Sorting out the Weibel-Palade body

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Sorting out the Weibel-Palade body

Uitpluizen van de Weibel-Palade body

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

General introduction

Ruben Bierings

Introduction

Vascular endothelial cells form the inner lining of the vessel wall and thereby help maintaining the structural integrity of the blood vessel. In addition, the endothelium provides the surface for and actively participates in the mediation of inflammatory responses and in the arrest of bleeding in the event of vascular damage. To effectively control the arrest of bleeding in case of vascular emergency, local circulation is temporarily obstructed by the formation of a hemostatic plug of activated platelets that are recruited to sites where the vessel is damaged, a process called primary hemostasis. During secondary hemostasis, proteins of the coagulation cascade promote the formation of a clot composed of fibrin strands that seals off the vessel, giving repair mechanisms the opportunity to reconstruct the damaged vessel. Inflammatory responses rely on rapid recruitment of leukocytes to areas where the vasculature has sustained damage and/or to sites of infection, and this a resultant of activating and adhesive events. Also, by loosening or tightening of the endothelial cell-cell contacts, local permeability of the vessel wall can be regulated to facilitate or prevent the extravasation of leukocytes to underlying tissue.

Endothelial cells also take part in these processes by releasing the content of their unique, cigar-shaped organelles called Weibel-Palade bodies (WPBs) [1]. These organelles function as storage vesicles for von Willebrand factor (VWF) [2], a multimeric adhesive glycoprotein crucial for platelet plug formation, the leukocyte receptor P-selectin [3,4] and a number of bioactive compounds that include angiopoietin-2 [5] (Ang-2) and the chemokines interleukin-8 (IL-8) [6,7] and eotaxin-3 [8].

This thesis is aimed at getting more insight into the molecular mechanisms that regulate storage of secretory proteins in WPBs as well as the mechanisms that control trafficking and exocytosis of these granules. In the following paragraphs the current understanding of the biogenesis of Weibel-Palade bodies and the processes that regulate their release are described in more detail.

Weibel-Palade bodies

WPBs are cigar-shaped electron-dense subcellular structures that are exclusively found in the cytoplasm of endothelial cells [1]. They function as secretory organelles that can rapidly deliver pre-stored amounts of secretory proteins in response to endothelial activation without the need of *de novo* protein synthesis. Subsequent studies indicated that WPBs are the endothelial storage compartment of VWF, P-selectin and a number of additional bioactive substances (see below). At the time of discovery it was already suggested that "these bodies are connected with vascular or blood physiology" [1]. The importance of release of WPBs for vascular homeostasis is underscored by the phenotypes that are associated with failure to release WPBs. Mice of which the endothelial cells are devoid of WPBs have a bleeding tendency that is caused by defective thrombus formation,

a direct result of impaired platelet recruitment [9]. Moreover, these mice display impaired leukocyte rolling, one of the steps in leukocyte extravasation, and they fail to recruit neutrophils to sites of inflammation. These defects can be attributed to failure to present P-selectin onto the cellular membrane through exocytosis of WPBs [10].

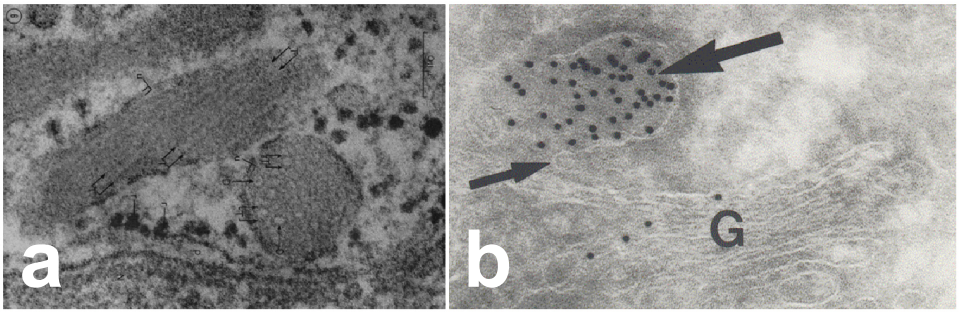

Figure 1: Weibel-Palade bodies are highly structured subcellular organelles that originate from the *trans*-Golgi network. (a) Electron micrographs showing transverse and oblique sections of Weibel-Palade bodies in endothelial cell of small pulmonary artery of a rat [1]. (reprinted with permission of The Rockefeller University Press). (b). Immunogold labeling of VWF in endothelial cells reveals a densely staining budding WPB (large arrow) that is still connected to the *trans*-Golgi network and the Golgi stack (G) by a limiting membrane (small arrow) [12] (reprinted with permission of Nature Publishing Group).

WPBs originate from the trans-Golgi network and their biogenesis is tightly connected to the biosynthesis of its main constituent, VWF. Several lines of evidence support the notion that VWF is the driving force behind the biogenesis of its own storage granule. Heterologous expression of VWF cDNA in non-endothelial cell types, irrespective of the availability of a regulated secretory pathway, induces the formation of pseudo-WPBs, vesicles that store VWF and that, at least in terms of morphology and secretion competence, resemble authentic WPB [11-13]. Conversely, in case of deficient VWF expression, such as canine type 3 von Willebrand disease (VWD) [14] or VWF knockout mice [9], no WPBs are formed.

Biosynthesis of von Willebrand factor

Von Willebrand factor (VWF) is a large adhesive, multimeric glycoprotein that is exclusively synthesized by endothelial cells and megakaryocytes [15,16]. It serves a crucial role in mediation of platelet adherence at sites of vascular injury. It also functions as carrier protein for the coagulation factor VIII (FVIII), by which it prevents its premature proteolytic degradation and clearance. VWD, a common hereditary bleeding disorder, is caused by abnormal functioning or deficient amounts of VWF resulting from mutations or gene deletions (reviewed in [17,18]). Expression of the approximately 180 kilobase VWF gene, which is located at the short arm of chromosome 12 [19-21],

yields a 2813 amino acid polypeptide (pre-pro-VWF), consisting of 22 amino acid signal peptide, a 741 amino acid propeptide and the mature VWF chain of 2050 residues [22] (Figure 2).

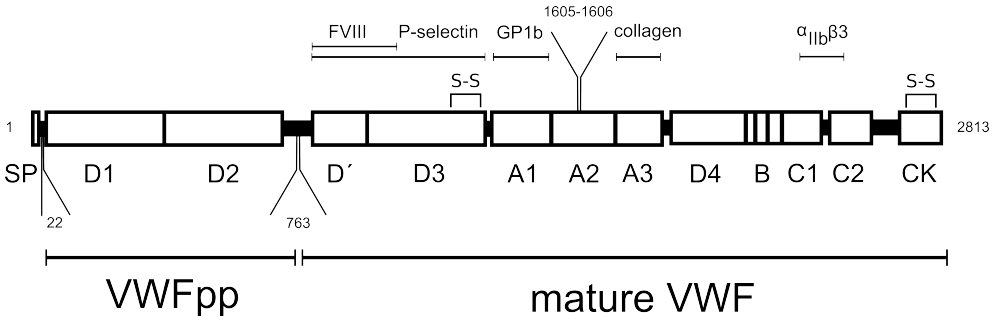

Figure 2: Domain structure of VWF. VWF is a 2813 amino acid long multidomain protein composed of repetitive A, B, C and D domains and a carboxyterminal cysteine knot domain (CK). Binding sites are indicated for FVIII, P-selectin, glycoprotein 1b (GP1b), collagen and αIIbβ3-integrin. The locations of intersubunit disulfide bonds are indicated (S-S). The locations for the signal peptide (SP) cleavage site (22), the propeptide cleavage site (763) and the peptide bond within the A2 domain, cleaved by ADAMTS-13 (1605-1606) are indicated.

The monomeric pro-VWF precursor is a multidomain protein that is composed of a number of repeating polypeptide regions, of which some have been associated with specific functions (Figure 2). After removal of the signal peptide in the endoplasmatic reticulum (ER) and addition of N-linked carbohydrates, the pro-VWF monomers undergo tail-to-tail dimerization through formation of disulfide bonds between their carboxyl terminal cysteine knot domains (Figure 3). Failure to dimerize, either by mutations that compromise the ability to form intermolecular disulfide bonds or by treatment with compounds that inhibit N-linked glycosylation, prevents translocation of pro-VWF from the ER to the Golgi apparatus [23,24]. In the Golgi cisternae additional post-translational modification steps take place, including modification of N-linked glycosyl moieties and addition of O-linked carbohydrates [25].

Upon reaching the *trans*-Golgi network (TGN), both mature VWF and the propeptide are sulfated at N-linked carbohydrates by the action of sulfotransferases [26]. In the gradually acidifying environment along the Golgi and TGN, multiple pro-VWF dimers associate into high molecular weight multimers by the formation of N-terminal sulfide bridges, a process also referred to as head-to-head polymerization. The propeptide performs a not yet fully understood function in the multimerization process, in which its putative oxidoreductase activity may facilitate the formation of intermolecular disulfide bridges in an (acidic) environment that generally does not allow rearrangement of sulfide bonds [27,28]. It has been hypothesized that the VWF propeptide contains an intrinsic disulfide isomerase activity that only develops at acidic pH [29], since alteration of the TGN environment with compounds that raise pH inhibit multimerization [23]. Finally, an endoproteolytic cleavage occurs at position 763, which releases the propeptide from the mature VWF protein.

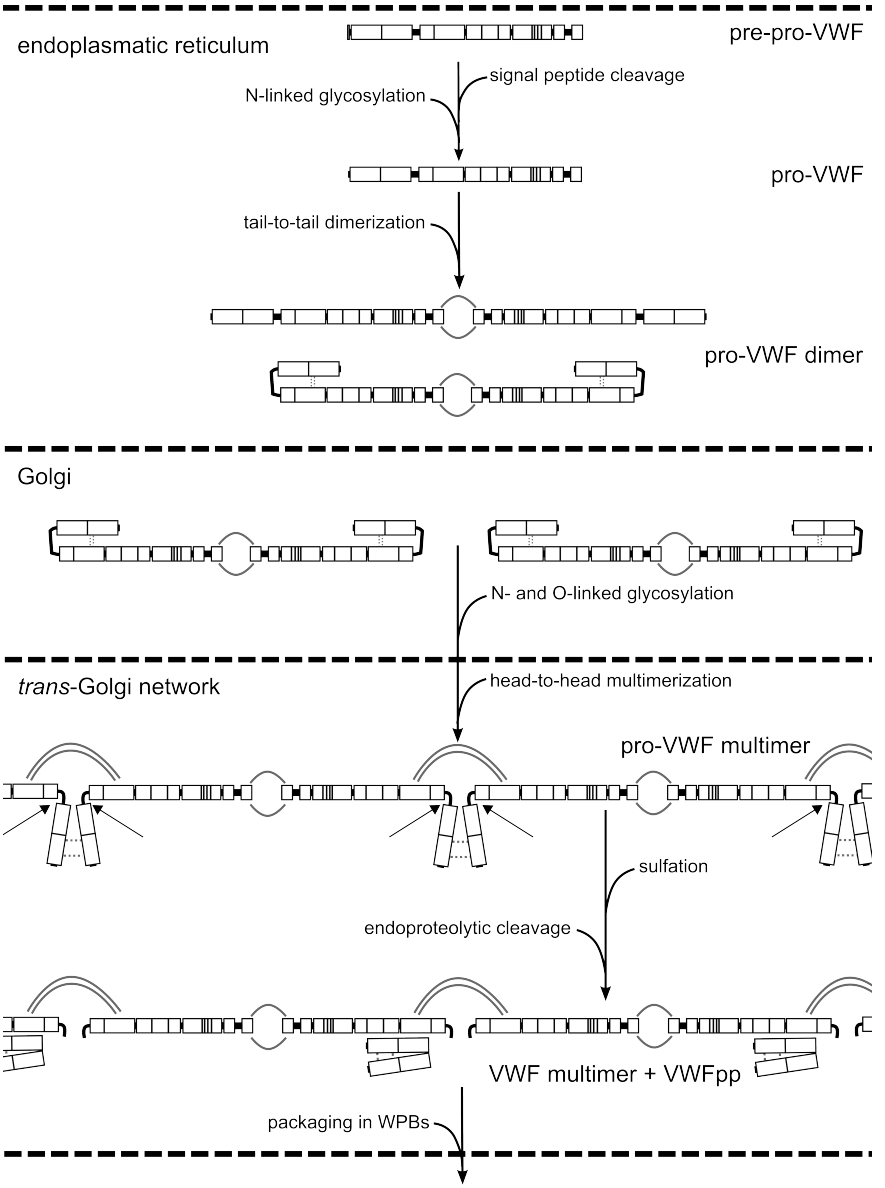

Figure 3: Processing of VWF and assembly of multimers in the secretory pathway of endothelial cells. Schematic representation of the various processing steps undergone by VWF. After signal peptide removal and N-linked glycosylation, pro-VWF dimers are formed in the endoplasmatic reticulum by the formation of intermolecular disulfide bridges between cysteine residues in the cysteine knot (CK) domain. In the *trans*-Golgi network further glycosylation takes place and multimers of pro-VWF dimers are formed by disulfide bonding of cysteine residues in the D'-D3 region. Finally, after sulfation of N-linked glycosyl groups, endoproteolytic processing removes the VWF propeptide (VWFpp) from mature VWF. On the left the respective cellular compartments are indicated. (adapted from [91])

At this site in pro-VWF a potential dibasic cleavage site is found (RKSR) that matches the cleavage consensus site recognized by pro-protein convertases such as PACE 4 or furin [30-32], the latter being a likely candidate for the endoprotease responsible for processing of pro-VWF inside the endothelial cell [33]. For a long time it has been thought that endoproteolytic cleavage occurs only after VWF and its propeptide jointly enter the forming granule, which would have been the simplest explanation for the 1:1 stoichiometry of mature VWF and propeptide present in the WPBs [34]. Later it was found that endoproteolytic processing does already occur rapidly following sulfation in the TGN, but that in the slightly acidic environment of the TGN a non-covalent association between propeptide and VWF keeps them closely linked after endoproteolytic cleavage; beyond the point that they enter the forming granule [35].

Apart from the regulated secretory pathway that involves storage in and subsequent stimulated release from WPBs (see below), VWF can also be released from endothelial cells constitutively. Endothelial cells grown in cell culture have been shown to release approximately 95% of *de novo* produced VWF in an uncontrolled fashion [34]. The observation that constitutively secreted VWF contains the same glycosylation and sulfation modifications as VWF that is stored in WPBs, indicates that constitutively secreted VWF undergoes the same processing steps and, up to the formation of the WPB, follows the same itinerary through the secretory pathway as VWF that is sorted to WPBs. The degree of multimerization of constitutively secreted VWF is lower than that found in WPBs [34], and we can therefore speculate that multimer size can serve as a sorting criterion for entry into the WPB. However, the significance of constitutively secreted low molecular weight VWF multimers is unclear, since in plasma only high molecular weight VWF multimers are found [36].

Weibel-Palade body components

In addition to VWF, the Weibel-Palade body provides a residence for an increasing number of other proteins. There are two criteria to tell them apart. Cargo proteins can be regarded as proteins that use the WPB as a vehicle for their regulated secretion into the circulation or presentation onto the cellular surface. They are generally located inside the vesicle, although some can be an integral part of the vesicle membrane. In addition, a number of accessory proteins have recently been identified, that are located on the outside of the WPB but that are not part of the actual delivery. Examples are Rab27a [37,38], Rab3D [39] and RalA [40], small GTP-binding proteins that take part in cytoskeletal transport (Rab27a), maturation (Rab3D) and exocytosis (RalA) of secretory vesicles, or the clathrin adaptor protein AP-1 which, together with clathrin, plays an essential role in the formation of WPBs at the TGN [41] and which is possibly responsible for the characteristic elongated shape of the WPB. Most of the currently known WPB components are of the cargo type [42,43], but since vesicle biogenesis and dynamics are complex processes we can speculate that also a significant number of

accessory proteins are (transiently) associated with the WPB.
The cargo-type proteins can be further classified according to their localization in the WPB: those that are found in the lumen of the WPB and those that are integral membrane proteins that are found in the enveloping membrane of the secretory vesicle. P-selectin [3,4], CD63/lamp3 [44] and α-1,3-fucosyltransferase VI (FucT-VI) [45] belong to the latter category.

Table 1: Composition of Weibel-Palade bodies

Protein	Biological function	References
Cargo integral membrane proteins		
P-selectin	inflammation, leukocyte extravasation	3,4
CD63/lamp3	cell adhesion, cell migration	44
α-1,3-fucosyltransferase VI (FucT-VI)	glycosylation	45
Cargo lumenal proteins		
von Willebrand factor (VWF)	WPB biogenesis, platelet adherence	2
von Willebrand factor propeptide (VWFpp)	WPB biogenesis, inflammation	92
angiopoietin-2 (Ang-2)	angiogenesis, inflammation	5
interleukin-8 (IL-8)	inflammation, neutrophil recruitment	6,7
eotaxin-3	inflammation, eosinophil recruitment	8
osteoprotegerin (OPG)	endothelial cell survival	56
endothelin-1 (ET-1)	vasoconstriction	57,58
tissue type plasminogen activator (t-PA)	fibrinolysis	59-61
coagulation factor VIII (FVIII)	coagulation	67
endothelin converting enzyme	proprotein processing	93
Accessory proteins		
RalA	WPB exocytosis	40
Rab3D	WPB maturation	39
Rab27a	vesicle trafficking	37,38
adaptor protein-1 (AP-1)	WPB biogenesis	41

As already mentioned, presentation of the leukocyte receptor P-selectin on the endothelial surface plays a vital role in the adhesion of leukocytes to inflamed endothelium. The intracellular trafficking of P-selectin has been partly elucidated and is, apart from VWF, best understood of all WPB residents. Several lines of evidence support the notion that VWF directs the storage of P-selectin in WPBs. Co-expression studies with VWF and P-selectin in the epithelial cell line T24 resulted in the formation of pseudo-WPBs that recruit P-selectin, while T24 cells not expressing VWF

were unable to store P-selectin [46]. In non-endothelial cell types equipped with a regulated secretory pathway, such as AtT-20 cells, heterologously expressed P-selectin is sorted to endogeneous (ACTH) storage granules [47], although upon coexpression of VWF P-selectin is again rerouted to pseudo-WPBs [48]. Furthermore, P-selectin has also been found to colocalize with VWF in α-granules of platelets, although its presence there does not depend on VWF [10]. Conversely, in the absence of a storage pathway or VWF to provide for a granule, P-selectin is targeted to lysosomes [9,14,49], and VWF-deficient endothelial cells consequently lack the ability to present P-selectin on their cellular surface and to recruit leukocytes following stimulation [10]. More recently it was found that P-selectin is recruited to WPBs from the TGN most probably owing to direct interaction between the extracellular or lumenal domain of P-selectin (which at that point is facing inwards in the TGN) and the D'-D3 region of VWF [50,51]. P-selectin is reinternalized following its presentation on the cellular membrane. Recognition of the KCPL- and the YGVF-motifs in the cytoplasmic tail of P-selectin by AP-3 and sortin nexin-17 respectively enables P-selectin to pass through the early and late endosome compartments, from where it recycles to the TGN for incorporation in newly forming WPBs [51-53].

The tetraspanin CD63/lamp3 is also not exclusively found in WPBs and it distinguishes itself from other WPB components in the alternative routing it follows from the TGN to the WPB. Instead of budding together with VWF into immature WPBs, CD63/lamp3 diverts to endosomes, from which it later on is included into mature WPBs [51,54]. Glycosyltransferases such as FucT-VI are commonly found in the Golgi apparatus, where they are responsible for glycosyl modification of secretory proteins. Possibly, it takes part in modification of WPB components and remains associated to some of them during vesicle biogenesis. Another possibility is that FucT-VI functions as a cargo-receptor for glycosylated WPB components, analogous to the mannose-6-phosphate receptor that sorts phosphomannosylated lysosomal hydrolases to lysosomes [55]. However, to date it has not been established why WPBs contain CD63 or FucT-VI.

Among the lumenal proteins we find, depending on the state of endothelial cell activation, the chemokines IL-8 [6,7], eotaxin-3 [8], angiopoietin-2 (Ang-2) [5], osteoprotegerin (OPG) [56] and endothelin-1 [57,58] (see Table 1), while some controversy exists whether tissue-type plasminogen activator (t-PA) also resides in the WPB [59-61]. The chemokines IL-8 and eotaxin-3 are structurally very similar peptides with chemotactic activity towards neutrophils (IL-8) and eosinophils (eotaxin-3). This suggests that upon stimulated release a chemotactic gradient is formed that attracts leukocytes. WPBs may not always contain IL-8 or eotaxin-3. Instead their expression needs to be upregulated, for instance, by proinflammatory cytokines as IL-1β (IL-8) and IL-4 (eotaxin-3) to induce their synthesis and subsequent packaging into WPBs [6-8,49]. This allows for dynamic regulation of the vesicle content by the endothelial cell in response to inflammatory conditions. Entry of IL-8 into the regulated secretory pathway has also been found to be dependent on expression of VWF.

Endothelial cells that have lost their ability to express VWF and that are devoid of WPBs do not store IL-8. Instead IL-8 is found in the Golgi apparatus [49]. Upon recapitulation of the storage system by reestablishment of VWF expression, formation of WPBs is restored and subsequently VWF and IL-8 do colocalize [49].

The angiogenic growth factors angiopoietin-1 and -2 (Ang-1 and Ang-2) are ligands for the Tie2 receptor kinase that is expressed on endothelial cells. Binding of Ang-1 to its receptor activates Tie2 and forces the endothelium into a vascular quiescent phenotype. Ang-2 is able to bind to the same region of the Tie2 receptor but fails to activate it, such that it functions as an antagonist of Ang-1 – Tie2 signaling and promotes destabilization and proliferation of the endothelium. The identification of a component of the Ang-1/Ang-2 - Tie2 ligand-receptor system in WPBs suggests that their release can potentially also elicit a change in the angiogenic profile of the local vasculature [5]. Possibly, Ang-2 antagonization of Ang-1 - Tie2 signaling provides for the destabilization and activation of the local vascular bed that may be needed in order to repair the damaged vessel wall. Additionally, it was found that Ang-2 also sensitizes endothelial cells for the proinflammatory cytokines TNF-α and IL-1β [62]. These cytokines upregulate the expression of a number of genes through the action of the primary transcription factor NF-κB. In quiescent, non-activated endothelial cells the Ang-1/Ang-2 ratio favors Ang-1-mediated Tie2 phosphorylation, a state that results in inhibition of the NF-κB pathway. However, local release from WPBs may shift this balance towards Ang-2 which in turn allows NF-kB to bind to promoter regions of cytokine responsive genes. Through this mechanism, Ang-2 may prime endothelial cells for expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) upon cytokine activation. These adhesion proteins promote leukocyte adhesion through their interaction with leukocyte-based integrins.

The presence of OPG and endothelin-1 in WPBs suggest that endothelial cells can participate in protection against vascular calcification or regulation of vascular tone by means of their WPBs. Furthermore, it has been shown that OPG can interact with VWF, a phenomenon that can possibly also be responsible for its targeting to WPBs [56,63].

An oddity in this list is the coagulation protein FVIII. Up to this point no cell type has been identified that produces both VWF and FVIII, or that contains both molecules in secretory vesicles like WPBs. This means that it is not a true WPB resident, except that FVIII contains a putative sorting signal that directs it to storage in (pseudo)-WPBs when heterologously expressed in cells that contain these vesicles (see below). Moreover, administration of the vasopressin analogue desmopressin (DDAVP) (reviewed in) leads to a rapid, simultaneous increase of VWF and FVIII levels in plasma, most probably as the result of cellular release (reviewed in [64]). Individuals with severe VWD type 3 are unresponsive for this treatment, which suggests that VWF and FVIII are released from the same storage compartment [65]. Unlike P-selectin, FVIII is not targeted to endogenous ACTH containing granules in AtT-20 cells. However, upon coexpression with VWF,

it is, like P-selectin, targeted to pseudo-WPBs. This is all the more intriguing since VWF and FVIII are two intimately linked proteins, both in function and physical interaction. In the circulation FVIII is found in complex with its chaperone VWF which prevents for the premature inactivation and/or clearance of FVIII. It has therefore been suggested that this interaction may also underlie the sorting of FVIII into (pseudo)-WPBs [66,67].

As described above, the diverse functions of its constituents underline the relevance of WPB exocytosis for physiological processes such as hemostasis, inflammatory responses and angiogenesis. It now is becoming apparent that hemostasis and inflammation are two tightly interconnected physiological processes, which goes further than sharing the instantaneous release of the WPB as the primary step in both the inflammatory and haemostatic response. Pertinent to this point is the increasing number of molecular and cellular participants that have independent functions in both hemostasis and inflammation (reviewed in [68]), which enable them to contribute to both pathways. Sharing components between the inflammatory and the hemostasis pathways indeed makes a lot of sense when one considers that both are defence mechanisms that will be mounted simultaneously in a lot of physiological and pathophysiological situations.

Indeed, the WPB contains all the necessary elements for an emergency package to be delivered following vascular perturbation.

Signaling cascades in Weibel-Palade body exocytosis

To adequately respond in the event of vascular trauma, endothelial cells need to be able to rapidly mobilize the pre-stored reserves of VWF and other components from the WPBs. A wide variety of secretagogues like thrombin, histamine, epinephrine and others (reviewed in [43,69]) are able to initiate signaling cascades that recruit WPBs to the plasma membrane and ultimately lead to exocytosis. Recent findings that WPBs can be released in response to invading bacterial pathogens (through binding of bacterial lipoproteins to Toll-like receptors) [70] or by an antibody against human leukocyte antigen (HLA) [71], suggest that WPB release also participates in innate immune responses or the pathology of transplant rejection, respectively.

In terms of the second messenger that these secretagogues use, we can classify them as Ca^{2+}- and cAMP-mediated agonists. In general, Ca^{2+}-mediated agonists like thrombin or histamine are prothrombotic, proinflammatory and vasoconstricting and provoke rapid, local release of VWF through exocytosis of the majority of the WPBs. Binding of secretagogues to G protein-coupled receptors leads to the activation of PLCγ, which hydrolyzes phosphatidylinositol 4,5 bisphosphate (PIP_2) into 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) [72-74]. The latter induces an increase in cytosolic free Ca^{2+} through release from intracellular stores, which readily associates with the calcium sensor calmodulin (CaM). Inhibition of Ca^{2+} signaling by calcium chelators or

inhibition of CaM resulted in inhibition of VWF release. This implicates the Ca^{2+}/CaM complex in the transduction of thrombin-induced WPB exocytosis [75,76].

