VAMP3 is associated with endothelial Weibel–Palade bodies and participates in their Ca^{2+}-dependent exocytosis ☆

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ABSTRACT

Weibel–Palade bodies (WPBs) are secretory organelles of endothelial cells that store the thrombogenic glycoprotein von Willebrand factor (vWF). Endothelial activation, e.g. by histamine and thrombin, triggers the Ca^{2+}-dependent exocytosis of WPB that releases vWF into the vasculature and thereby initiates platelet capture and thrombus formation. Towards understanding the molecular mechanisms underlying this regulated WPB exocytosis, we here identify components of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) machinery associated with WPB. We show that vesicle-associated membrane protein (VAMP) 3 and VAMP8 are present on WPB and that VAMP3, but not VAMP8 forms a stable complex with syntaxin 4 and SNAP23, two plasma membrane-associated SNAREs in endothelial cells. By introducing mutant SNARE proteins into permeabilized endothelial cells we also show that soluble VAMP3 but not VAMP8 mutants comprising the cytoplasmic domain interfere with efficient vWF secretion. This indicates that endothelial cells specifically select VAMP 3 over VAMP8 to cooperate with syntaxin 4 and SNAP23 in the Ca^{2+}-triggered fusion of WPB with the plasma membrane. This article is part of a Special Issue entitled: 11th European Symposium on Calcium.

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1. Introduction

The endothelium is a highly dynamic cell layer capable of responding to environmental signals by presenting either an anti-coagulant surface supporting unrestricted blood flow (normal condition) or a pro-thrombotic one that is capable of attracting leukocytes and platelets (activated condition). Hence, endothelial cells not only provide a physical barrier ensuring tightness of the vessel system but can also be considered gatekeepers of vascular homeostasis. They achieve this by sensing vascular perturbation and secreting in a tightly controlled manner soluble factors and membrane-associated proteins that regulate blood clotting, fibrinolysis and local inflammatory responses (for review see [1,2]). These factors are stored in different types of endothelial granules that are capable of undergoing stimulus-induced secretion. The most prominent of these secretory granules are the Weibel–Palade bodies (WPBs), large, cigar-shaped organelles that store the thrombogenic von Willebrand factor (vWF) and the leukocyte receptor P-selectin [3]. WPBs are formed

through a complex maturation process that is dictated by the maturation of vWF. Following initial emergence at the trans-Golgi network (TGN), WPBs acquire additional components from the endosomal system as well as cytoplasmically associated proteins such as the small GTPase Rab27a, thus sharing some characteristics with lysosome-related organelles such as pigment-storing melanosomes. Maturation of WPB is also accompanied by a movement of the organelles from the perinuclear region to more peripheral locations (for reviews see [4–6]).

To properly function in the control of thrombosis and the capture of platelets and leukocytes, the WPB constituents have to be released on demand. This is ensured by regulated exocytosis of WPB that can be triggered by a rise in intracellular Ca^{2+} or cAMP levels (for reviews see [7,8]). Typical secretagogues initiating the secretion of vWF are histamine and thrombin that act by elevating intraendothelial Ca^{2+}. Despite the prominent role of WPB constituents in the initiation of vascular thrombosis and leukocyte adherence comparatively little is known about the molecular machinery driving their regulated exocytosis. Several small GTPases have been implicated in the Ca^{2+}-evoked secretion of WPB. While Rab3D most likely functions in the maturation of the organelle [9], Rab27a participates in a peripheral storage of WPB acting as a negative regulator of secretion that prevents premature release [10]. Serving positive regulatory functions, RalA and its exchange factor RalGDS are required for efficient thrombin, i.e. Ca^{2+}-dependent, as well as epinephrin, i.e. cAMP-dependent, secretion of WPB [11–13]. Alpha-synuclein appears to

counteract RalA activity thus acting as a negative regulator [14]. Components of the cytoskeleton and cytoskeleton-regulating systems have also been reported to participate in regulating the motility and/or secretion of WPB, including the dynein–dynactin complex, the microfilament system and its regulator RhoA [4,15,16]. Furthermore, it has been shown that the formation of secretion-competent WPB requires the adaptor protein-1 (AP1) and its effectors clathrin, aftiphilin and gamma-synergin [17,18] and that protein phosphatase 2B appears to act as a negative regulator of WPB exocytosis since its inhibition triggers vWF release [19].

While many of the factors listed above affect the maturation and intracellular positioning/motility of WPB, little is known about the endothelial components acting at the actual site of fusion between WPB and the plasma membrane. This process appears to require specific changes in the plasma membrane phospholipid composition, specifically a phospholipase D1-mediated increase in phosphatidic acid (PA), and the action of the annexin A2-S100A10 complex that is capable of interacting with PA [9,20]. Finally, it has been shown that N-ethylmaleimide-sensitive factor (NSF) and proteins of the soluble NSF attachment protein receptor (SNARE) family participate in acute vWF release since NO-mediated nitrosylation of NSF inhibits the secretion of vWF [21]. Following up on these results we here set out to identify the SNARE complex acting in the Ca^{2+}-dependent exocytosis of WPB in human endothelial cells. We show that two v-SNAREs, VAMP3 and VAMP8, are present on WPB and that only VAMP3 forms a trans-SNARE complex with syntaxin 4 (Syx4) and SNAP23 and appears to be functionally required for WPB secretion.

