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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.

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

Abbreviations: HUVEC, human umbilical vein endothelial cells; NEM, N-ethylmaleimide; NSF, N-ethylmaleimide-sensitive factor; SLO, streptolysin O; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; Syx4, syntaxin 4; VAMP, vesicle-associated membrane protein; vWF, von Willebrand factor; WPB, Weibel–Palade body

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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