In contrast to Ca^{2+}-mediated agonists that mediate inflammation, thrombosis and vasoconstriction, activation by cAMP-mediated secretagogues has a largely opposing outcome: they lead to vasodilatation and by improvement of vascular barrier function and they prevent extravasation of leukocytes. Whereas Ca^{2+}-mediated agonists locally lead to WPB exocytosis, for instance, to promote platelet plug formation, cAMP-mediated agonists are most likely responsible for systemic regulation of plasma VWF (and FVIII) levels. In other secretory systems, like the pancreatic β-cell, it has been found that cAMP-mediated signaling in regulated secretion potentiates Ca^{2+}-dependent exocytosis [77], a phenomenon that has also been observed in endothelial cells [78-80].

Infusion of epinephrine or physical exercise lead to a rapid but transient increase in plasma VWF levels, due to regulated secretion [81-83]. Epinephrine binds to the endothelial expressed β2-adrenergic receptor and thereby activates cAMP-mediated signaling pathways that, amongst other effects, provoke the release of WPBs [78]. Administration of the synthetic vasopressin analogue desmopressin (DDAVP), which is used in clinical practice in patients suffering from coagulation disorders such as mild hemophilia A or type 1 von Willebrand disease, induces release of VWF (and possibly FVIII) from internal stores through activation of a cAMP-mediated signaling pathway [84]. Signaling through the vasopressin-2 receptor (V2R), which is expressed on endothelial cells of different origin, indeed leads to exocytosis of WPBs through cAMP in cultured endothelial cells expressing V2R [84]. Both the V2R and the β2-adrenergic receptor are G_s-coupled receptors that upon binding of their agonist activate intracellular adenylate cyclase to convert ATP into the second messenger cAMP. Inhibition of the cAMP-activated protein kinase A (PKA) in HUVECs results in reduced VWF secretion after stimulation with cAMP-mediated agonists, suggesting that PKA takes part in the signaling cascade leading to WPB exocytosis [80,84].

Exocytosis of WPBs in response to Ca^{2+}- and cAMP-raising agonists is accompanied by the activation of the small GTPase Ral [85,86], a GTP-binding protein that has previously been found associated with WPBs [40]. GTPases are molecular switches that cycle between an active, GTP-bound state and an inactive GDP-bound state. In its active state, Ral promotes vesicle exocytosis by activation of phospholipase D1 activity [87] and by its association with Sec5 and Exo84 [88], members of the exocyst complex that tether secretory vesicles to specific sites on the plasma membrane. Supporting its role in exocytosis, expression of the constitutively active mutant of RalAG23V is able to induce exocytosis of WPBs [85], as concluded from the reduction in the number of WPBs when it is expressed in endothelial cells. Furthermore, a cell permeable peptide comprising the carboxyterminal region of RalA, that is responsible for its interaction with CaM but probably also other factors, was found to inhibit WPB exocytosis [86]. Thrombin-induced activation of RalA proved to be dependent on CaM, as inhibition with the CaM inhibitor TFP resulted in reduced activation of RalA in response

to thrombin [85]. The difference in secretion kinetics that has been observed for Ca^{2+}- and cAMP-mediated secretagogues *in vitro* correlates with the activation profile for RalA: thrombin induces rapid but transient activation of RalA while epinephrine induces a slow but sustained activation of RalA.
A subset of the WPBs is able to escape regulated exocytosis in response to cAMP-mediated stimulation by perinuclear clustering at the microtubule organizing centre (MTOC), which involves retrograde transport of vesicles mediated by the dynein-dynactin complex [89,90].

Outline of this thesis

As described above, endothelial cells can participate in preservation of vascular homeostasis by release of WPBs that are packed with the necessary bioactive components. The studies performed in this thesis are aimed at gaining a better understanding of the two processes that are fundamental to this capability of the endothelium, namely sorting of secretory proteins to WPBs and regulated secretion through exocytosis of WPBs. To gain more insight in how endothelial cells modulate the content of their WPBs, we have investigated a possible chaperone function for VWF by which it governs entry of other constituents into the storage pathway. Furthermore, this thesis aims to unravel signaling cascades and identify cellular components that control the exocytosis of WPBs in response to activation of the endothelial cell.
In chapter 2, we have studied the entry of the chemokine IL-8 into the storage pathway of the endothelial cell in a quantitative manner. We have made the interesting observation that the molar amounts of IL-8 and VWF that enter WPBs are intimately linked. We postulate that this is due to a molecular interaction between these proteins in the acidic environment of the late secretory pathway. In chapter 3 we have found that the guanine exchange factor RalGDS regulates the release of WPBs in response to agonists that are mediated by the second messenger Ca^{2+}. Through its interaction with the Ca^{2+} sensor CaM, RalGDS activates the small GTPase Ral in thrombin-stimulated endothelial cells, which results in the exocytosis of WPBs. Chapter 4 describes a novel PKA-independent pathway through which endothelial cells regulate cAMP-mediated exocytosis of WPB. This depends on the activation of the GTPase Rap1 by the cAMP-GEF Epac. In chapter 5 we have found that WPBs in endothelial cells that mimic a phenotype induced by shear stress display less clustering at the MTOC in response to stimulation with agonists that raise intracellular cAMP.
Finally, in chapter 6 of this thesis our findings are summarized and we discuss them in the context of reports by others. A more general view is provided on the targeting of bioactive compounds to WPBs and regulatory mechanisms that exist in the endothelium to control release of these vesicles. Also, directions for future study are indicated.

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Chapter 2

Efficiency of von Willebrand factor-mediated targeting of IL-8 into Weibel-Palade bodies

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Abstract

BACKGROUND: After *de novo* synthesis in endothelial cells the chemokine interleukin-8 (IL-8) is targeted to endothelial cell-specific storage vesicles, the Weibel-Palade bodies (WPBs), where it colocalizes with von Willebrand factor (VWF). OBJECTIVE: In this study we investigated a putative regulator function for VWF in recruitment of IL-8 to WPBs. METHODS: We performed a quantitative analysis of the entry of IL-8 into the storage system of the endothelium using pulse-chase analysis and subcellular fractionation studies. RESULTS: Using pulse-chase analysis of IL-1β–stimulated human umbilical vein endothelial cells we found that a small part of *de novo* synthesized IL-8 was retained in endothelial cells after 4 hours. In density gradients of endothelial cell homogenates nearly equimolar amounts of VWF and IL-8 were present in subcellular fractions that contain WPBs. Furthermore we found that IL-8 binds to immobilized VWF under the slightly acidic conditions thought to prevail in the lumen of the late secretory pathway. CONCLUSIONS: These observations indicate that the sorting efficiency of IL-8 into the regulated secretory pathway of the endothelium is tightly controlled by the entry of VWF into WPBs.

Introduction

Endothelial cells contain typical, elongated vesicles, so-called Weibel-Palade bodies (WPBs) [1], which serve as a storage compartment for von Willebrand factor (VWF) [2], a large multimeric, adhesive glycoprotein involved in platelet plug formation after vascular damage. Following stimulation of endothelial cells with agonists that raise intracellular Ca^{2+} or cAMP-levels, such as thrombin or epinephrine, WPBs are transported to and fuse with the plasma membrane, thereby delivering their contents onto the cellular surface or into the circulation and subendothelial tissue. Besides VWF, the cargo of WPBs may consist of a wide variety of membrane and secretory proteins including P-selectin, angiopoietin-2, endothelin-1, osteoprotegerin-1 (OPG) and the chemokines interleukin-8 (IL-8) and eotaxin-3 (reviewed in [3]). Resting endothelial cells do not synthesize IL-8 or eotaxin-3 in significant amounts. *De novo* synthesis of these and other chemokines requires exposure of endothelial cells to cytokines such as IL-1β or IL-4. Subsequently IL-8 and eotaxin-3 are stored in WPBs. Thus, endothelial cells are able to adapt to dynamic changes in the microenvironment of the vasculature by modifying the composition of their WPBs (reviewed in [3]). Previously, we and others have shown that VWF can direct the storage of IL-8 and P-selectin in endothelial and non-endothelial cell types [4-6] and accumulating evidence indicates that a variety of other molecules are costored with VWF in WPBs. However, little is known about the nature and efficiency of the sorting process of IL-8. What portion of *de novo* synthesized protein destined for regulated secretion is targeted to WPBs? We have initiated experiments aimed at studying the

efficiency of sorting of IL-8 to WPBs after induction of its synthesis in primary endothelial cells. Using density gradient centrifugation of homogenates of endothelial cells, we made the novel observation that there is a linear relationship between the concentration of IL-8 and VWF in WPB-containing subcellular fractions. We show that, irrespective of the amounts of IL-8 and VWF synthesized, these proteins are targeted to WPBs in virtually equimolar amounts. A similar linear relationship is found between the amounts of VWF and IL-8 that are released from the WPBs by thrombin-stimulated HUVEC. We also demonstrate that IL-8 is able to bind to immobilized VWF under the slightly acidic conditions thought to exist in the lumen of the *trans*-Golgi network (TGN). These observations suggest a mechanism in which IL-8 interacts with VWF in the TGN prior to vesicle formation in such a manner that the amount of VWF that enters the forming WPB governs the sorting efficiency of the co-targeted IL-8.

Materials and Methods

Reagents and antibodies

Culture media, trypsin, penicillin and streptomycin were from Invitrogen (Breda, The Netherlands). IL-1β, IL-8, and polyclonal anti-human IL-8 were from Strathmann Biotech (Hannover, Germany). Monoclonal anti-IL-8 was from Sigma-Aldrich Chemie (Steinheim, Germany). Alexa 488- and Alexa 633-conjugated antibodies were from Molecular Probes (Breda, The Netherlands). Protein A-Sepharose, CNBr-activated Sepharose 4, Percoll and Pro-mix L[^{35}S]were from Amersham Biosciences (Buckinghamshire, UK).

Cell culture

Endothelial cells were isolated from umbilical veins and cultured essentially as described previously [7]. All experiments were performed with early passage HUVEC (passage 3-5).

Pulse chase analysis

HUVEC were seeded on 10 cm culture dishes (Nunc, Rochester, NY, USA), grown to confluency and were incubated overnight with 10 ng/ml IL-1β. Cells were preincubated for 60 minutes in culture medium lacking L-methionine and L-cysteine supplemented with IL-1β. Cells were labelled for 30 minutes with 0.9 mCi Pro-mix L[^{35}S], followed by chases for indicated time periods in normal culture medium without IL-1β. After radiolabeling, IL-8 was immunoprecipitated from medium and cell lysates using preformed anti-IL-8 - Protein-A-Sepharose complexes.

Subcellular fractionation

HUVEC were grown in 175 cm^2 flasks until they reached confluency, and were subsequently

incubated for 24 hours with 10 ng/ml IL-1β. Subcellular fractionation using Percoll density gradient centrifugation was performed essentially as described previously [8]. Density gradients were established by centrifugation in a Beckmann Optima ™ LX-100 XP ultracentrifuge equipped with the Ti50.2 fixed angle rotor for 30 minutes at 100,000g. 1.6 ml fractions were taken from bottom up and the density was determined by weighing or by optical refraction.

Secretion

HUVEC were grown on 6-wells plates. Upon reaching confluency they were incubated for 48 hours with or without 10 ng/ml IL-1β. After pre-incubation for 6 hours in serum free medium (20% serum replaced by 1% HSA) without IL-1β, cells were treated for 15 minutes with 2U/ml thrombin or with serum free medium. Levels of VWF, VWFpp and IL-8 released into the medium were determined by ELISA.

Assays

VWF-, VWFpp- and proVWF antigen were measured by ELISA as described previously [9,10]. IL-8 antigen was determined by ELISA (Sanquin, Amsterdam, the Netherlands).

Immunofluoresence

Endothelial cells were grown on gelatin-coated coverslips. After 24 hour treatment with or without 10 ng/ml IL-1β, cells were fixed with 3.7% formaldehyde for 15 minutes. VWF was visualized using CLB-RAg20 [11] and Alexa 633-conjugated anti-mouse IgG_{2b}. VWFpp was visualized using CLB-Pro17 [9], IL-8 was visualized using monoclonal anti-IL-8. For both, Alexa 488-conjugated anti-mouse IgG_1 was used as secondary antibody. Cells were embedded in Vectashield mounting medium (Vector Laboratories, Burlington, CA, USA) and analyzed by confocal microscopy using a Zeiss LSM510 (Carl Zeiss, Sliedrecht, the Netherlands). For both conditions 10-15 cells were randomly selected, images were generated by making optical sections (Z-stacks with 0.36 μm intervals). Maximal projections were analyzed using Image Pro Plus 6.0 (Media Cybernetics, Breda, the Netherlands) to quantify the number of IL-8-, VWFpp- and VWF- positive vesicles in single cells.

Recombinant VWF

Recombinant human VWF was expressed in HEK293 cells that were transfected with the pcDNA3.1+ wtVWF-plasmid, containing full length human VWF cDNA. VWF was purified by immuno-affinity chromatography using CLB-RAg20 coupled to CNBr-activated Sepharose 4. Protein concentration was determined by Bradford. VWF concentration was determined by ELISA. Purity of the VWF was assessed by SDS-PAGE under reduced conditions and silver staining, while

the multimeric composition was analyzed using agarose gel electrophoresis and Western blotting with DAKO-HRP.

IL-8 binding to immobilized VWF

Purified recombinant VWF (0.5 µg/well) was immobilized to microtiter wells (Maxisorp). Non-bound VWF was removed by washing 4 times with washing buffer containing 50 mM MES, 150 mM NaCl, 5 mM $CaCl_2$ and 0.1% Tween-20 (pH 5.5). Remaining binding sites were blocked with binding buffer (washing buffer supplemented with 10 mg/ml BSA). Wells were incubated with IL-8 (0–0.5 µM) for 2 hours at 37°C in binding buffer. In case of co-incubation with soluble VWF, 0.15 µM IL-8 was supplemented with 0.01–0.15 µM VWF. Non-bound IL-8 was washed away with washing buffer. Bound IL-8 was detected using biotin-labelled anti-IL-8 followed by streptavidin-polyHRP and was corrected for non-specific binding, determined by incubation of IL-8 in empty wells. Surface plasmon resonance (SPR) studies were performed on a Biacore3000 (Biacore AB, Uppsala, Sweden). 22 fmol/mm^2 purified VWF was immobilized on a CM5 sensor chip by amine-coupling as described by the manufacturer. As a control we used an uncoupled flow cell without VWF , which was subsequently blocked with ethanolamide. Binding to the control flow cell was typically less than 5% of a flow cell to which VWF was coupled. Binding of recombinant IL-8 to immobilized VWF was analyzed in 150 mM NaCl, 10 mM $CaCl_2$, 0.005% Tween-20, 5% glycerol and 50 mM Hepes (pH 7.4 and 6.7) or 50 mM MES (pH 6.2 and 5.5) for 4 minutes at 25°C with a flow rate of 20 µl/min. Dissociation was initiated upon replacement of IL-8 solution with buffer. For regeneration of the surface, each incubation was followed with 3 incubations using the same buffer supplemented with 1M NaCl. For calculation of affinity constants (K_D), responses at 240 seconds of association (R_{eq}) were plotted against protein concentration. If maximum binding was not reached during the first 240 seconds of association, we estimated maximum binding by fitting the association curves to an exponential association function. Non-linear regression was performed on resulting binding isotherms assuming a one site binding hyperbola ($R_{eq} = R_{eq}max * [IL\text{-}8]/(K_D+[IL\text{-}8])$.

Statistical analysis

Non-linear and linear regression analysis using the Deming method and Student's or paired t-test were performed with Graphpad Prism version 4.03 (GraphPad Software, San Diego, USA).

Results

Pulse-chase analysis of storage and secretion of IL-8 in endothelial cells.

Initially, pulse-chase experiments were performed to explore and compare the cellular retention of IL-8 and VWF. Unlike endothelial cells of different origin, such as human intestinal microvascular endothelial cells (HIMEC), resting HUVEC do not express IL-8 unless activated to upregulate IL-8 expression by proinflammatory cytokines such as IL-1β [12]. HUVEC were incubated for 18 h with IL-1β to induce IL-8 synthesis. Subsequently these cells were pulse-labelled and at various chase times labelled IL-8 from medium and cell lysates was immunoprecipitated and analyzed by SDS-PAGE and auto-radiography. During the first hour of chase IL-8 was almost completely secreted (Fig.1). After a chase period of 4 h only a small amount of IL-8 remained associated with cells. Apparently the relative amount of IL-8 that enters the storage pathway of IL-1β-stimulated HUVEC is low; virtually all of *de novo* produced IL-8 leaves the cell within 40 min following its synthesis.

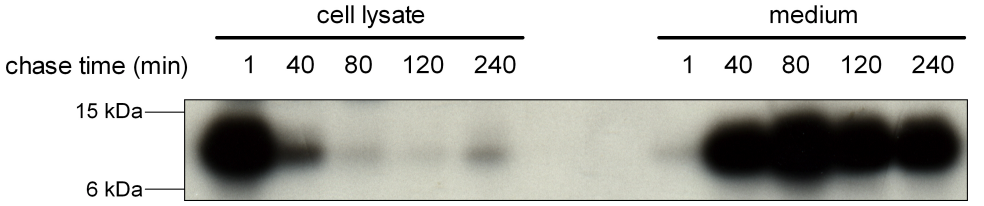

Figure 1: Release and retention of IL-8 in endothelial cells. Endothelial cells, incubated overnight with 10 ng/ml IL-1β to upregulate IL-8 gene expression, were metabolically labelled for 30 minutes, followed by chases in unlabelled medium for indicated time periods. IL-8 was immunoprecipitated from respectively conditioned media and cell lysates 1, 40, 80, 120 and 240 minutes after addition of unlabelled medium. Immunoprecipitates were subjected to SDS-PAGE under reducing conditions, after which autoradiography was performed.

IL-8 and VWF co-sediment in comparable amounts in WPB-containing subcellular fractions.

Next we determined the amount of VWF and IL-8 that is stored in WPBs after upregulation of IL-8 synthesis by incubation with IL-1β. Homogenates of IL-1β-stimulated HUVEC were subjected to density gradient centrifugation and the concentration of IL-8, VWF, VWFpp and proVWF were determined in the various fractions by ELISA. Figure 2A shows the analysis of a density gradient, which is representative of seven independent experiments (Table 1). IL-8 and VWF were found in three subcellular fractions: a dense fraction with a density ranging from 1.10-1.16 g/ml containing WPBs [8], a buoyant fraction with a density ranging from 1.06-1.09 g/ml, containing subcellular organelles of the secretory pathway (ER, Golgi, TGN) and vesicles containing proteins that constitutively leave the cell, and top fractions containing material from cell organelles that lysed during the cell disruption procedure. The dense fractions contain primarily mature VWF and

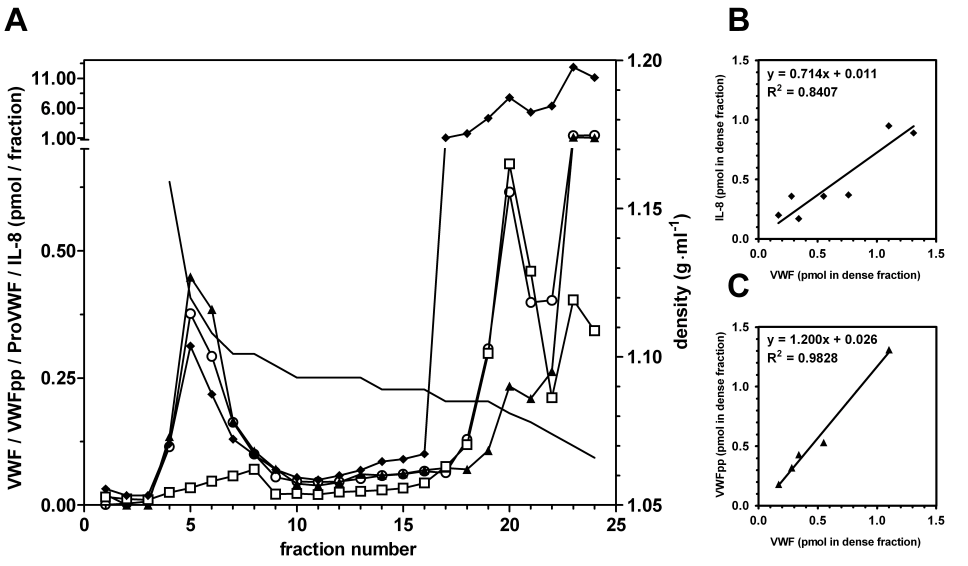

Figure 2: VWF, VWFpp, proVWF and IL-8 distribution in subcellular fractions. (A) Postnuclear supernatants of IL-1β-stimulated cells were fractionated by Percoll density gradient centrifugation. Shown is a representative experiment (indicated with ‖ in Table 1). VWF (○); VWFpp (▲); proVWF (□); IL-8 (♦); density (line). (B-C) Linear regression analysis of the total molar amount of VWF versus the total molar amount of IL-8 (B) or VWFpp (C) in the dense fraction. The equations for the plotted lines are given in the graphs (5–7 experiments, Table 1).

approximately equimolar amounts of VWFpp (Table 1). This is consistent with the observation that VWF and VWFpp are secreted in a 1:1 molar ratio after stimulation with agonists [9,13,14]. VWF that was found in the buoyant fractions is largely in its unprocessed, proVWF form (Fig.2A). In the experiment depicted in this figure, about 1.8% of the cellular IL-8 was found in the dense granules. The IL-8 concentration in these fractions was in the same order of magnitude as the concentration of VWF and VWFpp (0.2–1.0 pmol/fraction, Table 1). In this experiment the total amount of IL-8, VWF and VWFpp recovered after gradient centrifugation was 87, 95 and 91% respectively.

There was a considerable variation in terms of VWF and IL-8 production among the different HUVEC cell cultures following IL-1β stimulation. Not only did the total amount of VWF and IL-8 that was found in the total lysate fluctuate, but also the portion of VWF and IL-8 found in the dense fractions (Table 1). The variation in amount of VWF found in the dense fractions has been observed before [8]. It is reasonable to assume that this can be attributed to differences in VWF expression in various HUVEC cultures. In addition, individual HUVEC cultures responded differently to IL-1β stimulation. The total amount of IL-8 found in lysates of IL-1β-stimulated cells varied more than 30-fold (Table 1). However, this was not reflected by the amount of IL-8 found in the dense fractions. In fact, there was a significant linear correlation (P=0.0035) between the molar amounts of IL-8 and VWF that were found in the dense fractions. The WPB fraction,

on average, contained 0.7 moles IL-8 per mole VWF monomer (Fig.2B). Similarly there was a significant linear correlation (P=0.0009) between the molar amounts of VWF recovered in the WPB fractions and the molar amounts of propeptide found in these fractions (1.2 mole propeptide per mole VWF, Fig.2C). The difference between the molar ratio of VWFpp/VWF and IL-8/VWF was not significant (P=0.2450 by paired t-test).

dense fractions *					buoyant & cytosolic fractions †			total †		
VWF	VWFpp	IL-8	VWFpp VWF	IL-8 VWF	VWF	VWFpp	IL-8	VWF	VWFpp	IL-8
0.28 (7.9)	0.32 (25.1)	0.36 (4.5)	1.13	1.31	3.03 (85.6)	0.71 (56.2)	7.06 (87.8)	3.54	1.26	8.03 n.d.
0.76 (10.9)	n.d.	0.37 (7.6)	n.d.	0.49	5.87 (83.9)	n.d.	4.23 (85.8)	6.99 (90.8)	n.d.	4.93 (98.1)
1.31 (21.7)	n.d.	0.89 (1.8)	n.d.	0.68	4.40 (72.9)	n.d.	48.07 (94.8)	6.04 (90.9)	n.d.	50.7 (103.8)
1.10 (17.4)	1.31 (26.8)	0.95 (1.8)	1.19	0.86	4.78 (75.5)	3.11 (63.5)	49.53 (95.0)	6.34 (95.2)	4.89 (91.5)	52.11 (86.8)
0.17 (7.8)	0.18 (32.4)	0.20 (14.0)	1.07	1.17	1.84 (86.0)	0.22 (40.5)	1.02 (73.0)	2.14	0.55	1.39 n.d.
0.34 (8.0)	0.43 (20.2)	0.17 (10.3)	1.26	0.49	3.58 (85.4)	1.25 (59.1)	1.30 (80.3)	4.19	2.11	1.61 n.d.
0.55 (20.3)	0.53 (34.7)	0.36 (9.9)	0.96	0.64	1.76 (64.8)	0.64 (41.4)	2.55 (71.1)	2.72	1.54	3.59 n.d.
		average (± SD)	1.12 (± 0.11)	0.89 (± 0.35)						

Table 1. Summary of 7 independent subcellular fractionation experiments of IL-1β-stimulated HUVEC. VWF, VWFpp and IL-8 are given in total amounts (pmol) recovered in dense, buoyant and cytosolic fractions and, in brackets, as percentage of the total amount recovered from the gradient. Molar ratios of VWFpp/VWF and IL-8/VWF in the dense fractions are also listed. The buoyant and cytosolic fractions as described in the text have been combined. * Dense fractions are those with a density of 1.10-1.16 g·ml^{-1}; † buoyant and cytosolic fractions have a density of 1.09 g·ml^{-1} and lower (Fig.2). The molar ratio of IL-8 and VWF found in the dense fractions is shown for each experiment. ‡ For some experiments the total amounts of VWF, VWFpp and IL-8 recovered are also expressed as the percentage of the amounts that were loaded on the gradient. The experiment indicated with ‖ is shown in Fig.2. n.d., not determined.

IL-8 and VWF are released in similar amounts upon thrombin stimulation.