2. Materials and methods

2.1. Antibodies and plasmids

WPBs were stained with mouse monoclonal (clone F8/86) and rabbit polyclonal anti-human vWF-antibodies (Dako, Glostrup, Denmark). A rabbit anti-human vWF peroxidase conjugate (Dako) was applied for the quantification of secreted vWF. Rabbit polyclonal antibodies against human Syx4, SNAP23, VAMP3 and VAMP8 were obtained from Synaptic Systems (Göttingen, Germany). Mouse monoclonal anti-human Syx4 (clone 49) antibodies were from BD (Franklin Lakes, NJ) and mouse monoclonal antibodies against human vimentin from Dianova (Hamburg, Germany). Secondary anti-mouse and anti-rabbit antibodies coupled to Texas Red, Cy2 or FITC were purchased from Dianova and peroxidase conjugated secondary anti-mouse and anti-rabbit antibodies were from Dako.

The coding sequences of human VAMP3 and VAMP8 were cloned into pEGFP-N1 to generate plasmids encoding GFP-VAMP3 and GFP-VAMP8. For bacterial expression, the cytoplasmic domains of human VAMP3 (aa 1–78) and VAMP8 (aa 1–74) were subcloned into pGEX-4T1 (GE Healthcare, Chalfont St. Giles, UK). The eukaryotic expression vector encoding tetanus toxin light chain was kindly provided by Thomas Binz (Hannover Medical School) [22].

2.2. Cell culture, transfection and RNAi

Primary cultures of human umbilical vein endothelial cells (HUVECs) were established from umbilical cords and cultivated as described [9,23]. Transfection of HUVECs was carried out as described previously [9]. Nearly confluent cells between passages 2 and 5 were used in the experiments.

siRNA-mediated silencing of SNARE proteins employed RNA-duplexes with dTdT 3′-overhang on both strands (Sigma-Aldrich, St. Louis, MO). The Syx4 targeting siRNA has been described previously [24]. RNAi-mediated silencing of VAMP3 and VAMP8 was carried out in each case by transfection of equimolar amounts of two different targeting oligos. The sequences used were as follows: SNAP23 (NM_003825.2, 367–385, 5′-CUUUGAGUCUGGCAAGGCU-3′),

VAMP3 #1 (NM_004781.3, 2169–2189, 5′-CACUGUAAUCAC-CUAAAUAAA-3′), VAMP3 #2 (NM_004781.3, 1462–1482, 5′-CCCAAAUAUGAAGAUAAACUA-3′), VAMP8 #1 (NM_003761.3, 386–404, 5′-CCUCUUCAUUGUGCUCUUU-3′) and VAMP8 #2 (NM_003761.3, 237–255, 5′-GAAAACUUGGAACAUCUCC-3′). Control experiments used non-targeting control siRNAs (siGenome non-targeting siRNA; Dharmacon, Chicago, IL). Cells were transfected with 200 pmol of targeting or control siRNA by nucleofection.

2.3. Cell lysate preparation

Cells were lysed in ice-cold lysis buffer (20 mM N-2-hydroxyethyl-piperazine–N′-2-ethanesulfonic acid (HEPES), pH 7.4, 150 mM NaCl, and 0.5% Triton X-100, with complete EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland)) by passage through a 23 gauge needle. Lysates were then cleared by centrifugation for 10 min at 1000 g. The corresponding supernatants were supplemented with SDS (sodium dodecyl sulfate) sample buffer (50 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02% bromophenol blue, supplemented with fresh 50 mM DTT), boiled for 5 min at 95 °C and subjected to analysis by SDS-PAGE and immunoblotting.

2.4. Confocal microscopy

For analyzing the distribution of SNAREs by immunofluorescence (IF), cells were fixed and permeabilized with 35% dimethyl sulfoxide (DMSO)/methanol for 2 min at 4 °C. Blocking of samples was carried out in 10% fetal calf serum (FCS) before incubation with primary and the respective secondary antibodies, diluted in 2% bovine serum albumin (BSA)/PBS. Mounted coverslips were imaged by confocal microscopy (LSM510; Zeiss, Jena, Germany).

