilies play an essential role in budding and fusion of vesicles that move between different cellular compartments (Novick and Zerial, 1997; Chavrier and Goud, 1999). So far, a number of small GTP-binding proteins of the Rab-family have been identified in endothelial cells. An isoform of Rab5 has been cloned from an endothelial cell cDNA library (Wilson and Wilson, 1992). In addition, the presence of Rab1, Rab3b, Rab4, Rab6 and Rab8 in endothelial cells was established by immunoblotting (Karniguian et al., 1993).
### TABLE 1: Small GTP-binding proteins involved in regulated exocytosis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cell type</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rab3A</td>
<td>Synaptic vesicles</td>
<td>Geppert et al., 1998; Lledo et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oishi et al., 1998; Fischer von Mollard et al., 1991; Matteoli et al., 1991</td>
</tr>
<tr>
<td>Rab3a</td>
<td>Neuroendocrine cells</td>
<td>Johannes et al., 1994</td>
</tr>
<tr>
<td>Rab3a</td>
<td>Adrenal chromaffin cells</td>
<td>Holz et al., 1994, Lin et al., 1996</td>
</tr>
<tr>
<td>Rab3a</td>
<td>Insulin secreting cells</td>
<td>Regazzi et al., 1996; Johannes et al., 1998; Olszewski et al., 1994</td>
</tr>
<tr>
<td>Rab3a</td>
<td>RBL cells</td>
<td>Smith et al., 1997</td>
</tr>
<tr>
<td>Rab3a/b</td>
<td>Epithelial cells</td>
<td>Weber et al., 1996</td>
</tr>
<tr>
<td>Rab3b</td>
<td>Pituitary cells</td>
<td>Tasaka et al., 1998, Perez et al., 1994</td>
</tr>
<tr>
<td>Rab3c</td>
<td>Synaptic vesicles</td>
<td>Fischer von Mollard et al., 1994</td>
</tr>
<tr>
<td>Rab3d</td>
<td>Pituitary cells</td>
<td>Baldini et al., 1998</td>
</tr>
<tr>
<td>Rab3d</td>
<td>Mast cells</td>
<td>Tuvim et al., 1999; Roa et al., 1997</td>
</tr>
<tr>
<td>Rab3d</td>
<td>Pancreas acini</td>
<td>Valentijn et al., 1996, Ohnishi et al., 1997</td>
</tr>
<tr>
<td>Rab4</td>
<td>Pancreatic acini</td>
<td>Ohnishi et al., 1999</td>
</tr>
<tr>
<td>Arf-like</td>
<td>PC12 cells</td>
<td>Icard-Liepkalns et al., 1997</td>
</tr>
<tr>
<td>Arf6</td>
<td>Chromaffin cells</td>
<td>Caumont et al., 1998; Galas et al., 1997</td>
</tr>
<tr>
<td>Rho</td>
<td>Pancreatic beta cells</td>
<td>Kowluuru et al., 1997</td>
</tr>
<tr>
<td>Rac/Rho</td>
<td>Mast cells and PC12 cells</td>
<td>Price et al., 1995; Mariot et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Dense granules in platelets</td>
<td>Brown et al., 1998; Komuro et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Synaptic vesicles in nerve terminals</td>
<td>Bielinski et al., 1993</td>
</tr>
</tbody>
</table>

A subset of small GTP-binding proteins has been implicated in regulated exocytosis (Table 1). Despite considerable effort, the precise function of small GTP-binding proteins in these processes has only been defined in a few cases. The role of Rab3A in regulated exocytosis of synaptic vesicles at the nerve terminal has been extensively studied (Südhof, 1995; Bean and Scheller, 1997, Gonzalez and Scheller, 1999). Under quiescent conditions, Rab3A in its GDP-bound form is attached to guanine dissociation inhibitor (GDI) (Figure 2). Following displacement of Rab3A from its GDI by the action GDI displacement factor (GDF), Rab3A is translocated to the synaptic vesicle. This translocation occurs during or after formation of the vesicle. Association of Rab3A with the synaptic vesicle is stabilized by guanine nucleotide exchange factors (GEFs) which exchange GDP for GTP. Similar to other small GTPases, Rab3A, in its GTP-bound conformation, interacts with a distinct set of effector molecules. In the last few years many effector proteins have been identified for different Rab proteins.
Effector proteins specific for Rab3 are RIM and Rabphilin-3A (Wang et al., 1997; Shirataki et al., 1993). Both RIM and Rabphilin-3A contain Ca\(^{2+}\)-binding C2 domains suggesting their involvement in Ca\(^{2+}\)-dependent process of synaptic vesicle docking and fusion. Recently, the crystal structure of the Rab-binding domain of Rabphilin-3A with Rab3A was elucidated, providing more insight on the specificity of interactions between effector proteins and their Rab proteins (Ostermeier and Brünger, 1999). In addition, Rab3 interacts with calmodulin in a Ca\(^{2+}\)-dependent manner (Park et al., 1997; Coppola et al., 1999). Rab proteins are not solely responsible for targeting of vesicles to the appropriate membrane. The identification of SNARE proteins on both vesicle- and target-membrane led to the hypothesis that these proteins direct vesicle targeting and docking. A possible link between Rab3 and SNARE proteins was suggested but the presence of Rab3 in SNARE complexes could not be established yet (Johannes et al., 1996). Also a number of other proteins that are involved in docking of vesicles have been identified. These findings suggest that a complex series of