As there is a linear relationship between the amounts of VWF, VWFpp and IL-8 stored in WPBs of IL-1β–treated endothelial cells, it is to be expected that this is also reflected in the quantities of these proteins secreted when cells are stimulated to release the contents of their WPBs. To test this, IL-

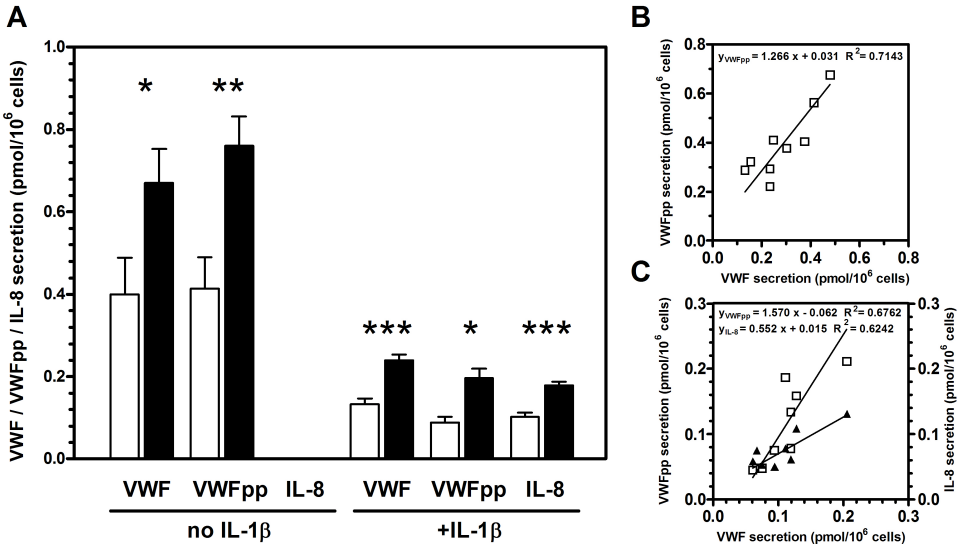

Figure 3: Regulated release of VWF, VWFpp and IL-8 from thrombin-stimulated endothelial cells. (A) HUVEC were treated for 48 hours with or without 10 ng/ml IL-1β as described in the Methods section. After 6 hours of pre-incubation in serum-free medium without IL-1β, cells were stimulated for 15 minutes with serum-free medium containing 2 U/ml thrombin (black bars) or medium alone (white bars). The concentration of VWF, VWFpp and IL-8 secreted in the medium were measured by ELISA. Error bars represent SEM. * P<0.05, ** P<0.005, *** P<0.0001 by Student's t-test. (B-C) Linear regression analysis of the amounts of VWF versus the amounts of VWFpp (□) and IL-8 (▲) released after thrombin stimulation (corrected for constitutive release) in resting (B) and IL-1β-stimulated (C) endothelial cells. The equations for the plotted lines are given in the graphs. Data represent the mean of at least 7 experiments.

1β-treated and untreated HUVEC were stimulated for 15 minutes with 2U/ml thrombin (Fig.3A). Indeed, a linear relationship was found between the amounts of VWF and IL-8 apparently released by IL-1β-treated endothelial cells in response to thrombin stimulation. On the average per mole VWF 0.6 moles IL-8 were recovered (*P*=0.0104) (Fig.3B). This is consistent with the observed molar ratio revealed by the density gradient fractionation studies. No detectable IL-8 secretion was found when endothelial cells were not subjected to IL-1β treatment. Treatment with IL-1β is known to decrease VWF expression [15], which is apparent from the reduction in VWF and VWFpp levels released from endothelial cells under these conditions (Fig.3A). As expected [9,14] also for VWF and VWFpp significant linear relationships were found in resting HUVEC (*P*=0.0039) as well as after IL-1β treatment (*P*=0.0028). The molar ratios VWF:VWFpp were 1:1.3 (no IL-1β) and 1:1.6 (+IL-1β) respectively (Fig.3B-C). The difference between the molar ratio of VWFpp/VWF and IL-8/VWF released was not significant (*P*=0.1505 by paired t-test).

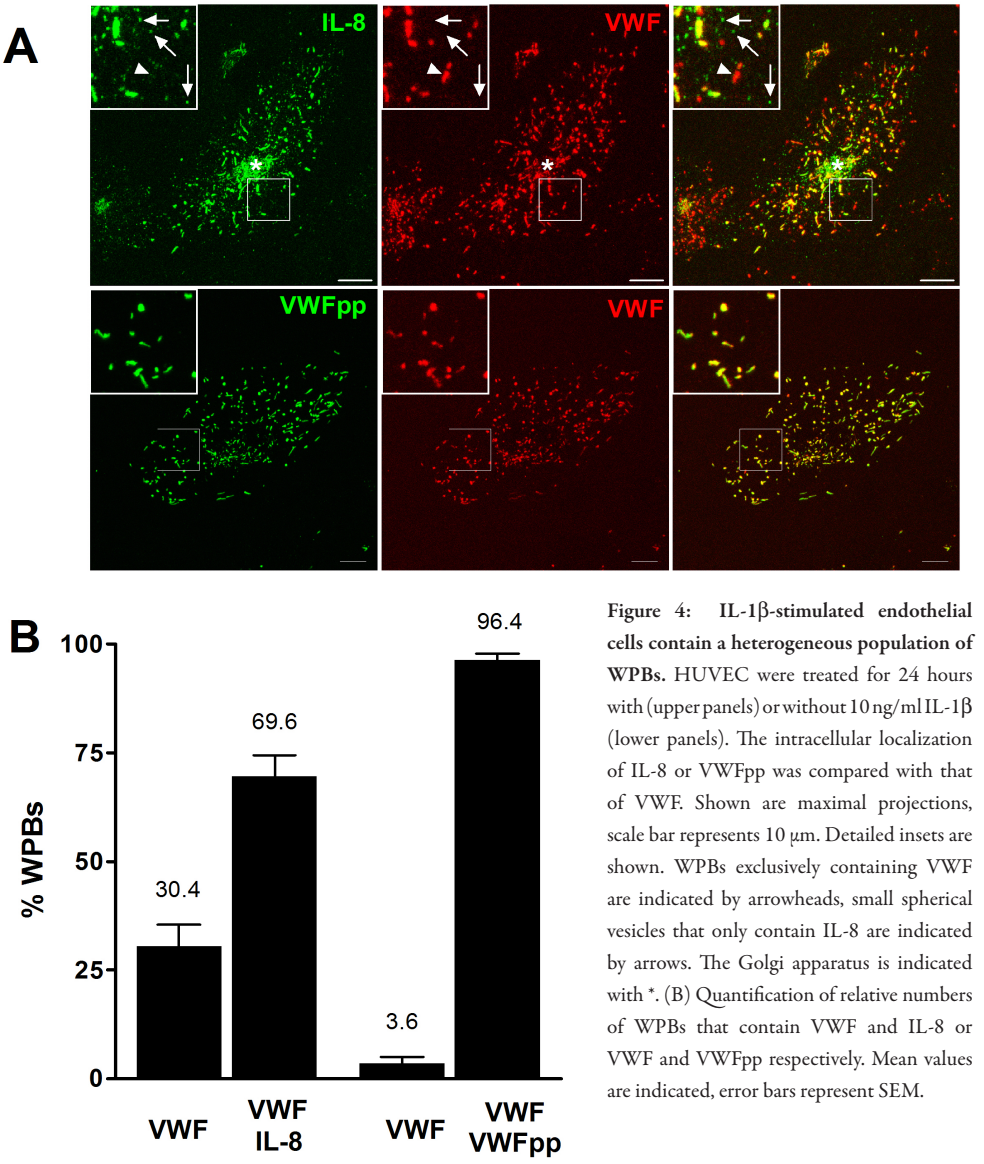

Figure 4: IL-1β-stimulated endothelial cells contain a heterogeneous population of WPBs. HUVEC were treated for 24 hours with (upper panels) or without 10 ng/ml IL-1β (lower panels). The intracellular localization of IL-8 or VWFpp was compared with that of VWF. Shown are maximal projections, scale bar represents 10 μm. Detailed insets are shown. WPBs exclusively containing VWF are indicated by arrowheads, small spherical vesicles that only contain IL-8 are indicated by arrows. The Golgi apparatus is indicated with *. (B) Quantification of relative numbers of WPBs that contain VWF and IL-8 or VWF and VWFpp respectively. Mean values are indicated, error bars represent SEM.

IL-8 and VWF are heterogeneously distributed in the WPBs of IL-1β–treated HUVEC.

The observation that about 0.7 mole IL-8 stored per mole VWF monomeric subunit is recovered in the WPB-containing fractions, raises the question whether IL-8 and VWF are equally distributed over all WPBs or, alternatively, that subpopulations of WPBs exist that contain different amounts of IL-8 and VWF. Previously we have shown that the latter possibility is likely the case [6]. To address this

issue in a quantitative manner we immunolocalized IL-8 and VWF in IL-1β-treated endothelial cells and quantified the number of WPBs that contain both VWF and IL-8 or VWF alone (Fig.4). IL-8 colocalizes with about 70% of the VWF-containing vesicles. In addition, IL-8 is found in structures typical of the late secretory pathway (Golgi, TGN), as well as in small spherical organelles that do not contain VWF (Fig.4A, upper panels). The latter are presumably vesicles of the constitutive secretory pathway as inhibition of *de novo* protein synthesis by prolonged incubation with cycloheximide results in the selective disappearance of these vesicles (not shown). Virtually all WPBs of resting endothelial cells contain VWF as well as VWFpp (Fig.4A, lower panels), a phenomenon predicted by the noncovalent association of VWF and VWFpp after the endoproteolytic processing events preceding sorting and storage of these proteins in WPBs [16]. Similarly, VWFpp shows virtually complete colocalization with all vesicles staining for VWF in IL-1β–stimulated endothelial cells (not shown).

IL-8 displays specific and saturable binding to VWF.

The simplest explanation for our observations that VWF and IL-8 are present in nearly equimolar concentrations in the WPBs is that VWF controls IL-8 targeting to storage organelles by stoichiometric interaction. To confirm potential intracellular interactions of IL-8 and VWF, we used an ELISA-based approach to detect binding of IL-8 to immobilized VWF in the presence of 5 mM Ca^{2+} at pH 5.5. This is a condition thought to prevail in the *in statu nascendi* secretory granule of (neuro)endocrine cells [17] but also at the interface of the *trans*-most Golgi cisternae and the forming *trans*-Golgi vesicles [18], the site where entry of both proteins could take place. We demonstrate that under these conditions IL-8 binds to VWF in a specific and saturable manner (Fig.5A). Soluble VWF competed with immobilized VWF for IL-8 binding in a dose-dependent manner (Fig.5B), demonstrating the specificity of the interaction. The late secretory pathway constitutes a gradually acidifying environment with overall pH of the respective subcellular compartments dropping from 7.2-7.4 (ER) to 6.2-6.4 (Golgi and TGN) and 5.2-5.5 inside the nascent vesicle [19]. Complementary to the approach described above, we explored the pH dependence of VWF – IL-8 interactions by SPR analysis to determine at which stage IL-8 and VWF are able to associate. VWF was immobilized to a CM5 sensorchip and was perfused with 200 nM IL-8 at various pH values (Fig.5C). While only minimal interaction of IL-8 with VWF was observed under neutral conditions, binding significantly increased at pH 6.2 and (less pronounced) at pH 5.5. For further analysis of the equilibrium binding isotherms under these conditions, we plotted the maximum binding response for a range of IL-8 concentrations as a function of those concentrations. We estimated an apparent K_D by fitting these data to a hyperbola using non-linear regression. This revealed that VWF and IL-8 bind with low affinity at pH 7.4 (K_D = 2.7 μM), with increased affinity under more acidic conditions (K_D = 0.6 μM and 0.2 μM at pH 5.5 and pH 6.2 respectively).

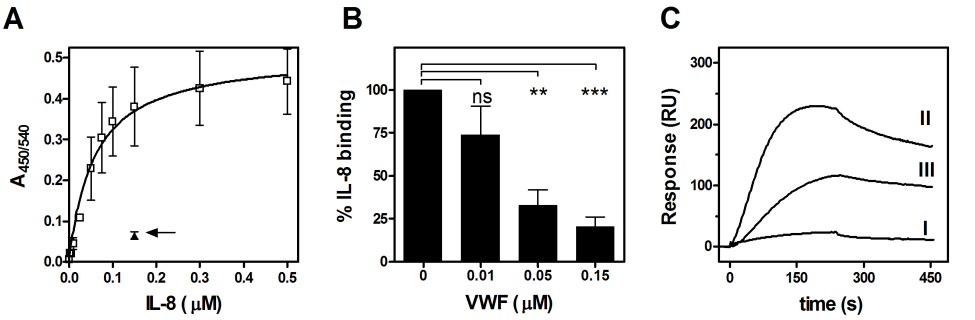

Figure 5: Interaction of IL-8 and VWF *in vitro*. (A) Immobilized VWF (□) was incubated with various concentrations of IL-8 (0-0.5 μM). In a competition experiment 0.15 μM IL-8 was co-incubated with 0.15 μM soluble VWF (▲, arrow). Data represent mean values (±SEM) of 5 experiments. Data were fitted in a non-linear regression curve assuming a one-site binding model. (B) 0.15 μM IL-8 was co-incubated with 0–0.15 μM soluble recombinant VWF. Bound IL-8 was expressed as the percentage of IL-8 bound in the absence of soluble VWF. Data represent mean values (±SEM) of 4 experiments. ** P=0.0051, *** P=0.0008 by paired t-test. (C) VWF immobilized onto a CM5 sensor chip was incubated with 200 nM IL-8 in neutral (pH 7.4, curve I) or acidic conditions (pH 6.2, curve II; pH 5.5, curve III). The amount of associated IL-8 is expressed as response units (RU) and is corrected for bulk refractive index changes and aspecific binding to an uncoupled control channel. Data shown are representative binding curves of 3-4 independent experiments.

Discussion

To obtain comprehensive information about the significance of the targeting of IL-8 to the regulated secretory pathway we have used subcellular density centrifugation as a technique to assess IL-8 sorting on a quantitative basis. We have made the interesting observation that the amount of *de novo* synthesized IL-8 that is sorted to WPBs correlates in a linear fashion with the amount of VWF associated with these organelles (Fig.2B, Table 1). On a molar basis IL-8 and VWF are stored in WPBs, isolated by density centrifugation, in nearly equal amounts. Also, the amounts of VWFpp and VWF recovered in the dense fractions were virtually equimolar (Fig.2C). As it has been demonstrated previously by different techniques that VWFpp is a typical WPB resident that, together with mature VWF, is stored in WPBs in equimolar amounts [9,14,16], this observation underscores the validity of the use of density gradient centrifugation to assess the molecular composition of WPBs in a quantitative manner. The observation that IL-8, VWF and VWFpp are secreted in similar amounts (Fig.3, also [9]) is in concert with the 1:1 stoichiometry of these molecules found in WPBs (Table 1). Differences in the molar amounts of IL-8 and VWF recovered in the medium might be due to differential binding of the proteins to the cell surface after release and/or, as discussed below, heterogeneity of the WPB population. The amounts of IL-8 synthesized after stimulation with IL-1β, as well as the amount of VWF synthesized under these conditions, varies considerably between

individual HUVEC isolates (Table 1). Nevertheless, we find that irrespective of the amounts of IL-8 (and VWF) synthesized, the molar concentration of IL-8 and VWF in WPBs is in the same order of magnitude (Fig.2B, Table 1). Apparently, the VWF-mediated targeting of IL-8 to WPBs is at a 1:1 stoichiometric level. This suggests specific interactions between these secretory molecules at the molecular level (see below). Depending on the amount of IL-8 synthesized, about 2–15% of the cellular IL-8 was associated with WPBs. Most of the IL-8 (and VWF) recovered was found in the buoyant fractions. Taking into account that the bulk of IL-8 is rapidly released after its synthesis (Fig.1), this indicates that only a minor part of newly synthesized IL-8 is targeted to WPBs. In this respect, the sorting efficiency of IL-8 in endothelial cells may seem inadequate. On the other hand, if one takes into account that VWF facilitates the entry of IL-8 into WPBs on a virtually equimolar basis (that is, one mole IL-8 per mole 220 kD VWF monomer), the observed targeting efficiency could be considered as significant and efficient. As relative low amounts of *de novo* synthesized IL-8 are sorted into WPBs, the physiological significance of our observations could be questioned. On the other hand, as the estimated IL-8 concentration in WPBs is in the μM range (calculated from the amount of IL-8 released upon stimulation and from the amount IL-8 found in dense fractions), this is sufficient to elicit an immediate inflammatory response in the vascular micro-environment. Therefore, the view that IL-8 storage could be considered as a mechanism to allow recruitment of this potent chemotactic agent to sites of vascular perturbation on demand remains a valid hypothesis [12,20]. We and others [6,12,20] have observed that endothelial cells, after prolonged treatment with IL-1β, contain subpopulations of WPBs that differ with respect to the presence of IL-8. Recent reports confirmed that distinct subpopulations of WPBs exist. For instance, P-selectin, a typical WPB resident, does not consistently co-localize with VWF [21]. Subpopulations based on CD63 content [22] or the presence of Rab27a [23] have also been reported. Under the experimental conditions studied here, we do not find IL-8-negative WPBs in close proximity to the TGN (Fig.4). We, therefore, assume that IL-8-negative WPBs represent vesicles that are formed before, or shortly after the onset of IL-1β–induced upregulation of IL-8 synthesis. Clearly, these vesicles constitute a subpopulation of WPBs. It would be of interest to know whether these senior vesicles are subject to differential regulation in terms of vesicle dynamics and/or secretion competence [21,24-26]. Since VWF expression is the driving force behind the biogenesis of its own storage vesicle [27], any co-segregated protein will, to some extent, be stored in proportion. However, our finding that not only a linear relationship exists between the molar amounts of VWF and IL-8 found in WPBs, but also that this ratio is virtually equimolar, suggests that each monomeric VWF subunit acts as a cargo-receptor, providing a single binding site for IL-8. The observation that IL-8 is able to bind to immobilized VWF under conditions that mimic the nascent granule in a specific and saturable manner (Fig.5), is in support of this view. We speculate that after upregulation of IL-8 synthesis, VWF multimers are saturated by IL-8, which thereby "gets a ride" into newly forming vesicles. Alternatively, IL-8 and VWF may both

enter the forming secretory granule without prior association. This will only occur after the budding event due to selective co-aggregation in the acidifying and condensing immature secretory granule. Subsequently, molar excess of IL-8 will be removed from the condensing vesicle, a mechanism which is consistent with the sorting-by-retention hypothesis [28]. However, our finding that also under less acidic conditions (pH 6.2) both proteins can interact suggests that the interaction may occur earlier in the TGN. The relative high concentrations of IL-8 and VWF in this compartment (see above) are probably sufficient to allow binding in a quantitative manner despite the apparent low affinity of this interaction.Direct interaction of VWF with other WPB components seems to be a more general phenomenon in sorting of secretory proteins to the regulated secretory pathway of the endothelium. A lumenal part of P-selectin interacts intracellularly with co-transfected VWF in HEK293 cells, where this interaction is sufficient to trigger recruitment of P-selectin to pseudo-WPBs [22,29]. Another recently discovered component of the WPB, OPG, also displays intra-cellular interaction with VWF, a feature that could play a role in its sorting to WPBs [30]. We conclude that by binding of IL-8 in the late secretory pathway, VWF actively recruits IL-8 to the newly forming WPBs. We postulate that this interaction determines the sorting efficiency of IL-8 into the WPBs. Similarly, the efficiency of the recruitment of other WPB residents could be governed by VWF. This would underscore the significance of VWF as a pleiotropic modulator of homeostasis.

Acknowledgments

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Chapter 3

Guanine exchange factor RalGDS mediates exocytosis of Weibel-Palade bodies from endothelial cells

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Abstract

The small GTP binding protein Ral has been implicated in regulated exocytosis via its interaction with the mammalian exocyst complex. We have previously demonstrated that Ral is involved in thrombin-induced exocytosis of Weibel-Palade bodies (WPBs). Little is known about intracellular signaling pathways that promote activation of Ral in response to ligand binding of G protein-coupled receptors. Here we show that RNAi-mediated knock-down of RalGDS, an exchange factor for Ral, results in inhibition of thrombin-induced exocytosis of WPBs, while overexpression of RalGDS promotes exocytosis of WPBs. A RalGDS variant lacking its exchange domain behaves in a dominant negative manner by blocking thrombin-induced exocytosis of WPBs. We also provide evidence that RalGDS binds calmodulin (CaM) via an amino-terminal CaM-binding domain. RalGDS association to CaM is required for Ral activation since a cell permeable peptide comprising this RalGDS CaM-binding domain inhibits Ral activation and WPB exocytosis. Together our findings suggest that CaM binding to RalGDS serves a functional role in Ral-dependent WPB exocytosis following stimulation with Ca^{2+}-raising agonists.

Introduction

Weibel-Palade bodies (WPBs) are endothelial cell-specific storage organelles [1] that contain a number of hormones, chemokines, enzymes and adhesive molecules that are rapidly released or recruited to the cell surface upon stimulation with specific agonists. Its main constituent, von Willebrand factor (VWF) [2], is also the driving force for the biogenesis of WPBs from the *trans*-Golgi network [3-5]. Current evidence suggests that VWF provides a platform for the co-segregation of a number of other bioactive compounds that include P-selectin, angiopoietin-2, and the chemokines inter-leukin-8 (IL-8) and eotaxin-3 into this intracellular storage compartment [6]. Release or surface presentation of WPB constituents enables the endothelium to control vascular homeostasis, by participating in diverse processes such as the arrest of bleeding, inflammatory responses and angiogenesis.

Regulation of WPB exocytosis by both Ca^{2+}- and cAMP-raising agonists involves signaling pathways initiated by agonist binding to G protein-coupled receptors (GPCRs) that ultimately result in fusion of WPBs with the plasma membrane [7]. Real time imaging of the secretory behavior has shown that rapid release of WPBs is observed following stimulation of endothelial cells with Ca^{2+}-raising agonists like thrombin or histamine [8,9]. Stimulation of endothelial cells with agents that raise intracellular cAMP, such as epinephrine or vasopressin, promote a relatively slow release of WPBs, whereas a subpopulation of WPBs escapes cAMP-mediated exocytosis by clustering at the microtubule organizing centre (MTOC) [8,10,11].

We previously reported that the small GTP-binding protein RalA co-sediments with WPBs on den-

sity gradients and multiple lines of evidence suggest that activation of Ral is a crucial step in both thrombin and epinephrine-induced exocytosis of WPBs [12-14]. Ral has been implicated in regulated release of secretory granules of various origin [15-18], playing a dual role in the process of exocytosis. Ral is involved in tethering secretory vesicles to specific sites on the plasma membrane through its GTP-dependent interaction with components of the exocyst complex [16]. In addition, Ral modulates exocytosis by enhancing ADP-ribosylation factor 6 (ARF6)-dependent phospholipase D1 activity [19], resulting in the formation of fusogenic lipids that promote membrane fusion.

The guanine exchange factor (GEF) involved in the activation of RalA in response to GPCR-mediated signaling in endothelial cells has not been identified. Recently, the activity of the RalGEF Ral-GDP dissociation stimulator (RalGDS) was found to be regulated by GPCR activation via receptor activation-mediated dissociation of RalGDS/β-arrestin complexes [20].

In this study, we investigated whether RalGDS is involved in Ral-mediated exocytosis of WPBs. We report here that siRNA-mediated knock-down of RalGDS markedly inhibited thrombin-induced WPB exocytosis and VWF secretion. Furthermore, a RalGDSΔ382-597 mutant lacking the catalytic exchange domain was found to act in a dominant negative fashion by inhibiting thrombin-induced exocytosis of WPB. Previously, we observed that activation of Ral by thrombin was delayed in the presence of inhibitors of calmodulin. We now provide evidence that calmodulin binds to RalGDS via an amino-terminal CaM binding site. A cell-permeable peptide comprising this CaM binding motif was found to prevent thrombin induced exocytosis of WPBs by inhibiting the activation of Ral. Together our results indicate that RalGDS plays a central role in the Ral-mediated, stimulus-induced exocytosis of WPBs from endothelial cells.

Materials and methods

Reagents and antibodies

Culture media, trypsin, penicillin, streptomycin and Oligofectamine were from Invitrogen (Breda, the Netherlands). Thrombin, Endothelial Cell Growth Supplement (ECGS), heparin and anti-α-tubulin monoclonal antibody (DM1A) were from Sigma-Aldrich Chemie (Steinheim, Germany). Anti-Ral and anti-GFP (JL-8) monoclonal antibodies were from BD Transduction Laboratories (Lexington, USA). Anti-actin monoclonal antibody (Ab1) was from Oncogene (Darmstadt, Germany). Anti-β-catenin (sc-7199) and anti-RalGDS (sc-25636) antibodies were from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-VWF monoclonal antibody CLB-RAg35 has been described previously [21]. Texas-Red-conjugated horse anti-mouse IgG and Vectaschield mounting medium were obtained from Vector Laboratories (Burlington, USA). Alexa488- and Alexa594-conjugated goat anti-mouse IgG and goat anti-rabbit IgG secondary antibodies and Alexa568-conjugated phalloidin were from Molecular Probes (Breda, the Netherlands). Calmodulin (CaM)-agarose was

from Stratagene (Amsterdam, the Netherlands). Glutathion-sepharose and streptavidin-sepaharose were purchased from Amersham Biosciences (Uppsala, Sweden). The RalGDS-derived peptide TAT-CaMBD was synthesized using Fmoc (N-(9-fluorenyl)methoxycarbonyl) solid phase chemistry and correspond to the sequence: biotin-YARAAARQARAG**VIYSISLRKVQLHHGGNKGQRWL**, in which the underlined sequence represents the TAT sequence, the sequence in bold represents the putative CaMBD of RalGDS, separated by a glycine linker. Chemiluminescence blotting substrate and Complete Protease Inhibitor Cocktail Tablets were from Roche Diagnostics (Mannheim, Germany). All chemicals used were of analytical grade.