2.5. Immunoprecipitation

In order to stabilize SNARE complexes, cells were treated for 15 min with 1 mM N-ethylmaleimide (NEM) in ice-cold PBS to inhibit NSF and thus prevent SNARE disassembly. Subsequently, cells were washed, and further incubated in PBS supplemented with 2 mM DTT for 15 min on ice. Cell extracts were prepared by scraping the 4 10^7 cells into lysis buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 0.2% NP-40, 1 mM EDTA, 1 mM EGTA, 1 mM $CaCl_2$, and 2 mM NEM, with complete EDTA-free protease inhibitor cocktail) and subsequent passage through a 23 gauge needle. The lysates were then cleared by centrifugation for 10 min at 1000 g and 4 °C. For immunoprecipitation (IP), a 700 μg cell extract was mixed with prewashed anti-mouse Dynabeads® (Invitrogen Dynal AS, Hamburg, Germany) coupled with 3 μg of the mouse monoclonal anti-human syntaxin 4 antibodies and incubated for 2 h at 4 °C. Immunocomplexes were washed five times with washing buffer (20 mM HEPES, pH 7.4, 300 mM NaCl, 0.2% NP-40, 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, and 1 mM $CaCl_2$) and analyzed by SDS-PAGE and immunoblotting.

2.6. Quantification of vWF secretion

HUVECs grown to confluency on collagen coated 24-well plates were starved overnight in basal medium (1% BSA/M199; PAA, Pasching, Austria). Regulated secretion was triggered by incubation with 20 μM histamine (Sigma-Aldrich) in basal medium for 20 min at 37 °C. Control experiments used secretagogue-free basal medium to measure the levels of constitutive secretion. Relative amounts of vWF in the cell culture supernatants were then determined by ELISA as described previously [20]. Potential differences in the releasable pool of vWF were prevented by using the same batch of HUVECs for each experiment and plating control and knockdown cells at similar confluencies. Results obtained with different batches of cells were

then compared with one another by calculating relative values for the stimulated secretion. Therefore, the constitutive secretion was set to 100%, and the values obtained with histamine-treated cells were then expressed as difference between constitutive and regulated secretion.

2.7. Recombinant expression and purification of dominant-negative VAMP3 and VAMP8

Escherichia coli (strain BL21 (DE3) pLysS) cultures were transformed with pGEX-4T1 constructs encoding GST or N-terminal GST-tagged cytoplasmic domains of VAMP3 or VAMP8 and grown to an A_{600} of 0.6. Recombinant protein expression was then induced by addition of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). After 3 h of cultivation, cells were harvested by centrifugation (5000 g for 10 min) and resuspended in lysis buffer (40 mM HEPES, pH 7.2, 300 mM NaCl, 10 mM β-mercaptoethanol, and 1.5 mM PMSF, with complete protease inhibitor cocktail). After lysing the cells by triple freeze/thaw cycles and sonication, the lysates were cleared by centrifugation for 1 h at 100,000 g. The respective supernatants were applied to a glutathione-Sepharose 4B (GE Healthcare) column equilibrated in PBS. After extensive washing with the same buffer, bound GST and GST-tagged VAMP3 and VAMP8 derivatives were eluted with 20 mM glutathione (AppliChem, Darmstadt, Germany) in 50 mM Tris–HCl, pH 8.0.

2.8. Ca^{2+}-evoked vWF secretion from streptolysin O-permeabilized HUVECs

On 24-well plate grown confluent HUVECs were starved overnight in basal medium and permeabilized as described [25] with some modifications. Cells were treated with 150 ng/ml streptolysin O (SLO) in EGTA-supplemented permeabilization buffer (20 mM HEPES, pH 7.2, 120 mM KCl, 20 mM potassium acetate, 4 mM $MgCl_2$, and 0.1% BSA) for 5 min on ice. Subsequently, unbound SLO was removed by washing with PBS without Ca^{2+}/Mg^{2+}, and 1 mM EGTA. The efficiency of the protocol was verified by analyzing the uptake of trypan blue and GST or GST-core SNARE fusion proteins into the permeabilized cells (not shown). Following permeabilization cells were incubated for 10 min at 37 °C with 30 μM of the purified VAMP3 or VAMP8 derivatives (rebuffered in permeabilization buffer with 1 mM EGTA). Regulated vWF secretion was induced by treating the permeabilized HUVECs for 20 min at 37 °C with Ca^{2+}-containing stimulation buffer (permeabilization buffer supplemented with 10 μM $CaCl_2$, 1 mM ATP, 3 mM creatine phosphate, and 1 μM creatine kinase). Control experiments used permeabilized cells incubated for 20 min with EGTA-containing permeabilization buffer to measure the levels of constitutive secretion. The relative amounts of secreted vWF in the cell culture supernatants were then quantified via the vWF-specific ELISA.

2.9. Statistical analysis

Graphs and associated statistics were generated with GraphPad Prism version 4.00 for Windows software (GraphPad Software, San Diego, CA) and values are given as mean ± s.e.m.. Significance of differences was calculated with Student's unpaired t test; ns, not significant; *P<0.05; **P<0.005; ***P<0.0005.