![Figure 2](image.png)

**Figure 2.** Model for cycling of Rab3A in regulated exocytosis. Rab in its GDP-bound form (oval marked with 'GDP') is solubilized in the cytosol associated with guanine nucleotide dissociation inhibitor (GDI). Upon action of GDI displacement factor (GDF), Rab dissociates from GDI and translocates to vesicle. GDP is exchanged for GTP mediated by guanine nucleotide exchange factor (GEF) which stabilizes the binding of Rab in its GTP-bound form (diamond marked with 'GTP') to the vesicle. Vesicle is recruited to the target membrane most likely mediated by interaction of Rab\(_{GTP}\) with a number of effector molecules. GTP is hydrolyzed to GDP and inorganic phosphate catalyzed by a GTPase activating protein (GAP) and fusion of vesicle and target membranes occur. The cycle is completed by recruitment of Rab\(_{GDP}\) from the membrane by GDI (Adapted from Bean and Scheller, 1997). For reasons of clarity, SNARE complexes and potential link of effector molecules to the cytoskeleton are not included.
interactions between proteins results in vesicle docking. The role of Rab proteins in this process is still not clear (Chavrier and Goud, 1999). The Rab protein in its GTP-bound conformation can recruit other proteins to the docking site or could regulate the formation of SNARE complexes. GTP hydrolysis mediated by a GTPase activating protein (GAP) occurs simultaneously or immediately after fusion of the vesicle with the plasma membrane. Recently, a GTPase-activating protein specific for Rab3 has been identified (Nagano et al., 1998). Subsequently, Rab3A in its GDP-bound form is retrieved from the plasma membrane by GDI and a new cycle can be initiated.

Next to Rab3A, several other small GTPases have been implicated in regulated exocytosis. A role for Rab3b in Ca\(^{2+}\)-dependent exocytosis in pituitary cells has been proposed based on studies with antisense oligonucleotides (Lledo et al., 1993). In addition, studies employing expression of constitutively active (GTP-bound) and inactive (GDP-bound) Rab3b variants have provided evidence for functional involvement of this small GTPase in regulated exocytosis in neuroendocrine PC12 cells (Weber et al., 1996). A similar approach has been used to define a role for Rab3D in regulated secretion in pituitary and mast cells (Baldini et al., 1998; Roa et al., 1997). Recently, Rab4 has been implicated in regulated secretion in pancreatic acini (Ohnishi et al., 1999). It should be noted that previous studies have assigned a function for Rab4 in endocytosis (van der Sluijs et al., 1992). In chromaffin cells, complexes of Arf6 and heterotrimeric G-proteins have been localized to secretory granules (Galas et al., 1997). A number of studies have suggested functional involvement of members of the Rho subfamily in signalling pathways that are linked to regulated secretion (see Table 1). Finally, subcellular fractionation studies have shown association of Ral with dense granules in platelets and synaptic vesicles at nerve terminals (Mark et al., 1996; Bielinski et al., 1993). Together these studies suggest that small GTP-binding proteins are involved in regulated secretion in a wide variety of cells.

**OUTLINE OF THIS THESIS**

The molecular mechanism underlying the regulated exocytosis of Weibel-Palade bodies by endothelial cells is largely unknown. In many cell types, regulated secretion is mediated by small GTP-binding proteins of the Ras superfamily (Table 1). In this thesis, we examined the role of small GTP-binding proteins in exocytosis of Weibel-Palade bodies. Throughout this study, we used cultured human umbilical vein endothelial cells (HUVEC) as a model. Limited information is available on the repertoire of small GTP-binding proteins in endothelial cells. First, the repertoire of small GTP-binding proteins in HUVEC was identified (Chapter 2). In this study, we have also investigated the subcellular localization of Rab30, one of the small GTPases identified in endothelial cells. In the course of this study, we identified a novel isoform of Rab6, which results from alternative splicing of Rab6A gene (Chapter 3). We have studied whether (1) there are differences in biochemical properties between these two isoforms and (2) whether there are differences in expression among cells and tissues. In Chapter 4, Weibel-Palade bodies were isolated and examined for the presence of small
GTPases on these storage organelles. Small GTPase RaLA was found to be associated with subcellular fractions containing Weibel-Palade bodies. Subsequently, the functional involvement of RaLA in exocytosis of Weibel-Palade bodies was studied in more detail by expression of RaLA variants in endothelial cells (Chapter 5). The results obtained from these studies are discussed within the context of findings reported by other investigators (Chapter 6).

LITERATURE


**Chapter 1**


**Schwartz, S.M.** (1978) Selection and characterization of bovine aortic endothelial cells. *In Vitro* 14: 966-980.