Cell culture

HEK293 cells were maintained in DMEM-F12 medium supplemented with 10% FCS, 100 units/ml penicillin and 100 mg/ml streptomycin. Transiently transfected HEK293 cells were obtained by electroporation using an AMAXA nucleofector in combination with the V5 cell-line nucleofector kit (AMAXA GmBH, Cologne, Germany). After transfection, cells were grown for 24-48 hours prior to further experiments. Endothelial cells were isolated from umbilical veins and cultured in medium containing M199, 20% fetal calf serum, 100 units/ml penicillin, 100 mg/l streptomycin, 33 mg/l L-glutamine, 12.5 mg/l ECGS and 50 mg/ml heparin. Human umbilical vein endothelial cells (HUVECs) were transfected by electroporation using an AMAXA nucleofector in combination with the basic nucleofector kit for primary human endothelial cells (AMAXA GmBH, Cologne, Germany). After transfection, HUVECs were grown on glass coverslips for 48 hours. Stimulation of endothelial cells with thrombin was performed in the following manner: HUVECs were washed two times with culture medium in which the fetal calf serum was replaced by 1% human albumin (serum-free (SF) medium). After washing, the cells were pre-incubated with SF medium for 1 hour. At the beginning of stimulation, the pre-incubation medium was replaced by SF medium containing 1 U/ml thrombin or 10 mM epinephrine and 100 mM IBMX. After stimulation, cells were fixed in 3.7% formaldehyde for 10 minutes. WPBs were visualized using CLB-RAg35 in combination with Texas-Red-conjugated horse anti-mouse IgG as a secondary antibody diluted in PBS; 1% BSA; 0.02% saponin. Cells were embedded in Vectashield mounting medium and analyzed by confocal microscopy using a Zeiss LSM510 equipped with the appropriate filters. Images were generated by making optical sections (Z-stacks with 0.36 μm intervals). Z-stacks of single cells were analyzed using Image Pro Plus 6.0 (Media Cybernetics, Breda, the Netherlands) to quantify the total number of WPBs in a single cell. Differences in WPB numbers were statistically analyzed using a Student's t-test.

siRNA and DNA constructs

A pool of 4 siRNA oligonucleotides directed against RalGDS (ON-TARGETplus # J-005193) was

purchased from Dharmacon, Inc (Lafayette, USA). A non-targeting siRNA (ON-TARGETplus # D-001810-01-05), was used as a control in these experiments. siRNA was delivered to HUVECs in two consecutive transfections using Oligofectamine (total concentration 100 nM) according to the manufacturer's instructions. Cells were left for 48 hours before assaying. GFP-RalA was constructed by cloning a KpnI-BamHI RalA PCR fragment into the KpnI/BamHI cut pEGFP-C1 vector from Clontech (BD Biosciences Europe, Erembodegem, Belgium). The GFP-RalGDS construct and the GFP-RalGDSΔ382-597 variant have been described previously [20].

Ral-activation assay

The amount of Ral that is activated upon stimulation was measured in a pull-down assay. The Ral binding domain (RalBD) of the putative Ral effector RalBP1/RLIP76 fused to a GST tag was expressed in IPTG-induced bacteria as described previously [22]. Purified GST-RalBD (100 mg/sample) was pre-coupled to 30 ml/sample of glutathione sepharose for 1 hour at 4 °C. The pre-coupled glutathione Sepharose was then washed 3 times with lysis buffer containing 15% (v/v) glycerol, 1% NP-40, 50 mM Tris (pH 7.5), 200 mM NaCl, 2.5 mM $MgCl_2$, 10 mM benzamidine, 100 nM aprotinin, supplemented with 1 protease inhibitor tablet per 50 ml. Cells, grown in 6-wells plates, were lysed in 400 µl lysis buffer. The activated, GTP-bound form of Ral was then isolated from cell lysates by incubation of 300 µl lysate with the RalBD pre-coupled glutathione sepharose for 1 hour at 4 °C. Finally, the Sepharose beads were washed 4 times with lysis buffer and analyzed on a 12.5 % SDS-PAGE gel by western blotting with anti-RalA monoclonal antibody.

CaM pulldown assays

For the CaM-pulldown assays, transfected HEK293 cells, grown in 6-well plates for 24 hours were lysed in 1 ml CaM-PD lysisbuffer/well containing 50 mM Tris (pH 8.0), 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgAc_2$, 1 mM imidazole, 1% Triton X-100 and 1 protease inhibitor tablet per 50 ml. Lysate (900 µl) was incubated with 100 µl of CaM-agarose for 1 hour at 4 °C. Subsequently, CaM-agarose was washed 4 times with CaM-PD lysis buffer and analyzed for the presence of GFP-RalGDS on a 7.5% SDS-PAGE gel by western blotting with an anti-GFP (JL-8) monoclonal antibody.

For the TAT-peptide pulldown from HUVEC lysate, 250 µg biotin-tagged TAT, TAT-Ral-c or TAT-CaMBD was coupled to 50 µl streptavidin-sepharose for 1 hour at 4 °C. The pre-coupled streptavidin-sepharose was then washed 3 times with CaM-PD lysisbuffer (see above). HUVECs were grown in 80 cm^2 flasks till confluency and then lysed in 1 ml CaM-PD lysisbuffer/flask. HUVEC lysate (900 µl) was incubated with the pre-coupled streptavidin-sepharose for 1 hour at 4 °C, washed 4 times with CaM-PD lysisbuffer and analyzed for the presence of CaM on a 12.5% SDS-PAGE gel by western blotting with an anti-CaM monoclonal.

Results

RalGDS knock-down reduces stimulus-induced WPB exocytosis

Expression of wild type RalA and the constitutive active mutant RalA-G23V resulted in secretagogue-independent loss of WPBs, while the dominant negative mutant RalA-S28N had no effect on WPB numbers [13]. In addition, a RalA-derived, carboxy-terminal peptide has been found to inhibit both thrombin and epinephrine-induced VWF secretion by inhibition of WPB-exocytosis [14], which implicates the carboxy-terminal hypervariable domain of RalA in the binding of crucial factors regulating this process. These previous results suggest that exocytosis of WPBs requires the

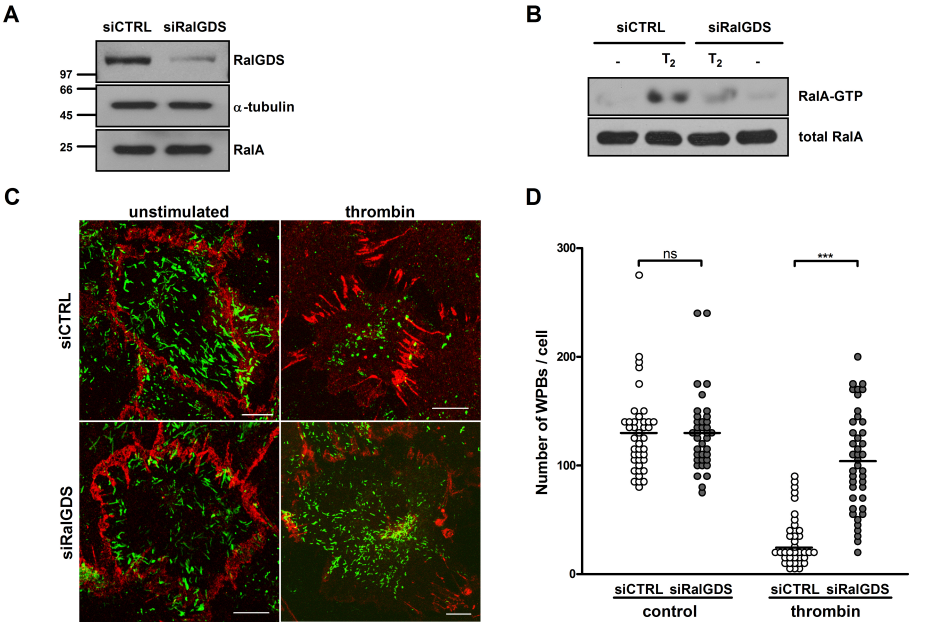

Figure 1. RalGDS knock-down by siRalGDS impairs thrombin-induced exocytosis of WPBs. (a) HUVEC were treated in 2 consecutive transfection rounds with a pool of four siRNA oligonucleotides directed against RalGDS (siRalGDS) or a control siRNA oligonucleotide (siCTRL). Western blot analysis 48 hours post transfection showed down-regulation of RalGDS expression, while α-tubulin and RalA remained unaffected. (b) Activation of RalA in siCTRL- and siRalGDS-treated HUVECs in response to thrombin was determined using a Ral-GTP specific pull-down. Cells were pre-incubated with SF-medium for 1 hour, after which they were stimulated for 2 minutes with 1U/ml thrombin (T2) or SF medium alone (-). All lysates contained similar amounts of RalA (lower panel).(c) Representative confocal images of siCTRL- and siRalGDS-treated HUVECs that were incubated for 45 minutes with 1U/ml thrombin or SF medium alone (unstimulated). WPBs were visualized by immunofluorescent staining of VWF (green) while staining of β-catenin (red) was used to delineate the cell membrane. Bars correspond to 10 μm. (d) Numbers of WPBs in individual cells were quantified as described in the Materials and methods section. Approximately 40 randomly selected cells from 2 independent experiments were counted for each experimental condition. ***P<0.0005 by Student's t-test.

activation of RalA. Small GTPases are activated by GEFs that induce GDP release and thus enhance GTP binding to the GTPase. A possible candidate responsible for the activation of Ral in endothelial cells is RalGDS, a well-studied, widely expressed exchange factor for Ral [23]. Activation of a G-protein coupled receptor (GPCR) following ligand binding results in the dissociation of inactive RalGDS/β-arrestin complexes enabling RalGDS to activate Ral [20]. Since both thrombin and epinephrine induce WPB exocytosis via stimulation of the GPCRs protease-activated receptor 1 (PAR 1) and β2-adrenergic receptor (β2-AR), respectively, we investigated whether RalGDS is involved in WPB exocytosis in HUVECs. First, we determined whether HUVECs express RalGDS by performing a RalGDS-specific PCR on cDNA acquired from three different HUVEC donors. These experiments showed expression of RalGDS mRNA in all three HUVEC samples (not shown). These results confirm gene expression data from the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo) that RalGDS is expressed in HUVECs.

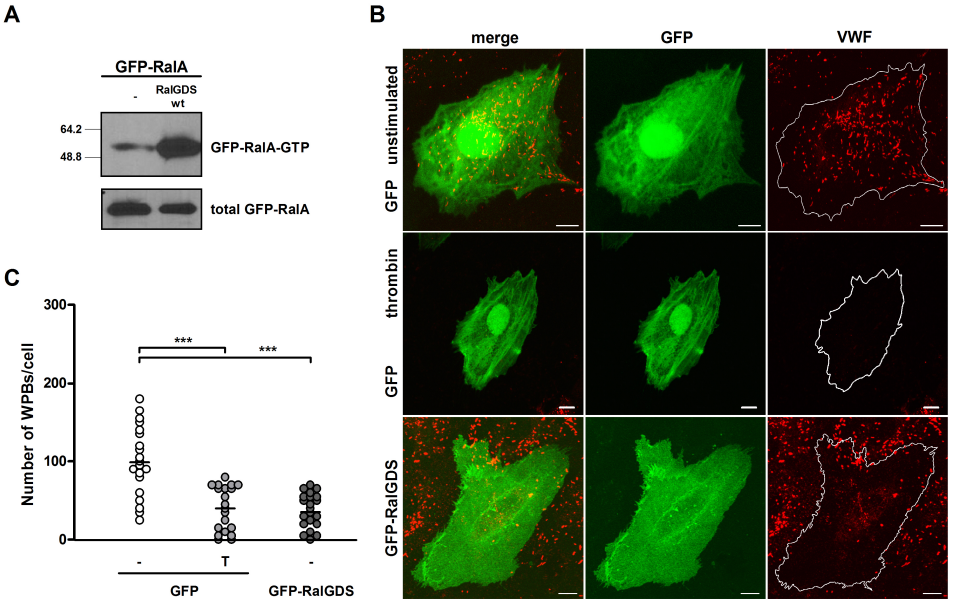

Figure 2. RalGDS induces RalA activation and WPB exocytosis. (a) Activation of GFP-RalA upon co-expression of GFP-RalGDS in HEK293 cells was determined using a Ral-GTP-specific pull-down. Total GFP-RalA levels are shown in the lower panel showing equal expression. (b) HUVECs were transfected with GFP (negative control) or GFP-RalGDS and grown for 48 hours. GFP-expressing cells were incubated with 1U/ml thrombin (T) or SF medium alone (-) for 45 minutes. WPBs were visualized by immunofluorescent staining of VWF (red). Shown are representative confocal images of HUVECs expressing GFP or GFP-RalGDS. Bars correspond to 10 mm. (c) The numbers of WPBs in individual, randomly selected GFP- and GFP-RalGDS-positive cells were quantified using confocal microscopy. ***P<0.0005 by Student's t-test.

To investigate whether RalGDS plays a role in exocytosis of WPBs, we downregulated RalGDS expression in HUVECs using RNA interference. Small interfering RNA's (siRNA) that target RalGDS (siRalGDS) were able to specifically knock down the expression of RalGDS by more than 80% when compared to endothelial cells that were treated with a control siRNA (siCTRL) (Figure 1A). Downregulation of RalGDS inhibited HUVECs in their ability to activate Ral in response to thrombin: thrombin-induced activation of RalA was ~70% reduced in HUVEC treated with siRalGDS when compared to siCTRL-treated cells (Figure 1B). We questioned whether knockdown of RalGDS in HUVECs is also able to inhibit stimulus-induced exocytosis of WPBs. HUVECs were transfected with siRalGDS or siCTRL and were subsequently stimulated with 1 U/ml of thrombin. Analysis of the number of WPBs per cell revealed a loss of WPBs upon thrombin stimulation in cells treated with siCTRL (Figure 1C and 1D), similar to that observed previously in untransfected HUVECs. However, in HUVEC treated with siRalGDS, the number of remaining WPBs upon thrombin stimulation was significantly higher compared to siCTRL-treated cells indicating that down-modulation of RalGDS results in inhibition of thrombin-induced WPB exocytosis (Figure 1D).

Dominant negative RalGDSΔ382-597 blocks stimulus-induced WPB exocytosis

Subsequently, we determined the effect of expression of RalGDS, that was tagged with GFP to allow for easy identification of transfected cells, on RalA activation and WPB exocytosis. First, we assessed whether GFP-RalGDS could enhance the activation of RalA *in vivo*. Expression of GFP-RalGDS in HEK293 cells clearly stimulated activation of exogenous co-transfected GFP-RalA in HEK293 cells (Figure 2A). Furthermore, GFP-RalGDS expression in HUVECs resulted in the formation of filopodia, shown by actin co-staining, which is reminiscent of RalA activation (Figure 3). The extent of filopodia formation was similar to that observed upon overexpression of GFP-RalA in HUVECs, whereas no filopodia were observed in HUVEC transfected with GFP alone. To study the effect of RalGDS overexpression on WPB exocytosis, HUVECs were transfected with GFP-RalGDS or GFP alone and the number of intracellular WPBs in GFP-positive cells was quantified. Expression of GFP-RalGDS resulted in a reduction in the number of WPBs per cell, similar to the one observed in GFP-expressing cells after stimulation with thrombin (Figure 2B and 2C). Stimulation of GFP-RalGDS-expressing cells with thrombin did not result in a further decrease in the number of WPBs (data not shown). These results show that overexpression of RalGDS promotes exocytosis of WPB.

To further confirm the involvement of RalGDS in RalA-regulated WPB release, we overexpressed the GFP-RalGDSΔ382-597 mutant, which lacks the catalytic exchange domain (Figure 4A) and subsequently analyzed RalA activation and WPB exocytosis in these cells. Expression of GFP-RalGDSΔ382-597 in HEK293 cells did not result in activation of RalA, in contrast to expression

of full length GFP-RalGDS (Figure 4B). Next we investigated whether GFP-RalGDSΔ382-597 was able to block thombin-induced exocytosis. In HUVECs, GFP-RalGDSΔ382-597 expression markedly reduced the thrombin-induced loss of WPBs per cell (Figure 4C), which suggests that GFP-RalGDSΔ382-597 is able to act as a dominant negative in stimulus-induced WPB exocytosis (Figure 4C). These results indicate that the exchange factor RalGDS regulates thrombin-induced exocytosis of WPBs.

RalGDS interacts with calmodulin

Previous work from our laboratory revealed that thrombin-induced RalA activation is inhibited by the Ca^{2+}-chelator BAPTA-AM (our unpublished observations) and the CaM inhibitor trifluoperazine (TFP) [13]. Binding of Ca^{2+}/CaM to the C-terminus of RalA has been shown to enhance RalA activation by 2-3 fold [24], however not to the extent observed in response to thrombin, suggesting that the combined action of CaM and a RalGEF is required for maximal activation of RalA. Here, we addressed whether RalGDS interacts with CaM during thrombin induced exocytosis of WPBs. To investigate this possibility in more detail we analyzed the amino acid sequence of the RalGDS protein for the presence of a CaM binding site using http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html [25]. A putative IQ-like CaMBD was found in the N-terminal region of RalGDS at position 75-97 (VIYSISLRKVQLHHGGNKGQRWL). This amino acid sequence contains 8 hydrophobic residues (VIILVLWL) of which 4 are located on one side of the helical wheel projection while the basic (RKKR) and other hydrophilic residues (YSSQHHGGNGQ) are on the other side of the wheel displaying the characteristic features of an amphiphilic helix (Figure 5A). To investigate whether RalGDS is indeed able to bind CaM, CaM-agarose beads were used to pull down lysates of HEK293 cells transfected cells with GFP (control) or GFP-RalGDS. Western blot analysis using an anti-GFP antibody showed that GFP-RalGDS was bound to the CaM-agarose whereas GFP alone did not (Figure 5B). As expected no binding of GFP-RalGDS to CaM was observed in the presence of 5 mM EDTA or EGTA (Figure 5C), indicating that the interaction between RalGDS and CaM is Ca^{2+}-dependent.

Cell permeable peptide corresponding to the putative CaMBD of RalGDS inhibits thrombin-induced WPB exocytosis

To further investigate the role of the putative CaM binding domain (CaMBD) of RalGDS, we designed a cell permeable peptide corresponding to amino acids 75-97 of the RalGDS protein. In addition to the putative CaMBD, this peptide contains the protein transduction domain (TAT) of human immunodeficiency virus, which enables the peptide to be transduced directly into the cell [26]. To see whether the TAT-CaMBD peptide could bind to CaM, we performed pulldown experiments from HUVEC lysate. As a control we used a TAT-Ral-c peptide containing the carboxy-

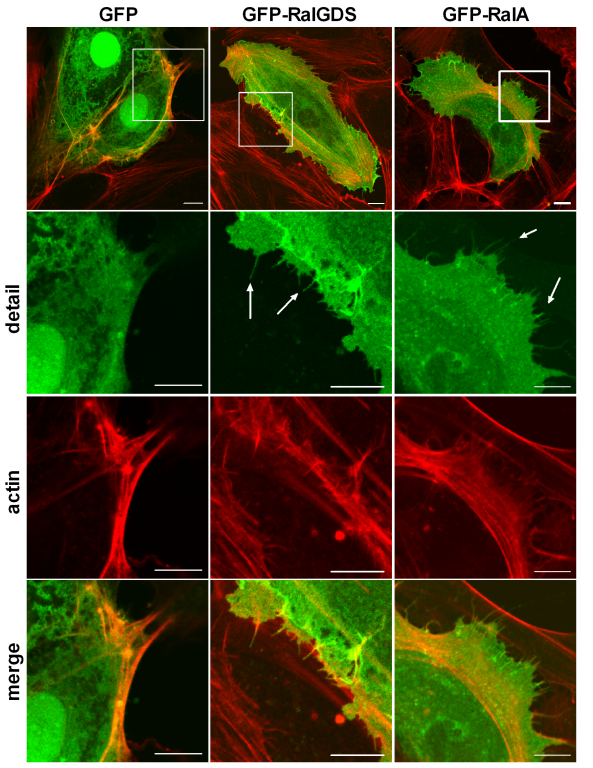

Figure 3. Expression of RalGDS or RalA induce filopodia formation in endothelial cells. Confocal images of HUVECs expressing GFP, GFP-RalA or GFP-RalGDS. After cells had grown for 48 hours on glass coverslips, F-actin was visualized by immunofluorescence using Alexa 568-conjugated phalloidin (red). Lower panels show magnifications of the areas within the boxes in the corresponding upper panels. Bars correspond to 10 µm. Expression of GFP-RalA and GFP-RalGDS induced the formation of filopodia reminiscent of RalA activation (arrows).

terminal 26 amino acids of RalA which is known to bind to CaM and a peptide containing only the TAT transduction domain. Western blot analysis using a monoclonal anti-CaM antibody showed that TAT-CaMBD is indeed able to bind endogenous CaM from HUVEC lysate (Figure 6A). As expected also the TAT-Ral-c peptide was capable of binding to CaM, although to a lesser extent, whereas no CaM binding was observed with the TAT-peptide.

Next, we investigated whether the TAT-CaMBD peptide could also interfere with thrombin-induced Ral activation and WPB exocytosis. HUVECs were preincubated for 30 minutes with 200 µg/ml TAT as a peptide control, 200 µg/ml TAT-CaMBD or serum free (SF) medium alone. Subsequently, these cells were incubated for 2 minutes with SF medium or thrombin (1 U/ml) and a Ral activation assay was performed. Thrombin induced a marked increase in the amount of active Ral in untreated and TAT-treated cells. However, treatment with TAT-CaMBD, greatly inhibited thrombin-induced Ral activation (Figure 6B). Consistently, TAT-CaMBD also effectively inhibited thrombin-induced WPB exocytosis, as the number of remaining WPBs after thrombin stimulation was significantly higher in the presence of TAT-CaMBD (Figure 6C). These results indicate that TAT-CaMBD effectively competes with endogenous RalGDS for CaM binding thereby preventing thrombin-induced Ral activation and WPB exocytosis from endothelial cells.

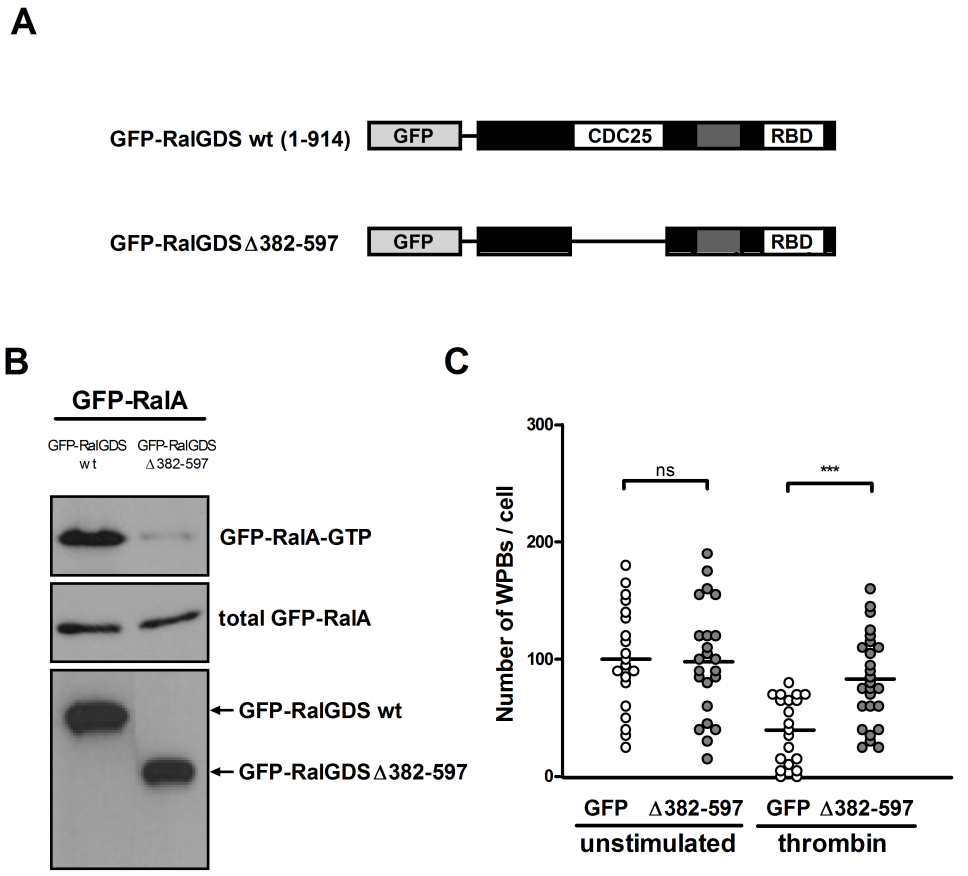

Figure 4. GFP-RalGDSD382-597 blocks stimulus-induced WPB exocytosis. (a) Schematic representation of GFP-tagged RalGDS and RalGDSΔ382-597, which lacks the catalytic CDC25 domain. (b) RalA activation upon co-expression GFP-RalGDS or GFP-RalGDSD382-597 with GFP-RalA was determined using a Ral-GTP-specific pull-down. Middle and lower panels show total GFP-RalA and GFP-RalGDS and GFP-RalGDSΔ382-597, respectively, to confirm equal expression. (c) HUVECs were transfected with GFP (negative control) or GFP-RalGDSΔ382-597 and grown for 48 hours. Cells were incubated with 1U/ml thrombin or SF medium alone (unstimulated) for 45 minutes. WPB numbers in GFP- and GFP-RalGDSΔ382-597-postive cells were quantified using confocal microscopy. ***P<0.0005 by Student's t-test.

Discussion

Ral is involved in exocytotic processes through its binding and regulation of the exocyst complex, required for targeting secretory vesicles to specific sites on the plasma membrane [16], and through activation of PLD, which promotes the fusion of plasma and vesicle membranes [19]. We have previously shown that RalA is involved in agonist-induced release of WPB in endothelial cells [13,14].

In this study we provide evidence that the Ral-specific GEF, RalGDS, is involved in exocytosis of WPBs. To our knowledge, this is the first identification of a RalGEF responsible for Ral-dependent exocytosis of dense core secretory granules. RalGDS is best known for its function in the Ras-Ral-GDS-Ral pathway, leading to potentiation of cellular transformation induced by oncogenic Ras [27,28]. Stimuli that are known to activate Ras, such as epidermal growth factor (EGF) or insulin, also lead to the activation of Ral, in a Ras-dependent manner [29]. Activation of this pathway is achieved by association of the C-terminal Ras binding domain (RBD) of RalGDS with membrane-tethered active Ras, which results in the translocation of RalGDS to the membrane and subsequent activation of its membrane-associated substrate Ral [30-32]. Deletion of the RBD region prevents RalGDS recruitment to the membrane and also impairs its ability to activate Ral [31]. Consistent with these findings we observed that a RalGDS variant with its 145 carboxyterminal residues removed (thus also lacking its RBD) is not targeted to the plasma membrane, does not activate Ral and does not lead to exocytosis of WPBs (data not shown). This observation suggests that targeting of RalGDS to the plasma membrane is essential for Ral mediated exocytosis of WPBs.