3. Results

3.1. A streptolysin O-permeabilized cell system to study endothelial cell secretion

To verify the involvement of NSF in regulated secretion in human umbilical vein endothelial cells (HUVECs) and to establish a permeabilized cell system for this secretory event that would allow the introduction of larger molecules into the cells, we made use of the

bacterial toxin streptolysin O (SLO). SLO had been used previously to generate permeabilized cells capable of mounting specific secretory responses (for reviews see [26,27]). HUVECs were permeabilized by activating membrane-bound SLO through warming up to 37 °C and exocytosis of WPB, which remained intact during this treatment, was triggered by incubating the SLO-treated cells in medium containing elevated Ca^{2+} (Fig. 1A). This protocol led to the Ca^{2+}-dependent release of vWF into the cell supernatant indicative of exocytotic fusion of WPB membranes with the plasma membrane (Fig. 1B). Next we introduced N-ethylmaleimide (NEM) known to inhibit NSF function into the permeabilized cells. Fig. 1B reveals that this treatment basically abolished the Ca^{2+}-triggered release of vWF from the permeabilized cells. Thus, at least in this minimal system WPB exocytosis is sensitive to NEM inhibition indicative of the involvement of NSF as observed previously in aortic endothelial cells [21].

3.2. WPB-associated SNARE proteins

Given an established function of NSF in Ca^{2+}-triggered WPB exocytosis (Fig. 1B and [21]) we next searched for endothelial SNARE proteins that could be involved in vWF secretion and thereby function as targets of NSF in the course of these events. We followed a candidate approach and probed immunoblots of HUVEC lysates with a panel of SNARE-specific antibodies. Fig. 2A shows that the t-SNAREs syntaxin 4 (Syx4) and SNAP23 as well as the v-SNAREs VAMP3 and VAMP8 are expressed in HUVECs. This differs to some extent from a previous report that revealed the presence of Syx4, SNAP23 and VAMP3 but failed to detect VAMP8 in endothelial cells from aortic origin [21]. We also carried out an immunofluorescence analysis that corroborated the plasma membrane localization of Syx4 and SNAP23. Interestingly, this analysis also showed that the two v-SNAREs identified in HUVECs, VAMP3 and VAMP8, are both predominantly found on WPB (Fig. 2B–D). The specificity of this observation was confirmed by recording the distribution of GFP-tagged VAMP3 and VAMP8 in live HUVECs, which again revealed their WPB association (not shown).

Given the specific WPB localization of VAMP3 and VAMP8 we next determined whether these proteins can engage in trans-SNARE complex formation with plasma membrane-resident Syx4 and/or SNAP23. Such complexes could represent substrates for the action of NSF and thus elements of the machinery mediating WPB exocytosis. Following NEM treatment to prevent NSF-mediated SNARE disassembly HUVECs were lysed and the lysates subjected to anti-Syx4 immunoprecipitation. Western blot analysis revealed the co-immunoprecipitation of SNAP23 and VAMP3 but not VAMP8 (Fig. 3). Thus, WPB-associated VAMP3, but not VAMP8, can engage in complexes with plasma membrane-resident Syx4 and SNAP23 identifying this SNARE complex as a possible intermediate in WPB exocytosis.

3.3. Involvement of WPB-associated SNAREs in Ca^{2+}-triggered vWF secretion

To provide evidence for a functional involvement in Ca^{2+}-dependent WPB exocytosis we next attempted to downregulate the SNAREs identified above by RNAi approaches. Efficient knockdown could be achieved by transfection of primary HUVECs with specific siRNA duplexes (Fig. 4A shows an example for SNAP23). HUVEC populations depleted of the individual SNAREs were then subjected to histamine stimulation and the amount of vWF acutely released was measured as compared to mock transfected control cells. None of the depletions had a significant effect on the histamine-induced secretion of WPB-resident vWF. One example is shown for SNAP23 in Fig. 4B. Although a slight decrease in the regulated vWF release was seen, the effects observed never proved to be statistically significant. Such lack of significant functional consequences of SNARE knockdown is not without precedence (see, for example, [28–30], and a detailed analysis
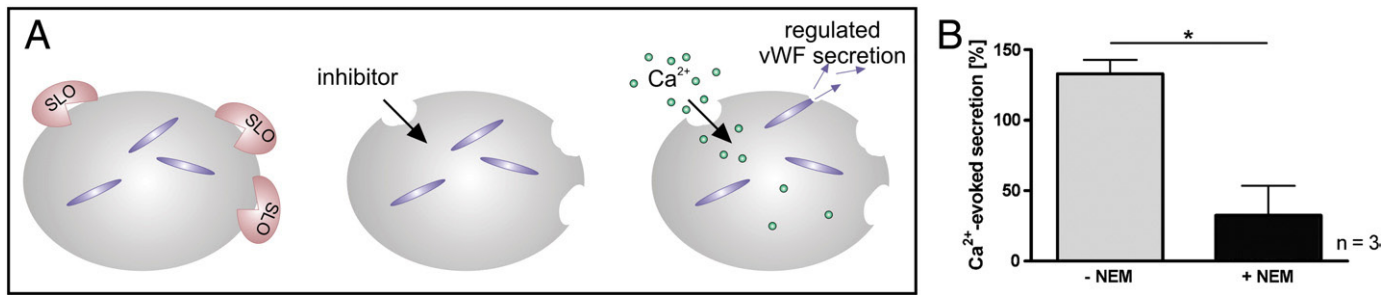
Fig. 1. Inhibition of the ATPase NSF reduces Ca^{2+}-evoked vWF secretion in HUVECs. (A) Schematic representation of the SLO-permeabilized cell system. HUVECs are permeabilized with SLO and subsequently incubated with potentially inhibitory molecules in the absence of calcium. The regulated vWF secretion is then induced by the application of 10 μm $CaCl_2$. (B) Selective NSF inhibition by NEM treatment interferes with the Ca^{2+}-triggered vWF secretion. After treating the SLO-permeabilized HUVECs with 2 mM NEM the cells were subjected to incubation with Ca^{2+}-containing stimulation buffer for 20 min. Control experiments used permeabilized cells incubated for 20 min with EGTA-containing permeabilization buffer to measure the levels of constitutive secretion. The acute release of vWF into the cell culture supernatant was then quantified by a vWF-specific ELISA. The relative Ca^{2+}-triggered increase of vWF secretion exceeding the constitutive level is given in percentage as the mean value ± s.e.m.. The effect on secretion was quantified in sets of independent experiments (number of experiments shown at the base of the graph). Student's unpaired t test was performed to evaluate statistical significance (*P<0.05).

of this phenomenon in [31]) and has been attributed to flexibility in the system allowing for functional compensation and the capability of low levels of SNARE proteins in maintaining proper organelle fusion. To corroborate this for endothelial cells, i.e. to show that lower levels of the SNARE complex identified above can mediate efficient WPB exocytosis, we also applied a different approach for SNARE downregulation and ectopically expressed the proteolytically active tetanus toxin light chain (TeNT LC) in HUVECs. TeNT LC is known to cleave VAMP3 (for review see [32]) and thus is capable of reducing the amount of active VAMP3 also in complexes that are potentially not sensitive to high turnover and thus the siRNA approach. Fig. 4C reveals that TeNT expressing HUVECs show a markedly reduced level of intact

VAMP3. However, despite this reduction histamine-stimulated vWF secretion is not affected indicating that the residual VAMP3 present is sufficient to support WPB exocytosis (Fig. 4D).