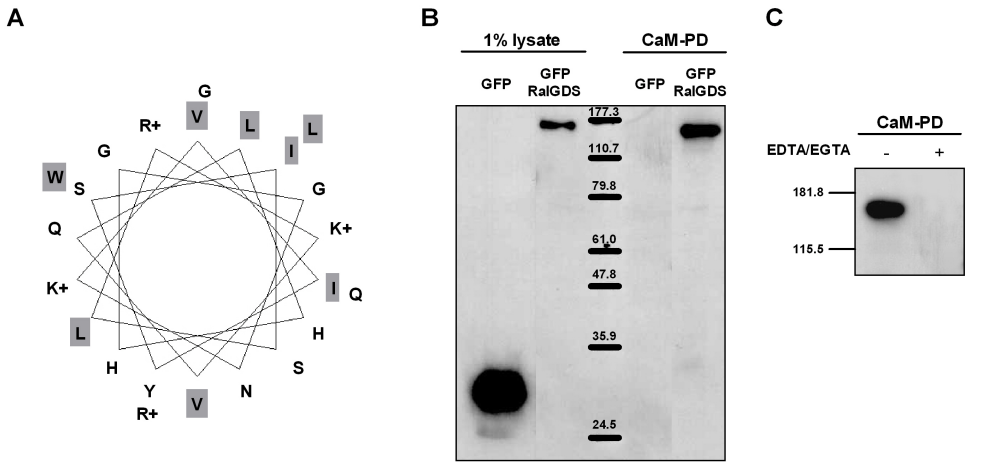

Figure 5. RalGDS binds CaM in a Ca^{2+}-dependent manner. (a) A putatative Calmodulin binding domain (CaMBD) was identified in the amino-terminal region of RalGDS (aa 75-97). An axial helical projection was generated from this amino acid sequence as outlined in the calmodulin target database [25]. Hydrophobic residues are boxed and basic residues are depicted by positive charge symbols. (b) CaM-pulldown (CaM-PD) from lysates of HEK293 cells expressing GFP or GFP-RalGDS using CaM-agarose as described in Materials and Methods. The CaM-agarose specifically pulled down GFP-RalGDS. 1% of the total lysate was used to show expression levels (left) (c) RalGDS binding to CaM is Ca^{2+}-dependent as the presence of 5mM EDTA/EGTA completely abolished this interaction.

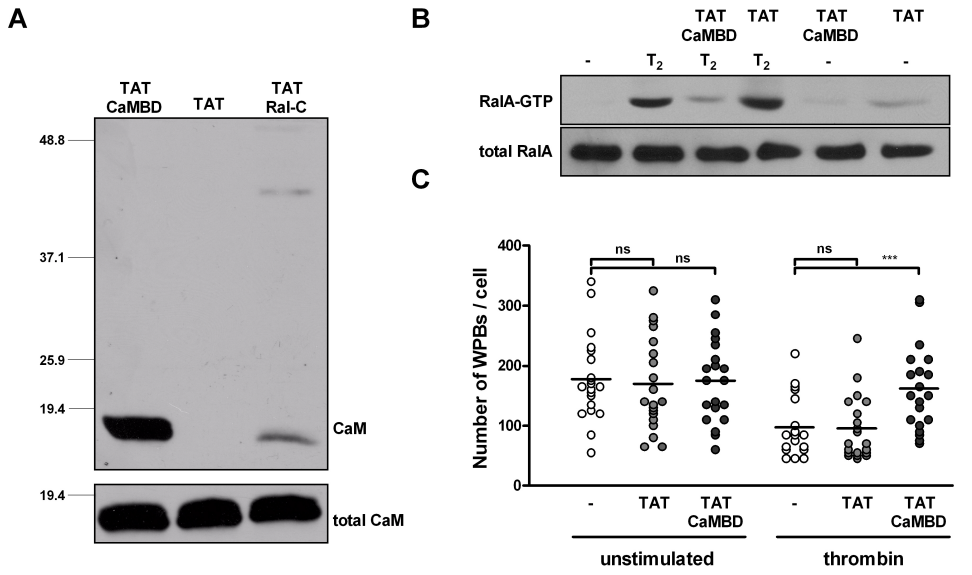

Figure 6. A cell permeable peptide comprising the N-terminal CaMBD of RalGDS inhibits thrombin-induced WPB exocytosis. (a) The biotin-labeled peptides TAT- CaMBD, TAT (negative control) and TAT-Ral-c (positive control) were coupled to streptavidin-sepharose and used in a pull down experiment from HUVEC lysate as described in Materials and Methods. The lower panel shows total CaM levels to show equal expression. (b) RalA activation in HUVECs that were pretreated with TAT-CaMBD or TAT (negative control) upon thrombin stimulation was determined using a Ral-GTP-specific pull-down. HUVEC were pre-incubated for 30 minutes in SF-medium in the presence or absence of 200 μg/ml TAT-CaMBD or TAT peptide. Subsequently, cells were stimulated with 1U/ml thrombin (T2) or SF-medium (-) for 2 minutes in the presence or absence of 200 μg/ml TAT-CaMBD or TAT peptide. The lower panel shows total RalA as a loading control. (c) HUVECs were pre-incubated for 30 minutes with SF medium, 200 μg/ml TAT or 200 μg/ml TAT-CaMBD. Subsequently, 1U/ml thrombin or SF medium (unstimulated) were added for 15 minutes. WPBs were visualized by immunofluorescent staining of VWF. The number of remaining WPBs per cell upon stimulation in absence or presence of TAT or TAT- CaMBD was quantified using confocal microscopy. ***P<0.0005 by Student's t-test.

Previously, the activity of RalGDS was found to be regulated by GPCR-signaling via receptor activation-mediated dissociation of cytosolic, RalGDS/β-arrestin complexes [20]. β-Arrestins are involved in desensitization and internalization of GPCRs [33] but they also regulate recruitment, activation and scaffolding of receptor-induced signaling complexes [32]. Reports that b-arrestins are linked to both protease activated receptor- and β2-adrenergic receptor-signaling [35-38] are consistent with a role for RalGDS/β-arrestin complexes in the regulation of exocytosis of WPBs.

The findings reported in the current study indicate that RalGDS interacts with CaM via an IQ-motif that is present in the amino-terminal part of RalGDS. Similarly, Ras-GRF1, CDC25^{Mm} and Ras-GRF2, guanine exchange factors for Ras, contain IQ-motifs that enable their interaction with CaM [39-42]. Different effects on GDP/GTP exchange have been observed following binding of these GEFs to CaM via their IQ-motifs. Activation of the guanine exchange factor Ras-GRF is medi-

ated by calmodulin (CaM) binding to an IQ motif in the amino terminal part of the Ras-GRF protein [40,42]. However, CaM binding to Ras-GRF2 did not enhance its activity [41] and binding of CaM to CDC25^{Mm} resulted in partial inhibition of the activity of this GEF [39]. The amino-terminal domains of Ras-GRF, Ras-GRF2 and CDC25^{Mm} restrain the actual exchange activity, since (partial) deletion of the N-terminal domains resulted in increased catalytic activity [39-41]. It appears that the amino-terminal domains act as an auto-inhibitory moiety; only when engaged by CaM and/or additional signaling factors, GDP/GTP exchange by the CDC25 domain of these GEFs is fully developed. The amino terminal domain of RalGDS is also involved in regulating its GEF activity [43,44]. The present study suggests that the interaction of CaM with the IQ-motif in the N-terminal domain of RalGDS positively contributes to its exchange activity. We can not fully exclude that depletion of CaM using the TAT-CaMBD peptide also interferes with interactions with other (yet unkown) factors that may contribute to Ral activation and/or WPB exocytosis, however this is less likely since the TAT-CaMBD peptide did not interfere with WPB exocytosis induced by cAMP-dependent agonists such as epinephrine (data not shown). Several observations are consistent with the concept that CaM enhances activation of Ral. Studies by Birch et al have shown that inhibition of CaM reduces thrombin-induced release of VWF from WPBs in minimally permeabilized endothelial cells [45]. In HUVECs treated with the CaM inhibitor trifluorperazine (TFP), thrombin-induced Ral activation was delayed and VWF secretion was significantly reduced [13]. Wang and coworkers have shown that Ral directly interacts with CaM [46] and, surprisingly, this interaction was shown to enhance GTP-binding to Ral by 2-3 fold [24]. The molecular mechanism underlying this effect remains to be clarified, but it was suggested that CaM induces a conformational change that promotes GTP-binding to Ral. Our data indicate that CaM plays an important role in Ral activation by binding to (and possibly activating) RalGDS. The role of CaM-mediated activation of RalGDS is underscored by the fact that downregulation of RalGDS impairs activation of Ral *in vivo*, which indicates that the interaction of CaM with Ral does not suffice for the rapid activation of Ral following stimulation of endothelial cells by thrombin. We propose that binding of Ca^{2+}/CaM to the exchange factor RalGDS relieves the auto-inhibitory property of the N-terminal domain and thereby enhances GTP-GDP exchange. Simultaneous binding of Ral and RalGDS to CaM may further enhance the efficiency of GTP-GDP exchange by RalGDS. Calmodulin also promotes thrombin induced activation of Ral in human platelets [47] which raises the possibility that RalGDS is also involved in thrombin induced Ral activation in platelets.

In conclusion, our study supports the hypothesis that thrombin induces WPB exocytosis through the activation of Ral by the Ral-specific GEF RalGDS, following activation of RalGDS by Ca^{2+}/CaM. Based on our findings, we propose the following model for RalGDS-mediated exocytosis of WPBs (Figure 7). The rise in intracellular levels of second messenger Ca^{2+} triggered by the stimulation of endothelial cells with thrombin, leads to an association of free Ca^{2+} with the calcium sensor

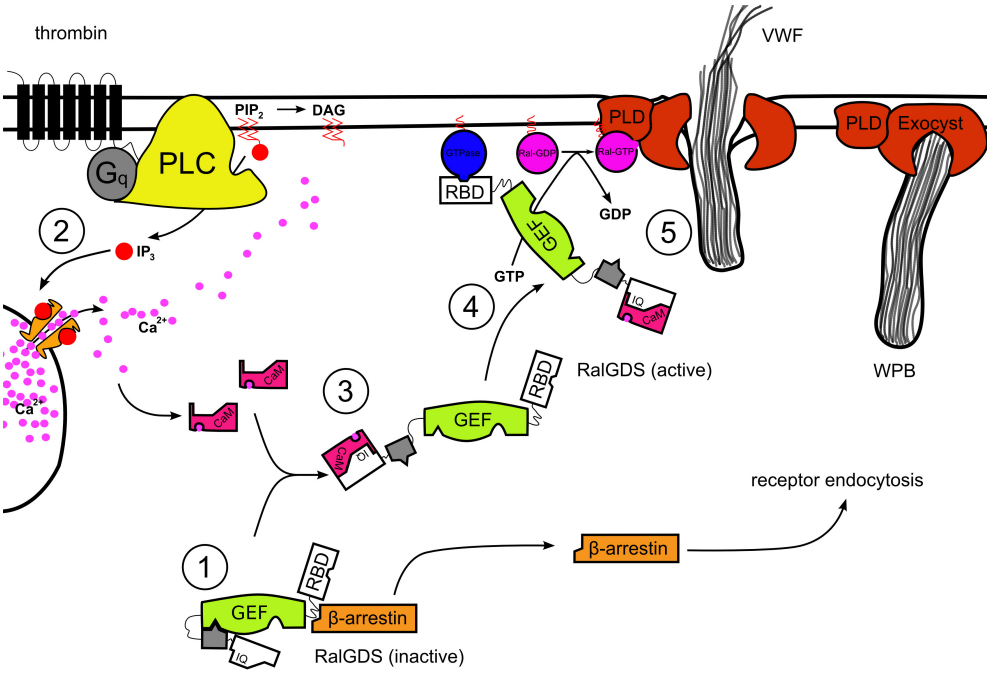

Figure 7. Proposed mechanism for the regulation of thrombin-induced WPB exocytosis by RalGDS and CaM. (1) In unstimulated endothelial cells, RalGDS is complexed with β-arrestin in the cytoplasm, with the N-terminal part of RalGDS shielding off its catalytic GEF domain.(2) Thrombin stimulation leads to the release of Ca^{2+} in a PLC-dependent-manner [48] and to the dissociation of the RalGDS - β-arrestin complex. (3) Ca^{2+}/CaM complex interacts with the IQ-motif present in the N-terminus of RalGDS inducing a conformational change that relieves RalGDS of auto-inhibition of its exchange activity. (4) Simultaneously, RalGDS translocates to the plasma membrane in a RBD-dependent manner by association of its RBD-domain with a membrane-tethered active GTPase. (5) Membrane-associated active RalGDS activates Ral, which mediates release of WPBs through the coordination of the exocyst complex and PLD-induced fusion of vesicular and plasma membrane.

CaM. The Ca^{2+}/CaM complex interacts with the calmodulin binding domain situated in the amino-terminal region of RalGDS. Through this interaction, Ca^{2+}/CaM sequesters the auto-inhibitory amino-terminal region of RalGDS thereby enhancing the exchange activity of the catalytic CDC25 domain of RalGDS. Simultaneously, the activation of an upstream GTPase that can interact with the carboxy-terminal RBD region of RalGDS leads to the translocation of the exchange factor to the plasma membrane where activation of Ral is needed to coordinate the exocyst complex and to promote PLD-induced fusion of vesicular and plasma membrane resulting in exocytosis of secretory granules.

Acknowledgements

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Chapter 4

Epac is involved in cAMP-mediated exocytosis of Weibel-Palade bodies in endothelial cells through the activation of the small GTPase Rap1

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Abstract

Endothelial cells contain specialized storage organelles designated Weibel-Palade bodies (WPB) that release their content into the lumen in response to specific agonists that raise intracellular Ca^{2+}, like thrombin, or cyclic AMP (cAMP), such as epinephrine and vasopressin. We have previously shown that cAMP-mediated WPB release is dependent on protein kinase A (PKA) and involves the activation of the small GTPase RalA. Here, we have investigated a possible role for other, PKA-independent cAMP-mediated signaling pathways in the regulation of WPB exocytosis, namely the exchange protein activated by cAMP (Epac) and its substrate, the small GTPase Rap1. siRNA-mediated knockdown of the cAMP-GEF Epac1 results in decreased epinephrine-induced WPB exocytosis. Epinephrine stimulation of endothelial cells leads to the activation of the small GTPase Rap1 in a PKA-independent fashion. In addition, siRNA-mediated knockdown of Epac1 completely abolished epinephrine-induced activation of Rap1 indicating that activation of Rap1 in response to epinephrine proceeds via Epac1. In contrast, thrombin-dependent activation of Rap1 was not inhibited in endothelial cells treated with siRNA for Epac1. We subsequently addressed whether activation of Rap1 is crucial for WPB release. Downregulation of Rap1 activation through expression of Rap1GAP effectively inhibits epinephrine-induced WPB exocytosis. Taken together, these data uncover a new pathway by which endothelial cells can regulate WPB exocytosis in response to agonists that signal through cAMP.

Introduction

Vascular endothelial cells provide a dynamic interface between circulating blood and underlying tissues that is critically involved in maintaining vascular integrity and homeostasis. The endothelium provides a surface for adhesion and subsequent extravasation of leukocytes to sites of inflammation. In addition, vascular endothelial cells are involved in the regulation of vascular tone, contribute to neo-vascularisation and mediate the formation of a platelet plug in the event of vascular damage. Rapid recruitment of bio-active components from intracellular storage pools have been shown to contribute to the critical role of endothelial cells in maintaining vascular homeostasis. A significant number of haemostatic components and inflammatory mediators originates from endothelial cell-specific, cigar-shaped organelles called Weibel-Palade bodies (WPBs) [1] that function as storage vesicles for von Willebrand factor (VWF) [2], a multimeric adhesive glycoprotein crucial for platelet plug formation, the leukocyte receptor P-selectin [3,4] and a number of bioactive compounds that include the chemoattractants IL-8 and eotaxin-3 (reviewed in [5]). Following stimulation with agonists that increase intracellular Ca^{2+} levels, such as thrombin or histamine, WPBs fuse with the plasma membrane, thereby releasing their content onto the cellular surface or into the circulation. More

recently, agonists such as epinephrine and vasopressin that raise intracellular levels of cAMP have been shown to promote the release of WPBs [6,7]. The physiological importance of this pathway is illustrated by the rise in VWF levels in patients with von Willebrand's disease and mild hemophilia A following administration of the vasopressin analogue desmopressin (DDAVP) [8] or epinephrine [9]. A subset of the WPBs is able to escape regulated exocytosis in response to cAMP-mediated stimulation by perinuclear clustering at the microtubule organizing centre (MTOC), which involves retrograde transport of vesicles mediated by the dynein-dynactin complex [10,11]. Previous work from our group has indicated that WPB exocytosis in response to cAMP-mediated agonists is partly controlled by a protein kinase A (PKA)-dependent signalling pathway which eventually leads to the activation of RalA [12], a small GTPase that co-sediments with WPBs in density gradients [13]. In its activated form, RalA has been shown to promote exocytosis through interaction with Sec5 [14,15] and Exo84 [16], components of the exocyst complex and by enhancing ARF-dependent phospholipase D1 activity [17]. Recent reports have documented signalling pathways independent of PKA that may be involved in regulation of cAMP-mediated secretory vesicle release. More specifically, the exchange protein activated by cAMP (Epac) has been implicated in cAMP-mediated vesicle exocytosis [18-20], suggesting that Epac may control PKA-independent signalling pathways leading to exocytosis. In this study we explored a potential role for the cAMP-GEF Epac in the regulation of WPB exocytosis in response to cAMP-mediated agonists.

Materials and Methods

Reagents and antibodies

Culture media, trypsin, penicillin, streptomycin and Oligofectamine were from Invitrogen (Breda, the Netherlands). Epinephrine, thrombin, forskolin, IBMX, BAPTA-AM, Endothelial Cell Growth Supplement (ECGS), heparin and anti-α-tubulin monoclonal antibody (DM1A) were from Sigma-Aldrich Chemie (Steinheim, Germany). 8-pCPT-2'-O-Me-cAMP (007) and Rp-8CPT-cAMPs were from Biolog (Bremen, Germany). Anti-β-catenin (sc-7199) and anti-Rap1 (sc-121) were from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-VWF monoclonal antibody CLB-RAg35 has been described previously [21]. The Epac1 mouse monoclonal antibody 5D3 [22] was a kind gift from Dr. J.L. Bos. Anti-myc mouse monoclonal 9E10 or rabbit polyclonal anti-myc were from Upstate (Charlottesville, USA). Alexa 488- and Alexa 594-conjugated goat anti-mouse IgG and goat anti-rabbit IgG secondary antibodies were from Molecular Probes (Breda, the Netherlands). Vectaschield mounting medium was from Vector Laboratories (Burlington, USA). Chemiluminescence blotting substrate and Complete Protease Inhibitor Cocktail Tablets were from Roche Diagnostics (Mannheim, Germany). All chemicals used were of analytical grade.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins and cultured in medium containing M199, 20% fetal calf serum, 100 units/ml penicillin, 100 mg/l streptomycin, 33 mg/l L-glutamine, 12.5 mg/l ECGS and 50 mg/ml heparin. Stimulation of endothelial cells with thrombin or epinephrine was performed in the following manner: HUVECs, grown in 6 wells plates, were washed two times with culture medium in which fetal calf serum was replaced by 1% human albumin (serum-free (SF) medium). After washing, the cells were pre-incubated with SF medium for 1 hour. At the beginning of stimulation, the pre-incubation medium was replaced by SF medium containing 1 U/ml thrombin or 10 mM epinephrine and 100 mM IBMX.

siRNA and DNA constructs

For siRNA-mediated knock down of Epac a previously reported siRNA [23] directed against the target sequence (CCATCATCCTGCGAGAAGA) was purchased from QIAGEN (Venlo, The Netherlands). A non-related siRNA, target sequence (TGAACATCGTTGGATAGGA), was used as a control in these experiments. siRNA was delivered to HUVECs in two consecutive transfections using Oligofectamine (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. Cells were left for 40 hours before assaying.. The myc-Rap1GAP construct was kindly provided by Dr. M.J. Lorenowicz. Myc-tagged Rap1AG12V and Rap1BG12V were obtained from the UMR cDNA Resource Center (www.cdna.org). HUVECs were transfected using an AMAXA nucleofector in combination with the Basic nucleofector kit for primary human endothelial cells (AMAXA GmBH, Cologne, Germany) and were grown on gelatin-coated glass coverslips for 48 hours before stimulation.

Immunofluorescence

Cells were fixed with 3.7% formaldehyde for 15 minutes. WPBs were visualized using anti-VWF mouse monoclonal antibody CLB-RAg35, while the plasma membrane at the intracellular junctions was stained using polyclonal anti-β-catenin. Myc-tagged proteins were detected using mouse monoclonal 9E10 or rabbit polyclonal anti-myc. Alexa 488- and Alexa 594-conjugated goat anti-mouse or goat anti-rabbit IgG were used as secondary antibodies. All antibodies were diluted in PBS; 1% BSA; 0.02% saponin. Cells were embedded in Vectashield mounting medium and analyzed by confocal microscopy using a Zeiss LSM510 microscope. Images were generated by making optical sections (Z-stacks with 0.36 μm intervals). Z-stacks of single cells were analyzed using Image Pro Plus 6.0 (Media Cybernetics, Breda, Netherlands) to quantify the total number of WPBs in a single cell. Differences in WPB numbers were statistically analyzed using a Student's t-test.

Rap1 activation assays

The amount of Rap1 activated upon stimulation was measured in a pull-down assay. The Ras binding domain (RBD) of RalGDS fused to a GST tag was expressed in IPTG-induced bacteria as described previously [24]. Purified GST-RBD (100 mg/sample) was pre-coupled to 30 µl/sample of Glutathione Sepharose 4B for 1 hour at 4°C. The pre-coupled Glutathione Sepharose was then washed 3 times with lysis buffer containing 15% (v/v) glycerol, 1% NP-40, 50 mM Tris (pH 7.5), 200 mM NaCl, 2.5 mM $MgCl_2$, 10 mM benzamidine, 100 nM aprotinin, supplemented with 1 protease inhibitor tablet per 50 ml. Following stimulation, cells grown in 6-wells plates, were lysed in 400 µl lysis buffer. The activated, GTP-bound form of Rap1 was then isolated from cell lysates by incubation of 300 µl lysate with GST-RBD pre-coupled glutathione sepharose for 1 hour at 4 °C. Finally, the sepharose beads were washed 4 times with lysis buffer and analyzed on a 12.5 % SDS-PAGE gel by western blotting with anti-Rap1 polyclonal antibody.

Results and discussion

Previously we and others have shown that exocytosis of Weibel-Palade bodies is mediated by elevated concentrations of cAMP that are due to triggering of G-protein coupled receptors of the G_s subtype, which eventually leads to the activation of Ral [6,7,12]. A crucial role for PKA in cAMP-mediated Ral activation and exocytosis of Weibel-Palade bodies has been established using specific inhibitors that return epinephrine-induced Ral activation to basal levels and simultaneously inhibit VWF secretion. Elevated concentrations of PKA inhibitors were needed to completely abolish WPB exocytosis, suggesting that a distinct PKA-independent pathway of WPB exocytosis exists that does not involve activation of Ral. Several studies have now shown that Epac, a cAMP-activated exchange factor for the small GTPases Rap1 and Rap2, is involved in regulated secretion. It has been previously shown that the Epac specific cAMP analogue 8-pCPT-2'-O-Me-cAMP (007) indeed promotes exocytosis of WPBs [12,25], but this is not accompanied by activation of Ral.

To further substantiate the involvement of Epac in regulated exocytosis in endothelial cells we utilized siRNA-mediated silencing of Epac1, using a previously described siRNAsequence [23]. Epac siRNA treatment of HUVECs resulted in downregulation of Epac to ~5% of its original level (Figure 1A). We addressed whether WPB exocytosis induced by binding of epinephrine to the β2-adrenergic receptor is dependent on Epac1. For this, we incubated siRNA-treated HUVECs with epinephrine or thrombin and quantified the number of residual WPBs using immunofluorescent staining of VWF. In accordance with previous data, a strong reduction in number of WPBs per cell was observed following incubation of endothelial cells with epinephrine (unstimulated: 136.6 ± 12.1; epinephrine: 61.2 ± 7.4) (Figure 1B, C). In the absence of Epac1 epinephrine-induced release of WPBs was virtually abolished as evidenced by the lack of reduction of WPB content (unstimulat-

ed: 132.8 ± 12.0; epinephrine: 122.6 ± 11.5) (Figure 1B, C). In contrast, thrombin-induced release of WPBs which is not mediated by cAMP but uses Ca^{2+} as second messenger, was not dependent on Epac1 (Figure 1C) as evidenced by the similar numbers of residual WPBs in Epac1 or control siRNA treated cells (control siRNA: 60.3 ± 10.9; Epac siRNA: 54.4 ± 7.3). These findings show that epinephrine-induced release of WPBs requires Epac1.