To overcome the potential problem of the siRNA approaches, i.e. the presence of residual SNARE protein levels sufficient to support WPB exocytosis, we employed the SLO system, which faithfully reproduced Ca^{2+}-triggered WPB exocytosis (see Fig. 1), to introduce high levels of SNARE mutant proteins that could act in a dominant-negative fashion. We chose mutants covering the cytosolic SNARE domains that engage in trans-SNARE coiled–coil interactions in the course of granule–plasma membrane fusion. Such cytosolic domains have been employed previously as dominant-negative tools
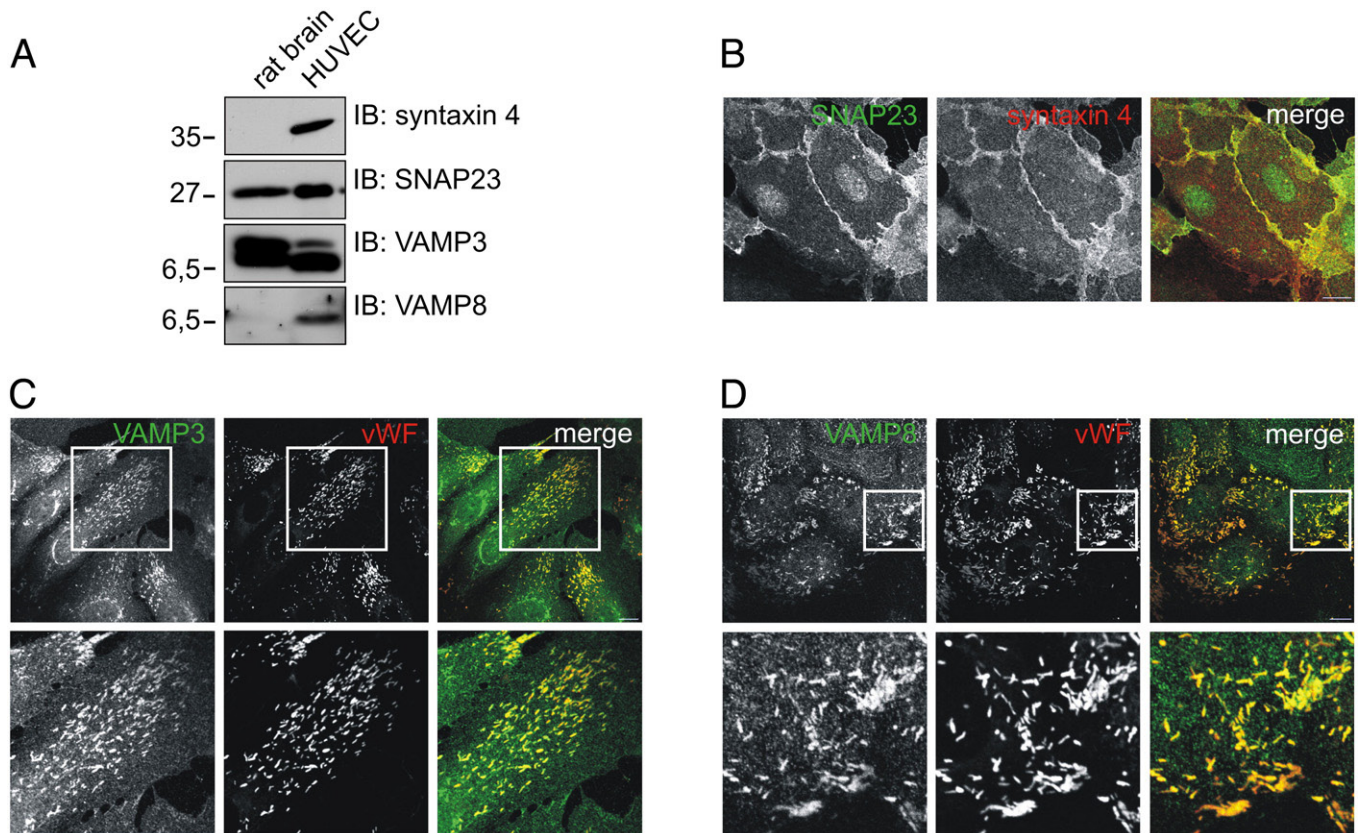

Fig. 2. Expression and subcellular localization of selected SNARE proteins in HUVECs. (A) Immunoblot revealing expression of Syx4, SNAP23, VAMP3 and VAMP8 proteins in HUVECs. Total lysates from rat brain tissue and HUVECs were analyzed by Western blot using polyclonal anti-human Syx4, SNAP23, VAMP3 and VAMP8 antibodies. Molecular weight standards are indicated on the left (kDa). (B) Immunofluorescence staining of the endogenous t-SNAREs Syx4 and SNAP23 and of the endogenous v-SNAREs VAMP3 (C) and VAMP8 (D). The subcellular localization of the two VAMPs is shown in comparison to that of vWF. Boxed images represent enlarged parts of the corresponding stainings. Scale bars represent 10 μm.
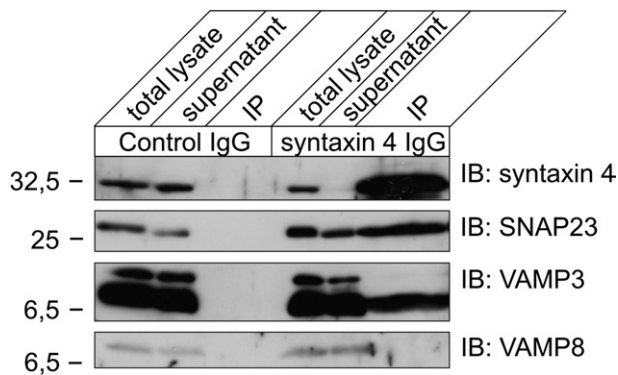
Fig. 3. Syntaxin 4, SNAP23 and VAMP3 form a complex in HUVECs. NEM-treated HUVEC lysates were subjected to immunoprecipitation using mouse anti-Syx4 or non-targeting isotype specific IgGs (control). Aliquots of the starting material (total lysate), the supernatant after immunoprecipitation and the immunocomplexes (IP) were probed by Western blot with polyclonal anti-Syx4, -SNAP23, -VAMP3 and -VAMP8 antibodies. Two parallel immunoprecipitations using the same total lysate were probed by Western blotting to determine the precipitation of either VAMP3 or VAMP8. Molecular weight standards are indicated on the left (kDa).

served as a negative control. WPB exocytosis was then induced by elevating the Ca^{2+} concentration and vWF release in response to the Ca^{2+} trigger was recorded by immunodetection of the protein in the cell supernatant. Fig. 5 reveals that cells loaded with the cytosolic mutant of VAMP3 showed a significantly reduced vWF secretion. In contrast, no inhibitory effect was observed when GST alone (not shown) or the cytosolic VAMP8 mutant were employed. Thus, in line with the co-immunoprecipitation experiments (Fig. 3) WPB-associated VAMP3, but not VAMP8, appears to be primarily involved in mediating Ca^{2+}-induced WPB exocytosis, most likely in conjunction with Syx4 and SNAP23.

competing with their endogenous counterparts in the formation of SNARE complexes, thereby inhibiting SNARE function in the course of membrane fusion events (see, for example, [33–35]). To focus on WPB exocytosis we concentrated on the two WPB-associated SNAREs identified above, VAMP3 and VAMP8. GST-tagged versions of the cytosolic VAMP3 and VAMP8 domains were expressed in bacteria, purified and introduced into SLO-permeabilized HUVECs. GST alone

4. Discussion

Acute secretion of granule contents enables endothelial cells to respond in a dynamic and tightly regulated manner to an altered vascular milieu. Factors released from endothelial cells by such means include the thrombogenic and inflammatory platelet and leukocyte receptors vWF and P-selectin as well as the fibrinolytic tPA. Several lines of evidence suggest that the morphology of the respective storage granules for vWF/P-selectin and tPA, respectively, and the mechanisms, regulation and molecular machineries underlying their exocytotic release differ from one another [9,20,36,37]. Such differences could equip endothelial cells with means to selectively drive the secretion of either thrombogenic or fibrinolytic granule contents in response to certain stimuli. Thus, understanding molecular details of the different release pathways is required to eventually control the thrombogenic or fibrinolytic responses of endothelial cells in a highly specific manner.
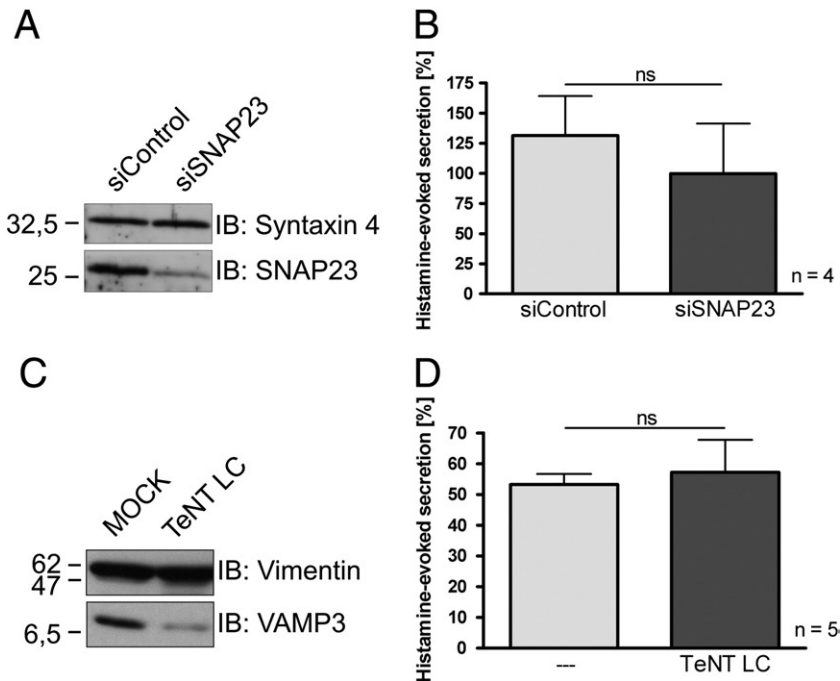