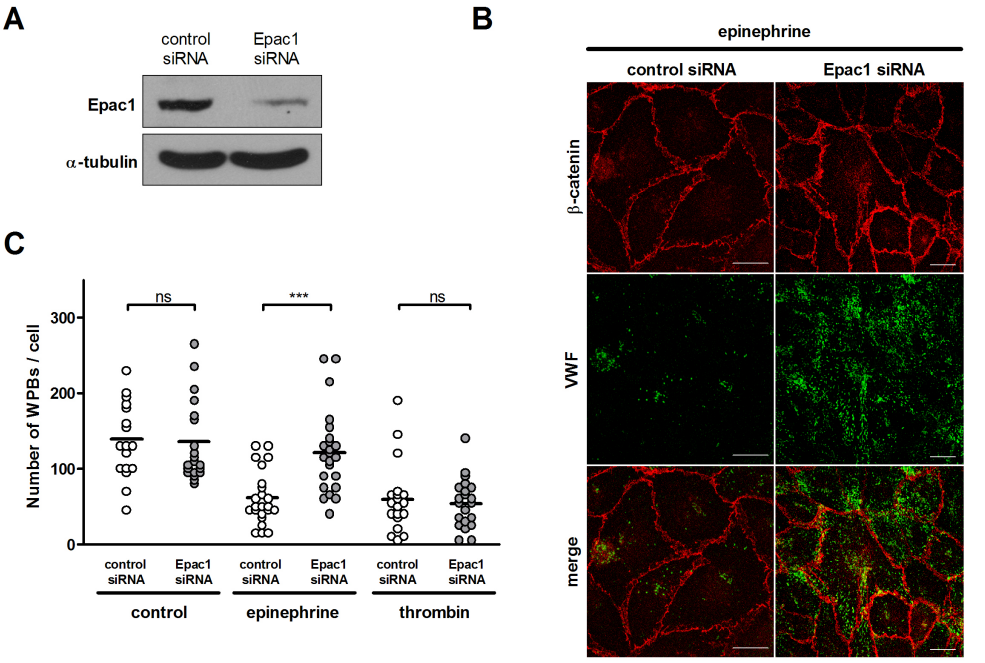

Figure 1: Downregulation of Epac expression inhibits epinephrine induced WPB release. (a) HUVEC were treated in 2 consecutive transfection rounds with a control siRNA oligo or a siRNA oligo directed against Epac1. Western blot analysis 48 hours post transfection showed downregulation of Epac1 expression. Levels of α-tubulin are shown as a loading control. (b) Control siRNA and Epac1 siRNA treated HUVECs were incubated for 45 minutes with 10 µM epinephrine and 100 µM IBMX, 1U/ml thrombin or SF medium alone (control). WPBs were visualized by immunofluorescent staining of VWF (green) while staining of β-catenin (red) was used to delineate the cellular membrane. Shown are representative confocal images of epinephrine stimulated endothelial cells. Bars correspond to 10 µm. (c) Numbers of WPBs in individual cells were quantified as described in the Materials and methods section. Approximately 20 randomly selected cells from 2 independent experiments were counted for each experimental condition. Bars represent mean values. ***P<0.0005 by Student's t-test.

Previous studies have shown that both 007 and the cAMP-raising compound forskolin can induce activation of the small GTPase Rap1 in HUVEC in an Epac-dependent manner [23,26]. cAMP-Epac-Rap1 signalling in endothelial cells regulates endothelial barrier function by promoting VE-cadher-independent tightening of cell-cell junctions [23,26,27]. Thrombin induced Rap1 activation is thought to serve as a negative feedback mechanism that counteracts thrombin-induced Rho activation such

that it can moderate vascular permeability [26]. We questioned whether receptor-mediated stimulation of WPB release would activate the Epac/Rap1 pathway. Similar as when challenged with 007 (Figure 2A) or the cAMP-elevating compound forskolin (not shown), endothelial cells activate Rap1 when stimulated with 10 µM epinephrine (Figure 2B): after 5 minutes a moderate but sustained increase in the amount of Rap1-GTP was observed. Epinephrine-induced Rap1 activation was, as expected, independent of PKA as shown by the lack of inhibition by the PKA-specific inhibitor Rp-8-CPT-cAMP. This result indicates that cellular stimulation of endothelial cells with the cAMP-elevating agonist epinephrine leads to the activation of Rap1. We also observed a sharp but transient increase in the amount of active Rap1 following incubation with 1U/ml thrombin (Figure 2C), confirming earlier findings by Cullere and co-workers [26]. The amount of active Rap1 was maximal after two minutes of thrombin stimulation and decreased to background levels after 10 minutes. These findings indicate that both epinephrine- and thrombin-induced exocytosis of WPB coincides with the activation of the small GTPase Rap1.

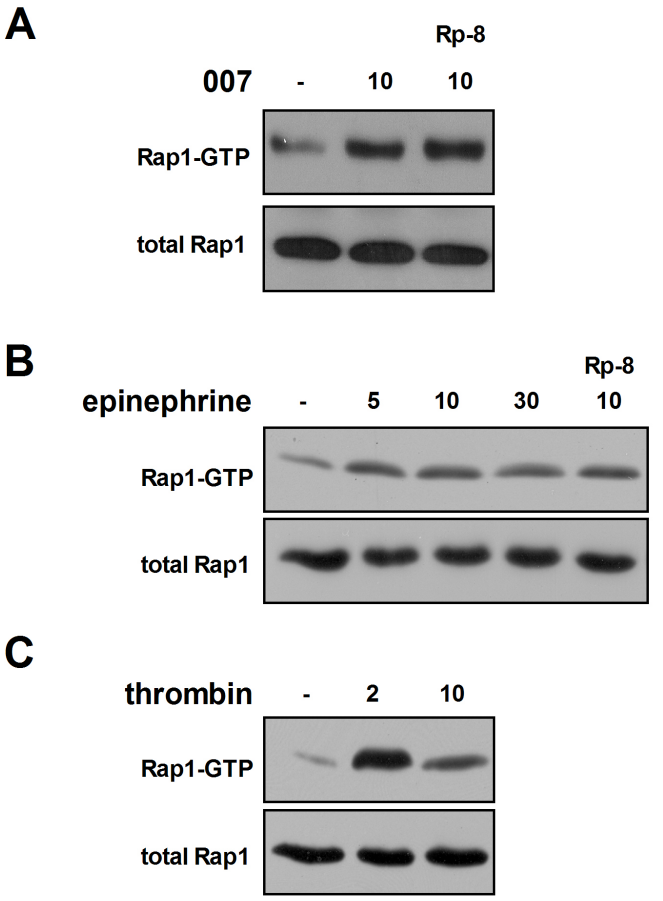

Figure 2: Stimulus induced WPB exocytosis is accompanied by activation of the small GTPase Rap1. Cells were pre-incubated with SF-medium for 1 hour. Lanes labeled with Rp-8 were supplemented with 500 µM Rp-8-CPT-cAMPs during pre-incubation. Subsequently, cells were stimulated with 100 µM 007 (a), 10 µM epinephrine and 100 µM IBMX (b) or 1U/ml thrombin (c) for the indicated perriods. Lanes marked with - represent cells that were left unstimulated. Activation of Rap1 in HUVECs was determined using a Rap1-GTP specificpull-down. Western blots of activated Rap1 demonstrate the activation of Rap1 by cAMP- and Ca^{2+}-mediated agonists. The total amount of Rap1 shown in the lower panels is used as loading control.

Remarkably, the kinetics of Rap1 activation in response to thrombin and epinephrine are very similar to that observed for the activation of the small GTPase Ral [12], suggesting that these two GTPases are activated in coordinated fashion. Subsequently, we addressed whether siRNA-mediated knockdown of Epac1 abolished epinephrine-induced activation of Rap1. In agreement with previous findings [23] knockdown of Epac1 did abolish the activation of the small GTPase Rap1 by epinephrine (Figure 3A). These findings confirm that Rapl is activated in an Epac dependent manner in endothelial cells upon stimulation with cAMP-elevating agonists [23,26,27]. Activation of Rap1 in response to thrombin was not affected by siRNA-mediated knockdown of Epac (Figure 3B), suggesting that Epac does not take part in the activation of Rap1 in response to agonists that use Ca^{2+} as a second messenger. Recently, the Ca^{2+} and diacylglycerol (DAG) activated exchange factor for Rap1, CalDAG-GEFI, has been shown to induce Rap1 activation in platelets [28] in response to thrombin. These findings raise the possibility that guanine exchange factors of the CalDAG-GEF family are involved in thrombin-induced Rap1 activation in endothelial cells.

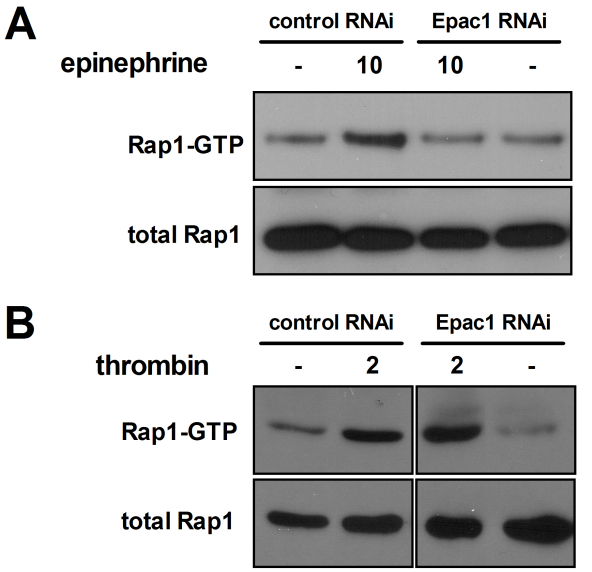

Figure 3: Impaired Rap1 activation in endothelial cells after downregulation of Epac1. HUVECs treated with Epac1 RNAi or control RNAi were pre-incubated with SF-medium for 1 hour. Subsequently, cells were stimulated with 10 μM epinephrine and 100 μM IBMX (a) or 1U/ml thrombin (b) for the indicated periods (- represents untreated cells). Activation of Rap1 in HUVECs was determined using a Rap1-GTP specific pull-down. Western blots of Rap1-GTP illustrate the inability of Epac1 downregulated endothelial cells to activate Rap1 in response to epinephrine (a), while thrombin-induced Rap1 activation remains unaffected (b). The total amount of Rap1 shown in the lower panels is used as loading control.

To further explore the potential role of Rap1 in regulated exocytosis of WPBs, we inhibited accumulation of active Rap1 by overexpression of Rap1GAP. Rap1GAP is a GTPase activating protein specific for Rap1, but with no GAP activity towards related GTPases such as Rap2 or Ras [29]. Earlier reports have shown that the amount of active Rap1 was strongly reduced in cells overexpressing Rap1GAP [30,31]. We transfected HUVECs with GFP or myc-tagged Rap1GAP and 48 hours post-transfection the cells were stimulated with epinephrine or thrombin. The number of residual WPBs was quantified in GFP- or Rap1GAP-expressing cells by immunostaining for VWF (Figure 4A).

Overexpression of Rap1GAP prevented epinephrine-induced exocytosis of WPBs (GFP: 59.0 ± 7.5; Rap1GAP: 122.5 ± 10.4) (Figure 4B). This indicates that active Rap1 is required for epinephrine-induced release of WPBs from endothelial cells. It also suggests that the role of Epac in WPB exocytosis depends on its ability to catalyze the GDP/GTP-exchange of its downstream effector Rap1. In contrast, overexpression of Rap1GAP did not block thrombin-induced release of WPBs (GFP: 66.0 ± 6.5; Rap1GAP: 54.6 ± 6.0) (Figure 4B), suggesting that activation of Rap1 is not essential for thrombin-induced release of WPBs.

Rap1 is found in two isoforms, Rap1A and Rap1B, that mainly differ in their carboxy-terminal tail. Overexpression of Rap1GAP should be able to inactivate both isoforms in vivo equally well [32]. However, gene expression data from the NCBI Gene Expression Omnibus (GEO) indicate that endothelial cells mainly express Rap1B (http://www.ncbi.nlm.nih.gov/geo). Overexpression of constitutive active (G12V) Rap1A or Rap1B did not result in release of WPB (data not shown). This suggests that the activation of Rap1, although an essential step in the Epac dependent-pathway of WPB release, is on itself not sufficient to promote release of WPB exocytosis. Recently it was found that Epac also catalyzes the activation of the small GTPase R-Ras in response to G-protein coupled receptor activation [33]. Activation of R-Ras stimulates the activity of phospholipase D (PLD), an enzyme that is also a prominent effector of RalA in exocytosis of secretory vesicles [17] and that takes part in the final stages of secretory vesicle exocytosis by promoting fusion of plasma and vesicle membranes. In addition, activation of R-Ras has also been implicated upstream of activation of RalA by the RalGEF Rgl2/Rlf [34]. This raises the possibility that Epac by virtue of its activation of an R-Ras/Rgl2 pathway promotes RalA dependent exocytosis of WPBs. However, previous findings from our laboratory have shown that activation of Epac by 007 does not result in RalA activation in endothelial cells [12]. Also, activation of RalA was found to be dependent on PKA arguing against a role for Epac mediated activation of R-Ras in regulated release of WPBs. Together with the data presented in the current study these findings support a model in which the epinephrine- and vasopressin-induced release of WPBs is controlled by both PKA and Epac signalling pathways (Figure 5). This study has shown that the cAMP-GEF Epac1 performs an essential function in the cAMP-mediated signalling cascade that controls WPB exocytosis. Triggering of WPB exocytosis by agonists that raise intracellular cAMP leads to the activation of Rap1 by the action of Epac1. In turn, activation of Rap1 is essential but not sufficient to induce cAMP-mediated WPB exocytosis. On the other hand, epinephrine-induced WPB exocytosis is also dependent on PKA-dependent activation of RalA [12]. Apparently, cAMP-mediated WPB exocytosis is the result of the concerted action of PKA-dependent and PKA-independent signalling processes that emanate from cAMP and that converge following the simultaneous activation of Rap1 and RalA. Pertinent to this point is the observation that epinephrine induces activation of Ral and/or Rap1 to a lesser extent than thrombin or 007 (Figure 2 and [12]). Thrombin is a prothrombotic and proinflammatory agonist that

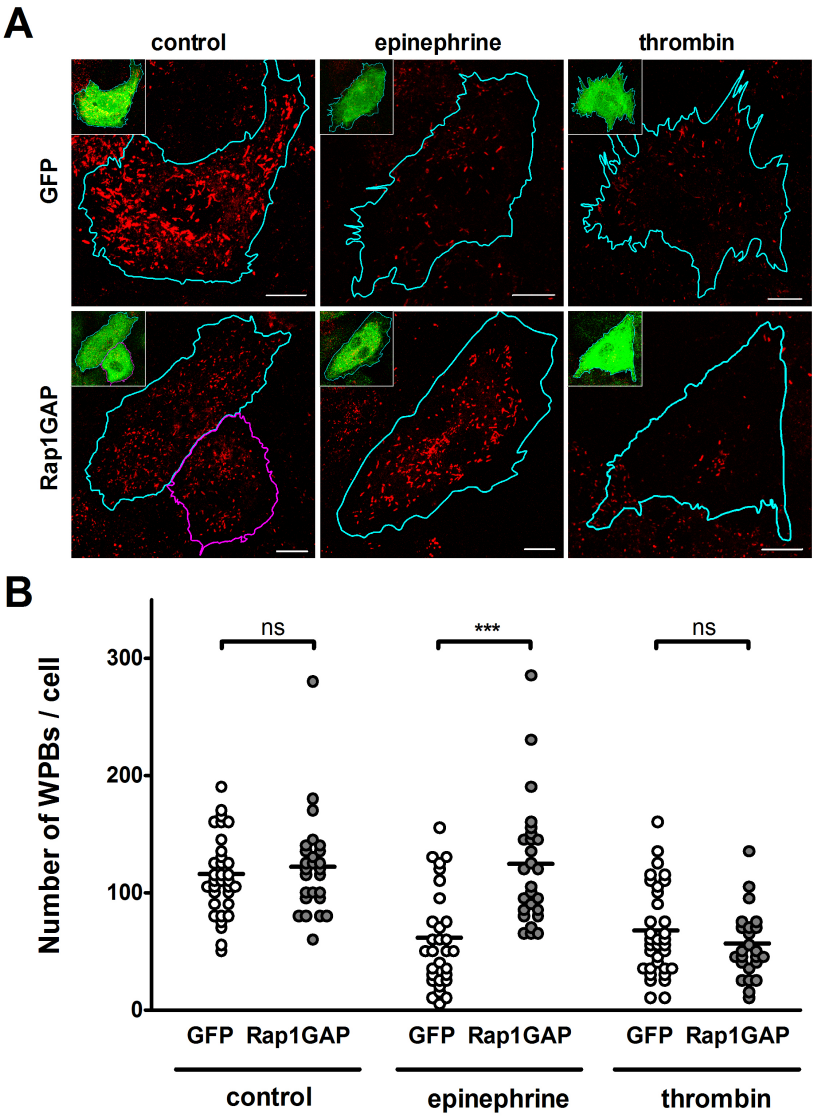

Figure 4: Activation of Rap1 is crucial for epinephrine induced WPB release. (a) HUVECs were transfected with GFP (negative control) or Rap1GAP carrying a myc tag and were grown for 48 hours. Cells were incubated with 10 µM epinephrine and 100 µM IBMX, 1U/ml thrombin or SF medium alone (control) for 45 minutes. WPBs were visualized by immunofluorescent staining of VWF (red), Rap1GAP expressing cells were visualized by immunofluorescent staining against the myc-tag. GFP or myc-staining (shown in detailed insets) were used to delineate the cellular contours. Shown are representative confocal images. Scale bars correspond to 10 µm. (b) Numbers of WPBs in individual GFP- or Rap1GAP-expressing cells were quantified as described in the Materials and methods section. Approximately 30 randomly selected cells from 2 independent experiments were counted for each experimental condition. Bars represent mean values. ***P<0.0005 by Student's t-test.

elicits a rapid, massive release of most of the endothelial cell's WPBs in the case of vascular emergency through excessive activation of RalA, which may be sufficient to promote exocytosis on its own without the specific need for a second signal to accomplish WPB release. Similarly, strong activation of Rap1 as induced by 007 treatment of endothelial cells may suffice to promote exocytosis without the need for simultaneous activation of RalA. cAMP-mediated agonists such as epinephrine and vasopressin are most likely involved in systemic regulation of VWF levels in plasma and unlike the vascular emergency agonist thrombin this may not require massive exocytosis of WPBs. Epinephrine also leads to much milder activation of both Rap1 and RalA and may therefore require the contribution of both pathways to promote exocytosis.

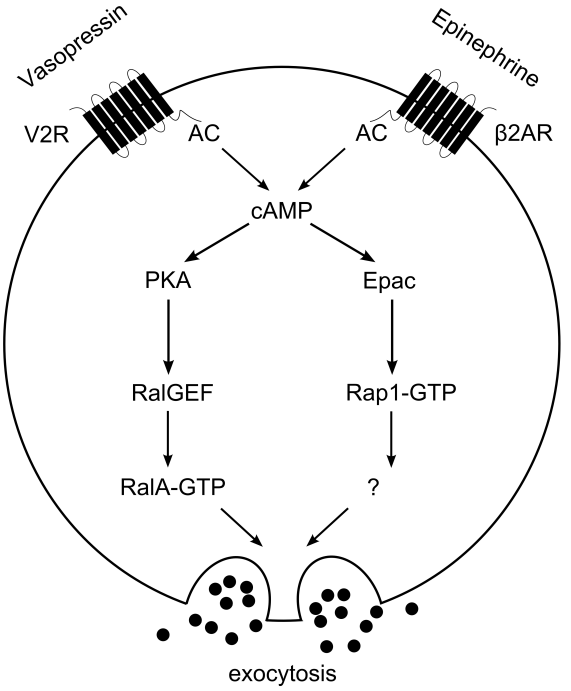

Figure 5: Signalling pathways that regulate WPB exocytosis. Stimulation of the β2-adrenergic receptor (β2AR) by e.g. epinephrine or the vasopressin-2 receptor (V2R) by vasopressin stimulates cAMP production by G_s-activated adenylate cyclase (AC). Emanating from cAMP, a PKA-dependent pathway induces the activation of the small GTPase RalA, probably by a yet to be identified RalGEF, while simultaneously Epac activates Rap1. Through a yet unknown mechanism active Rap1 together with active RalA promotes exocytosis of WPBs.

Rap1 has been implicated in exocytosis of secretory granules in a number of cellular systems [35-38]. Yet, how it is able to promote exocytosis is poorly understood. Rap1 has been described to interact with the the sarco/endoplasmatic reticulum Ca^{2+}-channel SERCA3B, by which it is thought to regulate calcium fluxes in platelets [39]. Furthermore, Kang et al. [18] have reported that stimulation of Epac1 using 007 in pancreatic β-cells is able to induce exocytosis through the release of calcium from internal stores. In this regard, β2-adrenergic receptor stimulation of endothelial cells is not accompanied by a rise in free intracellular Ca^{2+} [6], which makes it unlikely that the Epac1/Rap1 pathway promotes

WPB exocytosis through the release of Ca^{2+}. The events taking place downstream of Epac-mediated Rap1 activation that promote WPB exocytosis therefore remain to be determined.
In summary, the data presented in this work have uncovered a new pathway by which endothelial cells can regulate WPB exocytosis in response to agonists that signal through cAMP. Our findings implicate Epac1 as an important regulator of β2-adrenergic and vasopressin-2 receptor stimulated WPB exocytosis, and suggest that Epac1 may perform a prominent role in the systemic regulation of VWF levels in plasma.

Acknowledgments

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Chapter 5

The shear stress-induced transcription factor KLF2 prevents perinuclear clustering of Weibel-Palade bodies in endothelial cells

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Manuscript in preparation

Abstract

Endothelial cells release their storage organelles, Weibel-Palade bodies (WPBs) when stimulated with agonists that raise intracellular cAMP levels. A subset of Weibel-Palade bodies escapes exocytosis by perinuclear clustering at the microtubule organizing centre. In this study we investigated a possible involvement of KLF2, which translates the effect of prolonged shear flow on the endothelium into an atheroprotective gene expression profile, on clustering of WPBs. We find that endothelial cells that overexpress KLF2, display reduced perinuclear clustering of WPBs when stimulated with cAMP-dependent agonists. This result suggests that KLF2 regulates cellular components that are involved in retrograde transport of WPBs to the microtubule organizing centre.

Introduction

Endothelial cells are equipped with unique, elongated organelles called Weibel-Palade bodies (WPBs) [1] that function as storage vesicles for von Willebrand factor (VWF) [2], a multimeric adhesive glycoprotein crucial for platelet plug formation, the leukocyte receptor P-selectin [3,4] and a cocktail of vasoactive substances including the angiogenic growth factor angiopoietin-2 (Ang-2) [5] and the chemoattractants interleukin-8 (IL-8) [6,7] and eotaxin-3 [8]. Following stimulation with agonists that increase intracellular Ca^{2+} or cAMP levels, such as respectively thrombin or epinephrine, endothelial cells recruit these WPBs to the plasma membrane with which they fuse in a process called exocytosis, thereby releasing their content onto the cellular surface or into the circulation. Through release of WPBs, endothelial cells can participate in modulation of inflammatory responses and in the arrest of bleeding in the event of vascular damage. Real time studies of WPB dynamics using a GFP-tagged VWF variant revealed that a subset of the WPBs is able to escape regulated exocytosis in response to cAMP-mediated stimulation by perinuclear clustering at the microtubule organizing centre (MTOC) [9]. This retrograde transport of WPBs along microtubules is mediated by the dynein-dynactin complex in a protein kinase A (PKA) dependent manner [10]. Currently, the significance of this phenomenon is not understood, nor is it clear if the differential trafficking of subsets of WPBs represents a mechanism to selectively retain subsets of WPBs that may differ in composition or if it serves to limit the release of thrombotic and inflammatory mediators.

Krüppel-like factor 2 (KLF2) is a zinc finger transcription factor found in endothelial cells that has recently been shown to translate flow into activation or suppression of expression of its downstream targets. Prolonged exposure of endothelial cells to steady laminar shear stress induces the expression of KLF2 [11,12], which leads to an expression profile that is anti-inflammatory, anti-thrombotic, vasodilating and as such may be considered as atheroprotective [13]. Vascular regions that encounter turbulent flow, e.g. as occurs at aortic bifurcations, and that are prone to develop atherosclerotic le-

sions, display low levels of KLF2 expression [11]. Interestingly, in line with the atheroprotective action of flow, expression of KLF2 also leads to desensitization of endothelial cells to thrombin stimulation of WPB release, while remaining equally competent for activation by epinephrine.
In this study, we have addressed the consequence of KLF2 expression on cAMP-mediated signaling mechanisms in endothelial cells by further analyzing its effect on perinuclear clustering of WPBs. To this end, we have determined the degree of clustering of WPBs in a quantitative manner in cells overexpressing KLF2. Our results suggest that KLF2 indirectly regulates cellular components that are responsible for clustering of WPBs at the MTOC.

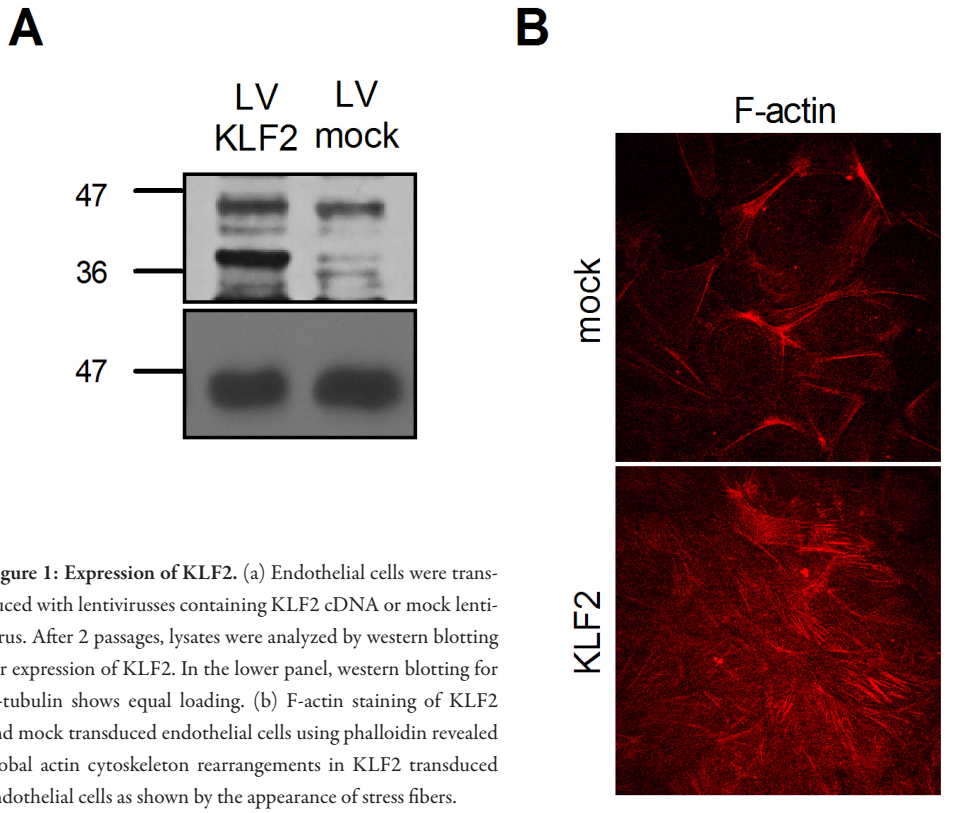

Figure 1: Expression of KLF2. (a) Endothelial cells were transduced with lentivirusses containing KLF2 cDNA or mock lentivirus. After 2 passages, lysates were analyzed by western blotting for expression of KLF2. In the lower panel, western blotting for α-tubulin shows equal loading. (b) F-actin staining of KLF2 and mock transduced endothelial cells using phalloidin revealed global actin cytoskeleton rearrangements in KLF2 transduced endothelial cells as shown by the appearance of stress fibers.

Materials and Methods

Reagents and antibodies

Culture media, trypsin, penicillin, streptomycin, okadaic acid and HRP-conjugated donkey anti-rabbit IgG were from Invitrogen (Breda, the Netherlands). Epinephrine, thrombin, forskolin, IBMX, Endothelial Cell Growth Supplement (ECGS), heparin and anti-α-tubulin monoclonal

antibody (DM1A) were from Sigma-Aldrich Chemie (Steinheim, Germany). Anti-β-catenin (sc-7199) was from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-VWF monoclonal antibody CLB-RAg20 and CLB-RAg35 have been described previously [14]. Alexa 488-, Alexa 594- and Alexa 633-conjugated secondary antibodies and phalloidin-BODIPY 655/665 were from Molecular Probes (Breda, the Netherlands). Vectaschield mounting medium was obtained from Vector Laboratories (Burlington, USA). Chemiluminescence blotting substrate and Complete Protease Inhibitor Cocktail Tablets were from Roche Diagnostics (Mannheim, Germany). All chemicals used were of analytical grade.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins and cultured in medium containing M199, 20% fetal calf serum, 100 units/ml penicillin, 100 mg/l streptomycin, 33 mg/l L-glutamine, 12.5 mg/l ECGS and 50 mg/ml heparin. Stimulation of endothelial cells with thrombin or epinephrine was performed in the following manner: HUVECs, grown in 6 wells plates, were washed two times with culture medium in which fetal calf serum was replaced by 1% human albumin (serum-free (SF) medium). After washing, the cells were pre-incubated with SF medium for 1 hour. At the beginning of stimulation, the pre-incubation medium was replaced by SF medium containing 1 U/ml thrombin or 10 mM epinephrine and 100 mM IBMX. Okadaic acid was used at 100 nM concentration during pre-incubation and stimulation.

Lentiviral transfections

Lentiviral KLF2 overexpression was performed by transducing HUVECs with lentiviral vectors expressing KLF2 cDNA or with empty lentiviral vectors as described [15].

Immunofluorescence

WPBs were visualized using anti-VWF mouse monoclonal antibody CLB-RAg35 or CLB-RAg20 16 and Alexa 594-conjugated goat anti-mouse IgG or Alexa-594-conjugated goat anti-mouse IgG_{2b} respectively. The cellular membrane was visualized by staining with anti-β-catenin polyclonal antibody and Alexa 633-conjugated goat anti-rabbit IgG as a secondary antibody. Microtubules and the MTOC were visualized using anti-α-tubulin mouse monoclonal antibody DM1A and Alexa 488-conjugated goat anti-mouse IgG_1. All stainings were performed in PBS supplemented with 1% BSA and 0.02% saponin. Cells were embedded in Vectashield mounting medium and analyzed by confocal microscopy using a Zeiss LSM510 (Carl Zeiss, Sliedrecht, the Netherlands) equipped with the appropriate filters. Images were generated by making optical sections (Z-stacks with 0.36 μm axial intervals).

Quantification of clustering

Image restoration and 3D analysis of Z-stacks of single cells was performed using Image Pro Plus 6.0 (Media Cybernetics, Breda, the Netherlands). Staining for β-catenin was used to select an area of interest that encompasses a single cell. The 3D Open filter was applied to separate narrowly connected WPBs. 3D data were rendered to a 3D Gaussian surface using the using the 3D Constructor module to automatically recognize the vesicles and MTOC and to determine their position. The distance of individual WPBs to the MTOC was calculated using

$$d_{WPB} = \sqrt{(x_{WPB} - x_{MTOC})^2 + (y_{WPB} - y_{MTOC})^2 + (z_{WPB} - z_{MTOC})^2}$$

Statistical analysis

Student's t-test was performed with Graphpad Prism version 4.03 (GraphPad Software, San Diego,USA).

Results

To establish a cell system in which we could address the role of KLF2 in regulation of clustering, we used lentiviral transduction to efficiently introduce KLF2 overexpression into HUVECs. Early passage HUVECs were transduced with either lentivirus containing KLF2 cDNA or with mock lentivirus. Western blot analysis of KLF2 expression from lysates obtained 2 passages after transduction, demonstrates increased expression of KLF2 in endothelial cells transfected with KLF2 lentivirus when compared to mock transfected endothelial cells (Figure 1A). Previously it has been reported that prolonged overexpression of KLF2 induces rearrangement of the actin cytoskeleton, which leads to the appearance of stress fibers. To verify that overexpression of KLF2 to the levels that we obtain leads to the phenotypic effects that have been described, we used phalloidin staining to visualize F-actin in lentiviral transduced endothelial cells. In contrast to mock transfected endothelial cells, KLF2 expression induced the formation of characteristic stress fibers (Figure 1B).

To address if KLF2 or one of its downstream targets is involved in the regulation of the clustering of WPBs, we undertook a quantitative analysis of clustering in KLF2 and mock transduced endothelial cells. Previously it was found that clustering of WPBs can be enhanced with okadaic acid, which is a potent inhibitor of dephosphorylation by protein phosphatases. To induce maximum degree of clustering, KLF2 and mock transfected cells were stimulated for 1 hour with the cAMP raising compound forskolin and IBMX in the presence of okadaic acid (OA) or were left for 1 hour with serum free medium alone. Subsequently, cells were fixed and immunofluorescent staining to visualize WPBs, microtubules and the cellular membrane was performed. Cells of similar dimensions were

randomly selected and three dimensional images were acquired; representative images of which are shown in Figure 2. In response to forskolin/IBMX/OA mock transfected endothelial cells displayed perinuclear clusters of the majority of the cell's WPBs that were localized to the MTOC as illustrated by the colocalization of these clusters with an intensely staining core of microtubules. Perinuclear clusters of WPBs were not found in mock or KLF2 transfected cells that were left unstimulated, instead WPBs were found dispersed throughout the cell. In endothelial cells that were

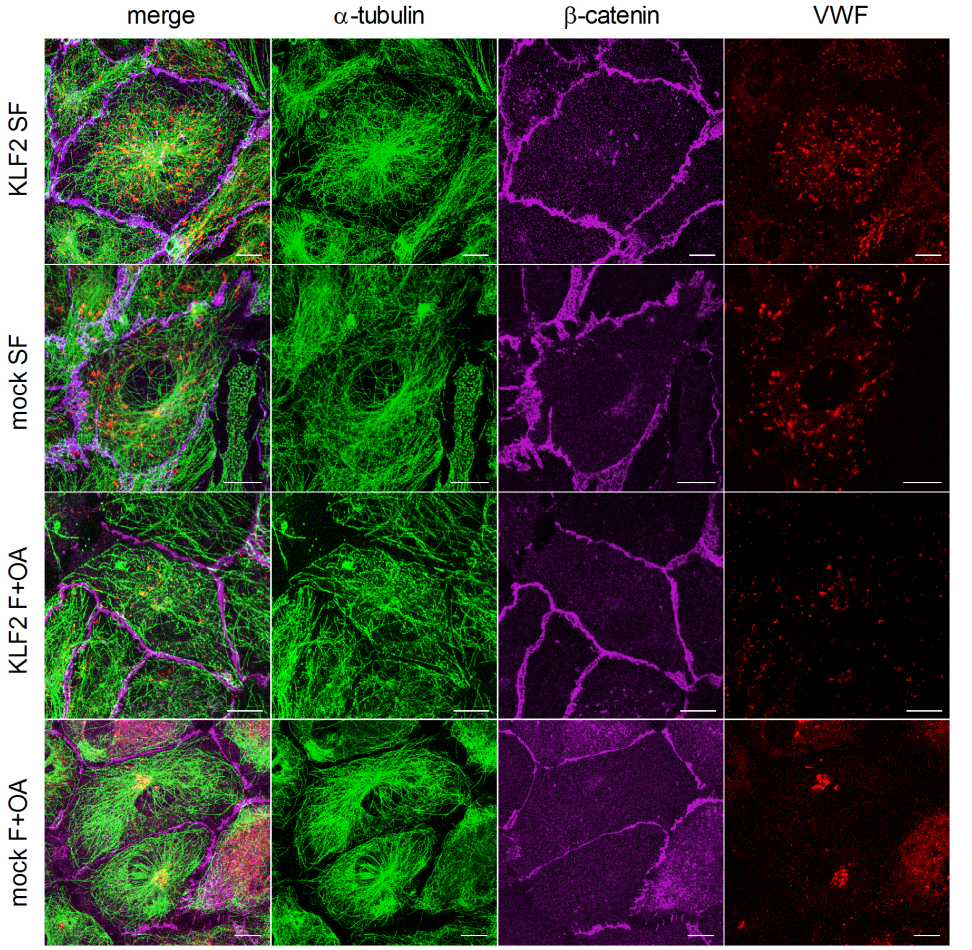

Figure 2A: KLF2 expression reduces cAMP-induced WPB clustering.
(a) Mock and KLF2 transduced endothelial cells were treated with 10μM forskolin/100 μM IBMX/100 nM okadaic acid (OA) for 1 hour or were left unstimulated. Subsequently, cells were fixed and immunostaining for VWF, β-catenin and α-tublin was performed to visualize WPBs, the cellular membrane and microtubules respectively. Shown are representative maximal projections of endothelial cells that were inspected by confocal microscopy. Bars represent 10 μm.

transduced with KLF2 some WPBs were indeed found to be associated with the MTOC, however most WPBs were still found throughout the cell. Treatment of endothelial cells with forskolin/IBMX/OA did not affect basic morphology of endothelial cells nor could we find any indication of extensive rearrangement or impairment of the microtubular network.

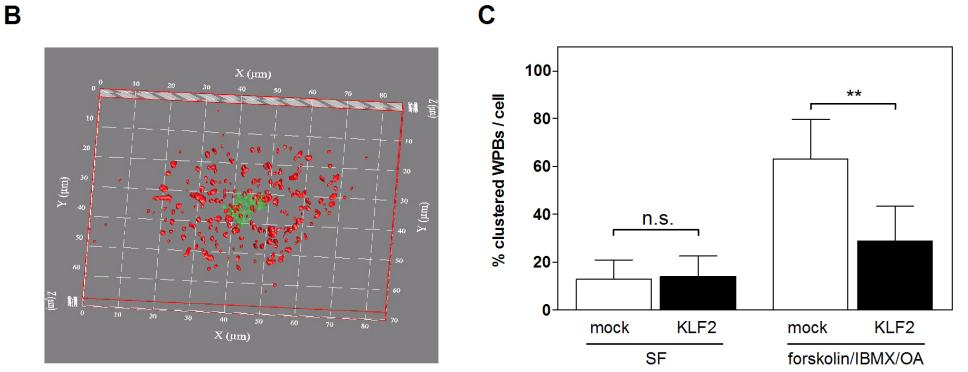

Figure 2B-C: Quantification of WPB clustering. (b) 3D rendering of an endothelial cell (KLF2 SF from Figure 2A) showing WPBs and the MTOC. (c) Quantification of clustering of WPBs. Expressed are the relative amounts of WPBs that were found within a region of 6 µm radius surrounding the MTOC. Bars represent SD and are the result of 10-15 cells.

To quantitatively determine the degree of clustering in these experiments, we determined the distance to the MTOC of all WPBs in 10 randomly selected cells for each condition by computer-aided image analysis (see Figure 2 and methods section). The average distance of WPBs to the MTOC in unstimulated cells was almost similar for mock and KLF2 transfected cells (mock: 14.2 µm +/- 0.9 µm; KLF2: 15.5 µm +/- 1.7 µm; P=0.5004). Treatment with forskolin/IBMX/OA induced retrograde transport of WPBs in mock transfected endothelial cells as illustrated by the decrease in average distance to the MTOC (6.6 µm +/- 0.8 µm). Expression of KLF2 leads to a significant inhibition of retrograde transport (9.8 µm +/- 0.9 µm; P=0.0279). Since the average distance to the MTOC is not a direct measure for the relative amount of clustered WPBs, we defined a region around the MTOC with a radius of 6 µm in which we consider a WPB to be part of a perinuclear cluster. We took a 6 µm boundary since this represents the distance of 2 longitudinally ordered WPBs. Using that criterion, we found no difference in the relative amount of WPBs in unstimulated cells that are clustered (mock: 12.9 % ; KLF2; 14.1% ; P=0.7523). Stimulation of mock transfected cells causes 63.2 % of the WPBs to retreat into a perinuclear cluster, which is significantly decreased (28.9 %; P=0.0010) upon KLF2 expression (Figure 2C). In conclusion we find that KLF2 expression leads to a decrease in the degree of perinuclear WPB clustering.

Discussion

The study presented here shows that retrograde transport mechanisms that are responsible for clustering of WPBs at the MTOC are inhibited by overexpression of the transcription factor KLF2. This suggests that downstream targets of KLF2 take part in the regulation of clustering. The molecular mechanism by which KLF2 prohibits clustering of WPBs is presently unclear. Clustering of WPBs is mediated by the dynein-dynactin minus-end motor complex and is dependent on phosphorylation induced by PKA [10]. Conceptually, this implies that KLF2 or its downstream targets negatively regulate the expression or activity of this kinase or promote the action or expression of protein phosphatases that counteract PKA induced phosphorylation. However, clustering of WPBs is not solely dependent on activation of PKA, since treatment of endothelial cells with the PKA-specific cAMP-analogue 6-Bnz-cAMP did not induce clustering on its own. This suggests that clustering may apart from phosphorylation by PKA also involve PKA-independent signaling events.

Also, for its ability to regulate motor protein-dependent organelle movement, PKA needs to be in close proximity or physical association with its regulatory targets. Minus- and plus-end motors that traffick intracellular organelles like WPBs, mitochondria [17] or melanosomes [18] along microtubules have been found to be part of multi-protein complexes, so called regulated motor units (RMUs) that integrate motor proteins, adaptor molecules and signaling components on the granular surface [19] and which also include PKA. This is achieved by interaction with so called A-kinase anchoring proteins, (AKAPs) that provide a binding site for PKA on the organelle surface. Instead of directly activating kinase or phosphatase activities, KLF2-induced mechanisms may also reduce clustering by targeting the formation of these complexes.

A number of studies support the concept that upregulation of KLF2 by shear stress promotes a functionally quiescent, anti-inflammatory and atheroprotective phenotype of the endothelium [13,20]. Our findings therefore imply that reduced clustering of WPBs is part of the atheroprotective phenotype that shear flow imposes on the endothelium. This is a rather unexpected finding, since one obvious function of WPB clustering could be to limit the release of thrombotic and inflammatory mediators. Through upregulation of thrombomodulin KLF2 indeed is able to desensitize endothelial cells to thrombin stimulation of WPB release, which complies with its anti-thrombotic and anti-inflammatory character [13]. On the other hand, the same study indicates that sensitivity of endothelial cells to epinephrine is not compromised by KLF2, while VWF expression and WPB biogenesis are even positively affected by KLF2 expression, suggesting that epinephrine induced WPB release does not contribute to atherogenesis or may even be beneficial for vascular health and therefore does not necessitate attenuation. An alternative explanation for the clustering phenomenon is that clustering of WPBs is not a mechanism to prevent excessive exocytosis but rather is used for "setting aside" a subset of WPBs with pro-inflammatory cargo. Supporting this hypothesis

are the observations that have been done in respect to the spatial subpopulations of WPB that can be mobilized by cAMP- or Ca^{2+}-mediated agonists: while cAMP-mediated agonists only mobilize peripheral WPBs for exocytosis, Ca^{2+}-mediated agonists are able to mobilize both peripheral and the more centrally located WPBs that require microtubular transport to the plasma membrane [21,22]. Cleator and colleagues have shown that endothelial cells differentially release P-selectin and VWF in response to agonists that use Ca^{2+} or cAMP as second messenger. Activation of endothelial cells with e.g. forskolin leads to selective release of WPBs that contain less or no P-selectin, in contrast to histamine which leads to release of WPBs irrespective of their content [23]. This observation raises the possibility that clustering of WPBs may as such represent a mechanism that diverts vesicles with pro-inflammatory cargo to a cellular region where they can only be mobilized by calcium-raising agonists. Expression of KLF2 has been shown to downregulate the expression of a number of pro-inflammatory components [13], some of which are present within WPBs. The reduced clustering of WPBs therefore most likely reflects a reduced necessity to prevent release of WPBs with pro-inflammatory cargo in endothelial cells overexpressing KLF2.

Acknowledgments

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Chapter 6

General discussion

Ruben Bierings

Co-targeting of secretory proteins to Weibel-Palade bodies

Over the past decade a large body of evidence has accumulated that suggests that, in addition to VWF and P-selectin, a number of other proteins, hormones and vascular modulators reside in Weibel-Palade bodies (WPBs) [1,2]. Up to this point not much is known about the significance of storage of these proteins in terms of sorting efficiency. We initiated studies aimed at studying the sorting efficiency of the WPB component IL-8 by endothelial cells on a quantitative basis. As described in chapter 2, we have found that only a fraction of *de novo* synthesized IL-8 is able to enter the regulated secretory pathway, and more intriguingly, that the molar amount of IL-8 that sorts to WPBs is limited by the amount of VWF that enters these granules. This suggests that VWF facilitates the sorting of IL-8 to newly forming WPBs by acting as a cargo-receptor. In that respect, we think that the sorting efficiency of a secretory protein in terms of molar fraction of newly synthesized protein as determined by pulse-chase analysis is a less relevant parameter to describe the efficiency with which a co-sorted secretory protein is targeted to the regulated secretory pathway, since it does not consider the limitations on storage capacity set by the availability of cargo-receptor. Rather should one speak of the efficiency with which a molecule fills up the available sorting capacity, in this case a yet unidentified IL-8 binding site on the VWF molecule that has been occupied to the full extent. When considering this aspect, we conclude that IL-8 sorts very efficiently to the regulated secretory pathway of the endothelium.

In our studies we did not address whether storage and regulated secretion of IL-8 by endothelial cells serves a physiological function. As originally proposed by Wollf and Utgaard, storage of IL-8 in WPBs may enable the endothelium to recruit neutrophils upon release of this chemokine from the WPB [3,4]. Although plausible, evidence for this is lacking. VWF deficient mice, which provide a model for severe von Willebrand's disease, do not produce WPBs and have, apart from several hematological defects, decreased leukocyte rolling and impaired neutrophil recruitment to sites of vascular injury and are less susceptible to develop atherosclerosis lesions [5-7]. VWF $^{-/-}$ mice phenocopy P-selectin $^{-/-}$ mice, their defects in leukocyte extravasation however can not be attributed to IL-8 [8]. Also VWF deficient pigs develop less atherosclerotic lesions [9]. In contrast, individuals that suffer from von Willebrand's disease (VWD) type 3 are not protected against atherosclerosis [10]. What complicates the interpretation of these observations is the fact that deficiency in VWF affects the release of a cocktail of vasoactive substances and as such it will be difficult to directly attribute a phenotype to the absence of IL-8 in WPBs. As discussed in chapter 2, the amount of IL-8 that is stored in WPBs can be sufficient to, upon release, establish a chemotactic gradient and to promote adherence of neutrophils to endothelial cells.

Endothelial cells produce and store a number of chemokines [11], but only two of them, the aforementioned IL-8 and eotaxin-3, a member of the CC-chemokine family, enter the WPBs.

Since these molecules all exhibit high, three-dimensional structural homology, this could provide a rationale to identify their sorting signal responsible for entering WPBs. It will be of interest to see if this signal indeed is also responsible for the interaction of IL-8 and eotaxin-3 with VWF.
We have recently explored the pH dependence of the interaction of IL-8 with VWF. Similar as observed for VWFpp, IL-8 associates with VWF in slightly acidic conditions in the presence of calcium. The greatly diminished binding of IL-8 to VWF at neutral pH makes it unlikely that upon release into the circulation, IL-8 will remain associated to VWF that is anchored to the surface of stimulated endothelial cells or the subendothelial matrix. Possibly IL-8 will, again analogous to VWFpp [12], quickly dissociate from VWF following exocytosis and subsequent exposure of WPB cargo to a neutral environment.
A more recently discovered component of the WPB, osteoprotegerin (OPG), also displays a physical interaction with VWF, intra- as well as extracellularly [13]. As shown in several reports [14,15], VWF can direct the storage of P-selectin to pseudo-WPBs. P-selectin has already been reported to bind to VWF, by which it promotes anchoring of strings of newly released ultralarge VWF multimers on the surface of endothelial cells [16], although this latter claim has been disputed recently [17]. In another study, Michaux and co-workers reported that recruitment of P-selectin variants lacking a functional cytoplasmatic domain to pseudo-WPB in HEK293 cells is dependent on the D`-D3 region of VWF. In combination with co-immunoprecipitation experiments of the VWF D1-D3 region with lumenal P-selectin, this suggests that binding between these proteins is necessary for P-selectin recruitment to WPB [18]. VWD type 2N mutations found in this region that affect the binding of FVIII to VWF also render VWF ineffective to co-target FVIII to storage vesicles in AtT20 cells, as found by Rosenberg et al [19]. If this means that there is a single site (or a group of nearby sites) on the VWF molecule that is responsible for the binding and co-targeting of a number of seemingly unrelated proteins is still speculative. The presence of a single site on VWF for binding of WPB cargo could provide an explanation for the observation that angiopoietin-2 and P-selectin storage in the WPB are mutually exclusive [20]. On the other hand, VWF has various ligand binding sites that could equally well promote simultaneous recruitment of multiple secretory proteins to WPBs.

Regulation of WPB exocytosis

In chapters 3 and 4, we have unraveled signaling cascades that regulate exocytosis of WPBs in response to endothelial stimulation with Ca^{2+}- and cAMP-mediated agonists. As was already earlier appreciated, exocytosis of WPBs through both Ca^{2+}- and cAMP-mediated pathways involves the activation of the small GTPase RalA [21,22]. The activation state of a GTPase is regulated by the action of guanine exchange factors (GEFs) and GTPase activating proteins (GAPs), hence for RalA activation dependent exocytosis of WPBs it is not unexpected that a GEF would catalyze

this reaction. Studies by Birch et al. and van den Eijnden-Schrauwen et al. [23,24] already implicated the calcium sensor calmodulin (CaM) in thrombin-induced VWF secretion, which nicely complements the finding that inhibition of CaM abrogated thrombin-induced RalA activation in endothelial cells [21]. RalA is known to contain a binding site for CaM in its caboxy-terminal tail, and association with CaM is able to increase GTP-loading of RalA about 3-fold *in vitro* [25,26]. A cell-permeabele TAT-peptide that includes this region was indeed able to inhibit thrombin-induced WPB exocytosis [22], supporting a scenario where interaction of CaM with RalA would be sufficient to trigger WPB exocytosis. However thrombin-induced RalA activation was not affected by the cell-permeable peptide corresponding to the CaM-binding domain of RalA (unpublished findings), while also cAMP-mediated WPB exocytosis was inhibited by this peptide. This makes it more likely that the carboxy-terminal domain of RalA harbors additional properties that are crucial for WPB exocytosis independent of its binding to CaM. These observations suggest that the interaction of RalA with CaM is at most secondary in the activation of RalA. This is supported by the notion that stimulus-induced RalA activation leads to a far more pronounced activation of RalA that cannot be explained solely by the interaction with CaM. More importantly, we have now identified a Ral-specific guanine exchange factor that is responsible for stimulus-induced activation of RalA and that by virtue of its interaction with CaM integrates the calcium sensing property of CaM resulting in a rapid activation of Ral following stimulation of endothelial cells with Ca^{2+}-raising agonists. This is based on our finding that a RalGDS variant lacking its catalytic domain acts as a dominant negative mutant in stimulus-induced WPB exocytosis, and more compelling, that endothelial cells deficient in RalGDS are unable to activate RalA and release WPBs when activated by thrombin. Analogous to our findings, fibroblasts from genetically altered mice that are deficient for RalGDS indeed are unable to activate RalA when stimulated with PDGF [27]. We have shown that RalGDS can interact with CaM through an N-terminal putative IQ-like CaM-binding site and that abrogation of this interaction inhibits Ca^{2+}-mediated but not cAMP-mediated (unpublished observations) WPB exocytosis and inhibits thrombin-induced RalA activation. Our data indicate that this interaction provides the connection between calcium release and RalA activation. Our data are also in line with a more generally proposed model where the N-terminal domain of RalGDS or other calcium activated guanine exchange factors, such as Sos, Ras-GRF, Ras-GRF2 and $CDC25^{Mm}$ [28-32], act as an auto-inhibitory moiety that prevents exposure of the catalytic domain of the exchange factor. Upon binding of an upstream factor to this moiety, i.e. CaM, a conformational change takes place that fully exposes the CDC25 domain, thereby unleashing the activity of RalGDS resulting in a rapid activation of Ral. When overexpressed in endothelial cells, RalGDS is found primarily on the cellular membrane. Earlier studies and our unpublished findings have indicated that localization of RalGDS depends on its Ras binding domain (RBD), a carboxyterminal region that binds to activated Ras GTPases. Association of membrane-tethered active Ras to the RBD activates the

RalGDS-Ral pathway by translocation of RalGDS to the membrane and subsequent activation of membrane localized Ral [33-35], leading to potentiation of oncogenic transformation [36,37]. Apart from active Ras the RBD of RalGDS can also interact with the small GTPase Rap1. We and others have shown that thrombin induces the activation of Rap1 in endothelial cells which has been proposed to negatively regulate RhoA induced cytoskeletal reorganizations that promote loss of endothelial barrier function [38]. Interestingly, thrombin-induced Rap1 activation peaks between 2 and 10 minutes following stimulation and then returns to basal levels. Similar kinetics have been described for the activation of the small GTPase Ral in endothelial cells in response to thrombin [21]. In platelets similar patterns of Ral and Rap1 activation were observed in response to i.e. thrombin, while the correlation between the activation of Ral and Ras was less obvious [39]. This would suggest that activation of Rap1 rather than Ras would promote translocation of RalGDS to the cellular membrane where its substrate Ral resides on pre-docked WPBs. Indeed, Rap1 binding to RalGDS has been shown to enhance Ral activation in reconstituted liposomes [33], but so far cellular evidence does not point to a role for Rap1 in activation of the Ral pathway [33-35] except for the Rap-Rgl-Ral pathway reported in *D. melanogaster* [40]. In this regard, it is interesting to note that the synthetic Epac-specific cAMP analog, 8-pCPT-2'-O-Me-cAMP (007) can potently activate Rap1 in endothelial cells (chapter 4), but does not result in the activation of RalA [22]. As also shown in chapter 4, counteracting the activation of Rap1 using overexpression of Rap1GAP did not reduce thrombin-induced WPB exocytosis. This can either mean that Rap1 is not responsible for recruitment of RalGDS to the cellular membrane or that recruitment to the cellular membrane is not critical for stimulus-induced activation of RalA and subsequent WPB exocytosis. Taken together, the findings reported in chapter 3 support a model for the RalGDS-mediated activation of RalA and regulation of WPB release in which Ca^{2+}/CaM contributes to the increase in activation potential of RalGDS while the RBD-dependent translocation ensures the effective utilization of the exchange activity by bringing exchange factor and substrate together (see Figure 1).