Fig. 4. Histamine-evoked vWF secretion is not affected significantly by siRNA-mediated SNAP23 downregulation. (A) Analysis of the efficiency of SNARE depletion. HUVECs were transiently transfected with 200 pmol of siRNA specific to SNAP23 or non-targeting control siRNA (siControl). 48 h after transfection, cell lysates were prepared and analyzed by Western blot using rabbit polyclonal anti-SNAP23 antibodies to reveal the knockdown efficiency. Probing with a polyclonal anti-Syx4 antibody was included as loading control. Molecular weight standards are indicated on the left (kDa). (B) Quantification of vWF secretion from histamine-stimulated HUVECs depleted of SNAP23 by siRNA treatment. 48 h after transfection with targeting siRNA or siControl, HUVECs were subjected to histamine treatment for 20 min. In parallel levels of constitutive secretion were determined by treating the cells with secretagogue-free basal medium. The acute release of vWF into the cell culture supernatant was then quantified by a vWF-specific ELISA. The relative histamine-triggered increase of vWF secretion exceeding the constitutive level is given in percentage as the mean value ± s.e.m.. The effect on secretion was quantified in sets of independent experiments (number of experiments shown at the base of the graph). Student's unpaired t test was performed to evaluate statistical significance (ns, not significant). (C) Analysis of selective VAMP3 degradation by tetanus toxin. HUVECs were transiently transfected with a construct encoding tetanus toxin light chain. As negative control a mock transfection was included. 24 h after transfection the efficiency of VAMP3 degradation was monitored by preparation of cell lysates and subsequent Western blotting of these lysates using rabbit polyclonal anti-VAMP3 antibodies. Probing with a monoclonal anti-vimentin antibody was included as loading control. Molecular weight standards are indicated on the left (kDa). (D) Quantification of vWF secretion from histamine-stimulated HUVECs depleted of the majority of intact VAMP3 by tetanus toxin treatment. 24 h after TeNT LC or mock transfection, histamine-triggered vWF secretion was quantified as described for SNAP23 depletion.
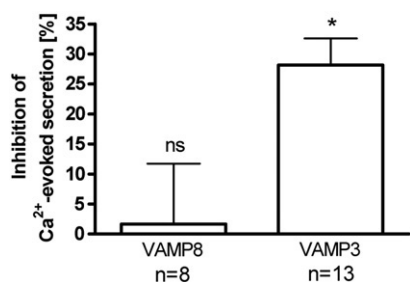
Fig. 5. Dominant-negative VAMP3 affects Ca^{2+}-evoked vWF secretion from SLO-permeabilized HUVECs. Quantification of the Ca^{2+}-evoked vWF secretion of SLO-permeabilized HUVECs treated with dominant-negative VAMP8 or VAMP3. After treating the SLO-permeabilized HUVECs with 30 μM GST, or the GST-tagged cytoplasmic domains of VAMP8 or VAMP3 the cells were subjected to incubation with Ca^{2+}-containing stimulation buffer for 20 min. Control experiments used permeabilized cells incubated for 20 min with EGTA-containing permeabilization buffer to measure the levels of constitutive secretion. The acute release of vWF into the cell culture supernatant was then quantified by a vWF-specific ELISA and values for the relative Ca^{2+}-triggered increase of vWF secretion (calculated as difference to the constitutive level which was set to 100%) were evaluated statistically by Student's unpaired t test (ns, not significant; *P<0.05). The graph shows the relative inhibition of Ca^{2+}-evoked vWF secretion in comparison to cells treated with GST alone in percentage as the mean value ± s.e.m.. The effect on secretion was quantified in sets of independent experiments (number of experiments shown at the base of each column).

Towards identifying core components of exocytotic pathways in endothelial cells we show here that two SNARE proteins, VAMP3 and VAMP8, are present on WPBs, the storage granules of vWF and P-selectin. Of those two, only VAMP3 forms a complex with two plasma membrane-resident SNAREs of endothelial cells, Syx4 and SNAP23, and inhibition experiments in SLO-permeabilized endothelial cells suggest that only VAMP3 contributes to the Ca^{2+}-regulated secretion of vWF. Although considered to primarily function in endosomal recycling, VAMP3 has also been shown to associate with and participate in the release of different types of secretory granules (for review see [32]). Most relevant to vascular haemostasis is probably the presence of VAMP3 on alpha-granules of platelets that also serve as storage organelles for both, vWF and P-selectin. VAMP8 was also found on platelet alpha-granules and targeting of VAMP3 and VAMP8 by introducing soluble protein mutants into SLO-permeabilized platelets had revealed that only VAMP3 appeared to be required for alpha-granule exocytosis [38]. Thus, in addition to their cargo, alpha-granules and WPB share two R-SNAREs that specifically associate with these organelles suggesting that common recruitment mechanisms are operating in platelets and endothelial cells. These results also suggest that VAMP3, but not VAMP8, is an important SNARE mediating the exocytosis of alpha-granules and WPB, respectively.

WPBs of HUVECs contain at least two v-SNAREs, VAMP3 and VAMP8, whereas aortic endothelial cells only appear to express VAMP3 [39]. Since VAMP8 has also been shown to engage in complex formation with Syx4 and SNAP23 (see, for example, [40]), endothelial cells have established a mechanism that selects one WPB-associated v-SNARE (VAMP3) over the other (VAMP8) for plasma membrane t-SNARE engagement and exocytotic membrane fusion. Furthermore, our findings raise the question about the function of WPB-associated VAMP8 since it is not involved in Ca^{2+}-regulated secretion. Given the complex biogenesis of WPB it is possible that VAMP8 functions in membrane fusion events occurring during WPB maturation. Future studies have to address this question.

Complexes of VAMP3, Syx4 and SNAP23 can be co-immunoprecipitated from endothelial cells indicating that VAMP3 forms a trans-SNARE complex with Syx4 and SNAP23 to support WPB exocytosis. This is in line with experiments in permeabilized aortic endothelial cells that showed an inhibitory effect of Syx4 antibodies on vWF secretion [21]. Future studies have to reveal other components of the SNARE machinery, e.g. associated Munc18, Munc13 or synaptotagmin isoforms that participate in the Ca^{2+}-dependent release of WPB.

Furthermore, it has ultimately to be established whether the same or different protein networks function in tPA granule exocytosis.

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