As noted above, cAMP-mediated WPB exocytosis also involves the activation of RalA. Previous work from our lab has indicated that cAMP-activated protein kinase A (PKA) acts upstream of Ral in the regulation of WPB exocytosis, as inhibition of PKA lead to reduced VWF secretion in epinephrine-stimulated endothelial cells, while completely abrogating the activation of RalA. In the light of the findings from chapter 3, the question arises if RalGDS also regulates cAMP-mediated WPB release through activation of RalA, and thereby forms the link between PKA and Ral activation. Indeed, a number of guanine exchange factors have been described of which the activity is regulated by phosphorylation by protein kinases, positively [41-45] as well as negatively [46]. It has been shown that RalGDS can be phosphorylated and that it contains several consensus PKA phosphorylation sites [47]. *In vitro* however, phosphorylation did not influence the activity of RalGDS towards its substrate [47]. *In vivo*, phosphorylation of RalGDS by PKC even inhibited

activation of Ral [48]. We failed to detect any consistent effects on cAMP-mediated WPB exocytosis or RalA activation in the absence of RalGDS, which leaves the exact mechanism by which PKA can promote RalA activation open for further study.

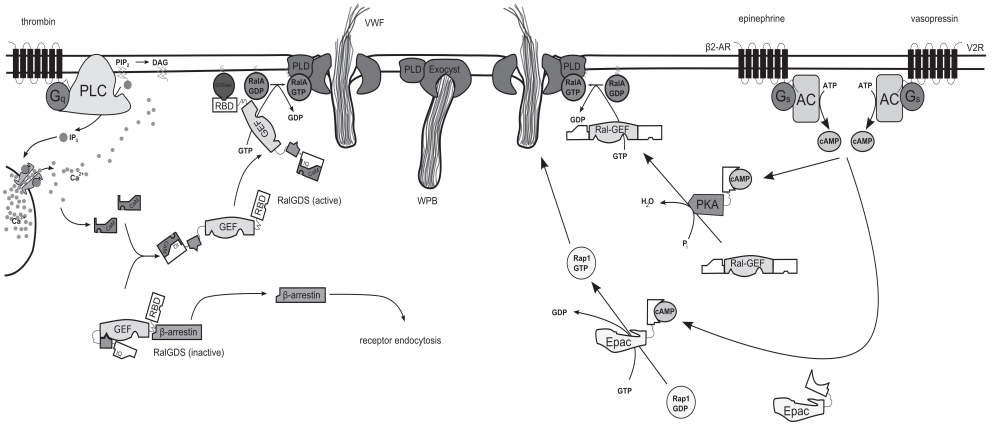

Figure 1: Proposed model for signaling pathways in Ca^{2+}- and cAMP-mediated WPB exocytosis. (Left) Endothelial stimulation by thrombin leads to hydrolysis of PIP_2 into IP_3 and DAG through the action of G_q-coupled phospholipase C (PLC). In turn, IP_3 releases Ca^{2+} from internal stores which readily associates with calmodulin (CaM). By interaction with an aminoterminal IQ-motif on RalGDS, the Ca^{2+}/CaM complex induces a conformational change in RalGDS, which exposes the catalytic GEF domain of RalGDS. Simultaneous RBD-dependent translocation to the plasma membrane brings RalGDS and RalA together, leading to the effective activation of RalA and promotion of WPB exocytosis. (Right) Binding of epinephrine or vasopressin to the β2-adrenergic receptor (β2-AR) or vasopressin-2 receptor (V2R), respectively, activates G_s-coupled adenylate cyclase (AC), leading to production of cAMP. cAMP-activated Epac catalyzes the activation of Rap1, which promotes WPB exocytosis through a currently unidentified mechanism. Simultaneous activation of PKA by cAMP leads to the activation of RalA, problaby by phosphorylation-dependent activation of a currently unidentified Ral-GEF. Cooperatively, Rap1-GTP and RalA-GTP promote WPB exocytosis. For further details and abbreviations see text.

Besides PKA another class of proteins, that of the cAMP guanine-nucleotide exchange factors (cAMPGEFs), commonly known as Epac proteins, are able to mediate cellular cAMP responses [49,50]. Of the 2 Epac variants, Epac1 and Epac2, only Epac1 is found in endothelial cells. In endothelial cells Epac1 has been shown to function in regulation of endothelial barrier function in response to elevation of intracellular cAMP [38,51-53]. As described earlier in this chapter, epinephrine-stimulated WPB exocytosis is mediated by the PKA-dependent activation of RalA. However some effects of cAMP-mediated signaling could not be attributed to PKA: elevated concentrations of the broad PKA inhibitor H-89 were needed to completely inhibit epinephrine-induced VWF secretion. Second, a synthetic cAMP analog that selectively activates Epac, 8-pCPT-2'-O-Me-cAMP (007), was able to induce WPB exocytosis in a RalA-independent manner. This prompted us to reinvestigate cAMP-mediated WPB exocytosis for the possible involvement of Epac1. Silencing of

Epac1 expression by RNA interference inhibited both epinephrine-induced WPB exocytosis as well as epinephrine-induced activation of Rap1. In support of a role for Rap1 in cAMP-mediated WPB exocytosis, we found that expression of Rap1GAP abolished epinephrine-induced WPB exocytosis. Based on these findings we propose a model in which β2-adrenergic and vasopressin-2 receptor stimulated WPB exocytosis is controlled by PKA-dependent and PKA-independent signaling pathways that are activated by elevated levels of cAMP (see Figure 1).

The mechanism by which Rap1 is able to promote exocytosis is still enigmatic. Presently, two mechanism have been described that potentially could explain the involvement of active Rap1 in exocytosis of WPBs. First, in platelets Rap1B has been reported to interact with sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA3B), a Ca^{2+}-channel on the endoplasmatic reticulum [54]. Resulting from this interaction, Rap1 may mobilize calcium from internal stores through calcium induced calcium release. Activation of Epac1 in pancreatic β-cells has reportedly lead to calcium spiking that was accompanied by release of serotonin from large dense core secretory vesicles [55]. Epac activated Rap2B has also been reported to stimulate the activity of phospholipase C ε (PLCε), generating the second messenger IP_3 which in turn can mobilize Ca^{2+} from internal calcium stores [56]. Our (unpublished) findings have indicated that 007-induced VWF secretion is sensitive to the calcium chelator BAPTA-AM, leading to a reduction in VWF secretion. This effect is downstream of the activation of Rap1, since activation of Rap1 was unaffected by BAPTA-AM treatment (unpublished observations). This points to a potential role for calcium in cAMP-mediated WPB exocytosis distal from the activation of Rap1. However, in accordance with work from Vischer and coworkers [57], we were unable to detect a rise in free cytosolic Ca^{2+} in 007- or epinehrine-stimulated endthelial cells.

Second, Maillet et al have found that Epac1-activated Rap1 regulates secretion of amyloid precursor protein, a key protein in Alzheimer's disease, by promoting the activation of the Rho-like GTPase Rac [58]. Rho-like GTPases are involved in establishment of cell polarity and regulate cytoskeletal rearrangments. Possibly a putative Epac-Rap1-Rac pathway is necessary for local changes to the cytoskeleton and cellular membrane to facilitate exocytosis of secretory granules. Complementary to such a mechanism is the finding that Rac1 regulates WPB exocytosis in aortic endothelial cells [59]. At present, Rap1's precise function in WPB exocytosis remains unclear.

KLF2

Krüppel-like factor 2 (KLF2) is a mechano-sensitive zinc finger transcription factor found in endothelial cells that has recently been shown to translate shear stress into activation or suppression of expression of its downstream targets. Prolonged exposure of endothelial cells to steady laminar shear stress induces the expression of KLF2 [60,61]. Expression of KLF2 within the vasculature seems to correlate with the resistance to atherosclerosis: steady laminar flow such as occurs at vascular

sites that are known to be relatively protected against atherosclerosis, induces the expression of KLF2, while endothelial cells situated in the vicinity of aortic bifurcations, areas that are prone to develop atherosclerotic lesions, are lacking in KLF2 expression [60,62]. Upregulation of KLF2 expression results in an expression profile that is anti-inflammatory, anti-thrombotic, vasodilating and promotes endothelial quiescence [63]. Further evidence that KLF2 has a pivotal role in imposing an atheroprotective phenotype on the vascular bed is provided by the beneficial effects of lipid-lowering 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, commonly known as statins, which are widely used to prevent cardiovascular disease. Independent from their cholesterol lowering capabilities, statins decrease vascular inflammation and improve endothelial function through changes in vascular gene expression, in a KLF2 dependent manner [64-67]. In chapter 5, we have found that endothelial cells that overexpress KLF2 do no longer cluster their WPBs at the microtubule organizing centre (MTOC) in response to a cAMP raising agonists. This observation indicates that, as part of the vasoprotective phenotype it imposes, KLF2 inhibits the perinuclear clustering of WPBs. Moreover, it shows that retrograde transport mechanisms that are responsible for clustering of WPBs at the MTOC are regulated by downstream targets of KLF2. Recent findings also place statins and the KLF2 pathway into the regulation of WPB exocytosis. KLF2 positively regulates the expression of endothelial nitric oxide synthase (eNOS). Apart from its vasodilatory action, nitric oxide (NO) has been shown to decrease platelet adherence and inhibit vascular inflammation [68]. In an attempt to explain part of the beneficial effects of statins and NO as a result of impeded WPB exocytosis, Matsushita and coworkers showed that S-nitrosylation of N-ethylmaleimide-sensitive factor (NSF) by NO prevents disassembly of the SNARE-complex [69]. The SNARE complex operates at the final stage of exocytosis, by catalyzing the fusion of vesicle and plasma membrane. Most probably, blocking the disassembly of the SNAREs after fusion prevents the recycling of the SNARE-complex leading to failure to facilitate subsequent exocytotic events. In support of this theory, WPB exocytosis was impaired in endothelial cells when treated with an NO donor. Complementary to this finding, Simvastatin was also found to inhibit WPB exocytosis in an eNOS-dependent manner [70]. However, some controversy still exists with respect to this issue, as other reports were unable to confirm eNOS involvement in the regulation of WPB exocytosis [71,72]. Moreover, overexpression of KLF2 in endothelial cells did not decrease the number of WPBs released upon stimulation with thrombin or forskolin [73], arguing against a scenario in which KLF2-mediated upregulation of eNOS by statins would interfere with vesicle exocytosis.

Future directions

From a quantitative point of view, we determined the sorting of IL-8 to the WPB as very efficient and our findings suggest that the amount of IL-8 that can be stored in and released from WPBs is sufficient to support a role in vascular homeostasis. The reality is that, up to this point, no *in vivo* evidence exists that underscores the physiological significance of IL-8 storage. More careful inspection of patient groups or model systems with a disturbed ability to produce or release WPBs (VWD type 3, VWD dogs, etc), with a focus on (subtle) phenotypes that could be associated with a compromised ability to present IL-8 through regulated release, may provide insight into the physiological relevance of storage of IL-8 in WPBs. We have determined that the capability of IL-8 to sort to WPBs relies on its association with VWF, however the regions on either molecule that take part in this interaction have not been defined. Possibly, insight into the molecular determinants that allow a protein to associate with VWF to facilitate its sorting to the WPB may provide a means to target heterologously expressed proteins to the regulated secretory pathway of the endothelium, e.g. in the case of gene therapy.

Endothelial cells contain subpopulations of WPBs in terms of age or cargo. We hypothesize that these subsets of WPBs behave differently with respect to intracellular localisation, vesicle dynamics in resting cells, secretion competence after stimulation with different agonists and the previously observed perinuclear clustering phenomenon of WPB after stimulation with cAMP-raising agents. It will be of interest to clarify the mechanisms that enable the endothelial cells to differentially regulate its WPBs.

Our studies on signaling cascades that regulate WPB exocytosis have defined a role for RalGDS and Epac1. These findings have raised a number of new issues. At present, the mechanism by which PKA can promote the activation of RalA has not been determined. Also, it is not clear how the small GTPase Rap1 promotes exocytosis of WPBs.

The current list of known WPB components is the resultant of serendipity and (well)-educated guesses; a systematic approach to explore the complete inventory of this unique organelle has yet to be performed. Apart from additional cargo molecules that may provide presently unappreciated connections of WPB release to other physiological functions, knowledge of which molecules (transiently) associate with this vesicle may provide invaluable clues on the processes that regulate formation, trafficking and release of this versatile storage compartment within endothelial cells.

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Summary

Summary

Vascular endothelial cells form the inner lining of the vessel wall and thereby help maintaining the structural integrity of the blood vessel. In addition, the endothelium provides a surface for and actively participates in the mediation of inflammatory responses and in the arrest of bleeding in the event of vascular damage. Endothelial cells also take part in these processes by releasing the content of unique, cigar-shaped organelles called Weibel-Palade bodies (WPBs). These organelles function as storage vesicles for von Willebrand factor (VWF), a multimeric adhesive glycoprotein crucial for platelet plug formation, the leukocyte receptor P-selectin and a number of bioactive compounds that include the chemokines interleukin-8 (IL-8) and eotaxin-3. To adequately respond to events like vascular trauma, endothelial cells are equipped with a complex signaling machinery that can regulate exocytosis of WPBs in response to cellular activation. The studies presented in this thesis were performed to gain more insight in the two processes that are fundamental for the endothelial cell's ability to participate in vascular homeostasis, namely sorting of secretory proteins to the WPBs and regulation of WPB release in response to vascular challenge.

Chapter 1 presents an overview of the current knowledge on the formation of WPBs and gives an inventory and functional description of the WPB constituents. Furthermore the regulatory mechanisms that control exocytosis of WPBs are described.

The WPB provides the storage compartment for a number of secretory proteins, however not much is known about the significance of storage of some of these proteins in terms of efficiency with which they can enter this granule. Therefore we initiated a quantitative analysis of the entry of IL-8 into the storage pathway in the endothelium, which is described in **chapter 2**. Pulse-chase analysis of IL-8 sorting in endothelial cells indicated that only a minor fraction of the *de novo* synthesized IL-8 is able to enter the storage compartment of the endothelium, while the vast majority leaves the cell through constitutive secretion. However, from density gradient subcellular fractionation we found that the amounts of IL-8 that is stored by endothelial cells is limited by the amount of VWF that enters the WPBs, since the amounts of IL-8 and VWF found in the dense fractions of endothelial cells or that were released upon cellular stimulation were virtually equimolar. In support of this we observed that IL-8 and VWF interact under conditions that prevail in the acidic environment of the late secretory pathway. These data suggest that VWF controls the sorting efficiency of IL-8 and other WPB constituents by acting as a cargo receptor.

Exocytosis of WPBs depends on activation of the small GTPase RalA. In **chapter 3** we have studied the regulatory networks that control WPB exocytosis and RalA activation in response to Ca^{2+}-mediated agonists in more detail. We have identified a crucial role for the Ral-specific guanine exchange factor RalGDS in thrombin-induced WPB exocytosis. Downregulation of RalGDS expression using RNA interference inhibited thrombin-induced WPB release and thrombin-induced

activation of RalA. In line with this, overexpression of RalGDS leads to unprovoked release of WPBs, which was accompanied by a marked increase of basal RalA activation. Overexpression of a mutant form of RalGDS that has lost its ability to catalyze the activation of RalA thereby behaving in a dominant negative manner, inhibited the stimulus-induced exocytosis of WPBs. Thrombin-induced WPB exocytosis is mediated by the second messenger Ca^{2+}, which upon release from intracellular stores readily associates with the calcium sensor calmodulin (CaM). In this chapter we also provide evidence for the presence of a putative CaM binding site in the amino-terminal part of RalGDS. Disruption of the CaM – RalGDS interaction by the use of a cell-permeable peptide comprising this region leads to the reduction of thrombin-induced RalA activation and inhbits WPB exocytosis. Together these findings indicate that RalGDS is critically involved in the regulation of Ca^{2+}-mediated WPB exocytosis through the activation of RalA.

Another class of secretagogues, that includes epinephrine and vasopressin, is able to induce WPB exocytosis by raising intracellular levels of the second messenger cAMP. Previously we have found that cAMP-mediated WPB exocytosis partly depends on the action of the cAMP-activated protein kinase A (PKA), leading to activation of RalA. Cellular cAMP responses can also be mediated by PKA-independent signaling pathways, such as through the exchange protein activated by cAMP, Epac. In **chapter 4** we have explored whether Epac is also involved in the regulation of cAMP-mediated WPB exocytosis. Downregulation of Epac1 expression by RNAi effectively inhibited the release of WPBs in response to epinephrine. Epac is a guanine exchange factor for the small GTPase Rap1. We show that thrombin- as well as epinephrine-induced WPB exocytosis is accompanied by the activation of Rap1. Downregulation of Epac1 abrogated epinephrine-induced Rap1 activation, whereas thrombin-induced Rap1-activation was unaffected, which indicates that Rap1 activation in response to cAMP-mediated agonists is mediated by Epac1. Furthermore it was found that downregulation of active Rap1 by overexpression of Rap1GAP, a GTPase activating protein specific for Rap1, results in the inhibition of epinephrine-induced WPB exocytosis. These data show that Epac1 mediates exocytosis of WPBs through the activation of Rap1.

Stimulation of endothelial cells with agonist that raise intracellular cAMP-levels also leads to retrograde transport of WPBs to a perinuclear region around the microtubule organizing centre (MTOC). Previously, it was found that this perinuclear clustering of WPBs is mediated by the dynein/dynactin motor protein complex and depends on phosphorylation of an as yet unidentified component by PKA. In **chapter 5** we have studied the clustering of WPBs in more detail. We found that endothelial cells that overexpress the mechano-sensitive transcription factor KLF2 display reduced clustering of WPBs in response to cAMP-mediated agonists. This suggests that downstream targets of KLF2 are involved in regulatory mechanisms that control retrograde transport of WPBs. Finally, in **chapter 6**, the findings presented in the different chapters are discussed with reference to data collected by other investigators.

Samenvattting

Samenvatting

Vaatwandendotheelcellen vormen de binnenbekleding van de vaatwand en zorgen daar onder andere voor de structurele integriteit van het bloedvat. Daarnaast neemt het endotheel actief deel in het mediëren van ontstekingsreacties en het bloedstelpingsproces in het geval van vasculaire schade. Endotheelcellen participeren in deze processen door de inhoud van hun unieke, sigaarvormige organellen, genaamd Weibel-Palade bodies (WPBs), uit te scheiden. Deze organellen dienen als opslag granules voor von Willebrand factor (VWF), een multimeer adhesief glycoproteïne dat een cruciale rol vervult bij de vorming van een bloedplaatjes prop, de leukocyten receptor P-selectin en een aantal bioactieve stoffen waaronder de chemokines interleukin-8 (IL-8) en eotaxin-3. Om adequaat te kunnen reageren in het geval van vaatwand schade zijn endotheelcellen uitgerust met een complex regulatie mechanisme dat de exocytose van WPBs controleert in het geval van cellulaire activatie. Het onderzoek dat in dit proefschrift is beschreven is gericht op het verkrijgen van meer inzicht in de twee processen die van essentieel belang zijn voor de endotheelcel om te kunnen participeren in de instandhouding van vasculaire homeostase, namelijk de sortering van secretie eiwitten naar de WPB en de regulering van WPB exocytose in het geval van vasculaire activatie.

Hoofdstuk 1 geeft een overzicht van de huidige stand van zaken wat betreft de vorming en inhoud van WPBs en gaat dieper in op de functionele eigenschappen van de WPB constituenten. Verder worden de regulatoire mechanismen die betrokken zijn bij de controle van WPB exocytose beschreven.

De WPB is een opslag compartiment voor een aantal secretie eiwitten, echter, voor sommige van deze eiwitten is er niet veel bekend omtrent de kwantitatieve en fysiologische significantie van hun opslag. Daarom hebben we in **hoofdstuk 2** een kwantitatieve analyse uitgevoerd van de opslag van IL-8 in het opslag compartiment van de endotheelcel. Pulse-chase analyse van IL-8 sortering in de endotheel cel gaf aan dat slechts een klein deel van het nieuw aangemaakte IL-8 zijn weg vindt naar de WPB, terwijl de bulk aan IL-8 constitutief uitgescheiden de cel verlaat. Echter, met behulp van subcellulaire fractioneringen vonden we dat de hoeveelheid IL-8 dat wordt opgeslagen door endotheelcellen wordt gelimiteerd door de hoeveelheid VWF die wordt opgeslagen in WPBs: de molaire hoeveelheden IL-8 en VWF die werden aangetroffen in dichte fracties van endotheelcellen of die werden uitgescheiden na endotheelcel activatie met behulp van thrombine zijn nagenoeg equimolair. Bovendien vonden we dat IL-8 en VWF een interactie met elkaar aangaan in de zure condities zoals ze zich voordoen in de secretie machinerie en de juist vormende WPBs. Deze bevindingen suggereren dat VWF de opslag efficientie van IL-8 en mogelijk andere WPB constituenten controleert door een rol te vervullen als cargo-receptor.

Exocytose van WPBs hangt af van de activatie van het kleine GTPase RalA. In **hoofdstuk 3** hebben we het regulatoire netwerk dat WPBs exocytose en RalA activatie in het geval van stimulatie

met Ca^{2+}-gemedieerde agonisten controleert gedetaileerd bestudeerd. Daarbij vonden we dat de Ral-specifieke guanine exchange factor RalGDS een cruciale rol vervult in de regulatie van thrombine-geïnduceerde WPB secretie. Verlaging van de expressie van RalGDS met behulp van RNA interferentie (RNAi) verstoorde zowel thrombine-geïnduceerde WPB exocytose als thrombine-geïnduceerde RalA activatie. Complementair hieraan vonden we dat overexpressie van RalGDS leidt tot ongestimuleerde secretie van WPBs, wat tevens vergezeld gaat met een sterke verhoging van de basale hoeveelheid actief RalA in een rustende cel. Een mutante RalGDS variant die, door het ontbreken van een katalytisch domein, niet langer in staat is om RalA te activeren, gedroeg zich bij overexpressie als een dominant negatieve mutant door stimulus-geïnduceerde WPB exocytose te inhiberen. Thrombine-geïnduceerde WPB exocytose wordt gemedieerd door de second messenger Ca^{2+}, wat, nadat het uit intracellulaire opslagplaatsen wordt losgelaten, onmiddelijk associeert met de calcium sensor calmoduline (CaM). In dit hoofdstuk tonen we aan dat RalGDS een mogelijke CaM bindingsplaats bevat in the aminoterminale deel van het eiwit. Verstoring van interactie tussen CaM en RalGDS met behulp van een celpermeabel peptide leidde tot een reductie in de activering van RalA na thrombine stimulatie en verhinderde bovendien de thrombine-geïnduceerde WPB exocytose. Deze resultaten te samen geven aan dat RalGDS een cruciale rol vervult in de exocytose van WPBs en de activatering van RalA.

Een andere groep van stimuli, waaronder epinefrine en vasopressine, induceren WPB exocytose door de intracellulaire concentratie cyclisch AMP te verhogen. Eerder onderzoek heeft uitgewezen dat cAMP-gemedieerde WPB exocytose afhankelijk is van cAMP-geactiveerd protein kinase A (PKA), wat leidt tot activatie van RalA. Cellulaire cAMP effecten kunnen ook gemedieerd worden via PKA-onafhankelijke routes, zoals bijvoorbeeld via de exchange protein activated by cAMP, kortweg Epac. In **hoofdstuk 4** hebben we onderzocht of Epac ook betrokken is bij de regulatie van cAMP-gemedieerde WPB exocytose. Sterke verlaging van de expressie van Epac1 met behulp van RNAi resulteerde in een inhibitie van WPB secretie na prikkeling met epinephrine. Epac is een guanine exchange factor voor het kleine GTPase Rap1. We laten tevens zien dat zowel thrombine- als epinefrine-geïnduceerde WPB exocytose worden vergezeld door activatie van Rap1. Na verlagen van Epac1 expressie wordt Rap1 niet meer geactiveerd na epinephrine stimulatie, terwijl thrombine-geïnduceerde Rap1 activatie niet wordt beïnvloed. Dit duidt erop dat activatie van Rap1 na stimulatie met cAMP-gemedieerde agonisten via Epac1 verloopt. Verder vonden we dat overexpressie van Rap1GAP, een GTPase activerend eiwit specifiek voor Rap1, epinefrine-geinduceerde WPB excoytose tegengaat. Dit toont aan dat Epac1 WPB secretie reguleert via de activatie van Rap1.

Stimulatie van endotheelcellen met agonisten die de intracellulaire cAMP niveau's verhogen leiden ook tot een terugwaarts transport van WPBs naar een perinuclaire regio rondom het microtubule organizing centre (MTOC). Eerder onderzoek had uitgewezen dat deze perinuclaire clustering van

WPBs wordt gemedieerd door het dynein/dynactin motor eiwit complex en afhankelijk is van fosforylatie van een tot nu toe onbekende component door PKA. In hoofdstuk 5 hebben we de clustering van WPBs opnieuw bestudeerd. We vonden dat endotheelcellen die de mechanosensitieve transcriptie factor KLF2 tot expressie brengen hun WPBs in mindere mate clusteren bij stimulatie met cAMP-gemedieerde agonisten. Dit suggereert dat een of meerdere van de eiwitten waarvan de expressie wordt gereguleerd door KLF2 betrokken zijn bij de regulatie van terugwaarts transport van WPBs.

Tenslotte worden in **hoofdstuk 6** de resultaten uit de voorgaande hoofdstukken bediscussieerd in combinatie met bevindingen van andere onderzoekers.