Molecular mechanisms involved in Weibel-Palade body exocytosis
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Depletion of Weibel-Palade bodies in endothelial cells overexpressing the RUN domain containing protein RUFY-3

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Abstract

Weibel-Palade bodies are rod shaped organelles in endothelial cells that function as storage organelle for multiple bioactive components that include von Willebrand factor, angiopoietin-2 and IGFBP7. Upon stimulation of endothelial cells with cAMP raising agonists like epinephrine or calcium raising agonists like thrombin WPBs release their content in the vascular lumen. Previously, we have shown that exchange protein activated by cAMP, Epac1 and the small GTPase Rap1 are involved in cAMP-mediated release of WPBs. Moreover, our data revealed that the Rac guanine exchange factor PREX1 acts downstream of Epac1-Rap1. In the latter study we also identified the small GTP binding protein Rab33A as a potential binding partner of the Ras binding domain of RalGDS. In accordance with previous data we show in this study that GFP-Rab33A can target to WPBs when overexpressed in endothelial cells. We also show that the Rab33A effector RUFY3 can bind to a signaling complex that interacted with the Ras binding domain of RalGDS. Binding of RUFY3 to the Ras binding domain of RalGDS was dependent on prior stimulation of endothelial cells with epinephrine. Analysis of binding of a series of domain constructs derived of RUFY-3 revealed that the RUN-domain was able to bind to the RBD of RalGDS upon incubation of endothelial cells with epinephrine. We next evaluated the effect of overexpression of RUFY3 and its individual domains on WPB homeostasis. Unexpectedly, overexpression of full length RUFY3 but not its individual domains resulted in a complete absence of WPBs. Together these data suggest that a RalGDS-Rap1-RUFY3 axis may promote accelerated turnover of WPBs.

Keywords
Weibel-Palade bodies/RUFY3/Rab33A/Rab33B/von Willebrand Factor
Introduction

Weibel-Palade bodies (WPBs) are elongated storage granules that contain fine tubules and that are exclusively present in endothelial cells [1,2]. It is now well established that WPBs harbour a number of inflammatory components that control leukocyte adhesion [1,3,4]. In addition, the presence of angiopoietin-2 and IGFBP7 as well as studies in VWF deficient model systems have suggested a role for WPBs in regulation of angiogenesis [5-8]. The dynamics and release of WPBs are controlled by a collection of regulatory proteins that include members of the family of small GTPases [1,9]. The small GTP binding protein RalA has been shown to control the stimulus-induced release of WPBs by virtue of its crucial role in the assembly of the exocyst complex [10-12]. The small GTPase Rab27A has been localized to the outer membrane of WPBs and subsequent studies revealed that Rab27A effector MyRIP regulates anchoring of WPBs to the actin cytoskeleton thereby preventing premature release of these organelles [13-15]. More recently, the Rab27A effector Slp4A was identified as a positive regulator of WPB exocytosis [16]. Apart from Rab27A a number of other small GTPases of the Rab-family have also been implicated in WPBs dynamics. Overexpression of Rab3D has been shown to result in targeting of this small GTPase to WPBs [17]. Also, endogenous Rab3B has shown to participate in localizing the Rab27A effector Slp4-A to WPBs [16]. A complete Rab screen revealed that overexpressed Rab3A, Rab27A, Rab15, Rab33A and Rab37 localized to WPBs [18,19]. We have previously shown that activation of the small GTPase Rap1 by the exchange protein activated by cAMP (Epac) controls epinephrine-induced VWF release [20]. More recently, we showed the phosphatidylinositol-3,4,5-triphosphate-dependent Rac exchange factor 1 (PREX1) acts downstream of Rap1 [21]. We hypothesized that simultaneous activation of Rac1 and the small GTPase RalA serves to coordinate cytoskeletal and phospholipase D mediated membrane rearrangements that facilitate cAMP-dependent WPB exocytosis [11,20-22]. Apart from PREX1 we also identified the regulatory β-subunit of phosphatidylinositol 3-kinase (PI3K) and Rab33A as possible downstream targets of Rap1. Here we explored whether Rab33A and its close relative Rab33B can be targeted to WPBs. We also show a potential role for the Rab33A effector RUFY3, that is capable of interacting with Rap1-GTP via its RUN domain.
Experimental procedures

Reagents and antibodies
EBM-2 and EGM-2 were from Lonza (Verviers, Belgium). M199 and RPMI1640, trypsin, penicillin, and streptomycin were from Invitrogen (Breda, the Netherlands). Epinephrine, thrombin, forskolin, 3-isobutyl-1-methylxanthine (IBMX), Endothelial Cell Growth Supplement (ECGS) and anti-α-tubulin monoclonal antibody (DM1A) were from Sigma-Aldrich Chemie (Steinheim, Germany). Mouse monoclonal anti-GFP antibody (clone JL-8) was used to detect GFP in Western-blot analysis (Clontech, California, USA). Polyclonal anti-Rap1 antibody (sc-121) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Glutathione Sepharose 4B was from GE Healthcare Europe GmbH (Diegem, Belgie). Chemiluminescence blotting substrate and Complete Protease Inhibitor Cocktail Tablets were from Roche Diagnostics (Mannheim, Germany). All chemicals used were of analytical grade. Anti-VWF monoclonal antibody CLB-RAg20 has been described previously [23]. The enzyme-linked immunosorbent assay (ELISA) for VWF and VWF propeptide has been described previously [24]. Alexa 488- and Alexa 568-conjugated goat anti-mouse IgG were from Invitrogen.

Cell culture
Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza and cultured in EGM-2 medium enriched with 10% fetal calf serum as described previously [21]. Stimulation of endothelial cells with secretagogues was performed in the following manner: HUVECs, grown in 6-well plates, were washed two times with serum-free medium (SF medium: M199 and RPMI1640 (1:1); 0.3 mg/ml L-glutamine; 100 units/ml penicillin; 100 mg/l streptomycin). Following pre-incubation a mixture of 10 μM epinephrine and 100 μM IBMX was dissolved in SF medium and added to cells.

Construction of RUFY3 variants
Full length RUFY3 cDNA clone 5265230 was obtained from Open Biosystems (Thermo Fisher Scientific, Open Biosystems Products, Huntsville, AL). Fusions of monomeric EGFP (mEGFP) with RUFY3 and RUFY3 derived domains were produced using ligation-independent cloning into the mEGFP-LIC vector (essentially as described previously [16]). Amplification of full-length RUFY3 was performed using PCR primer RUFY LIC-primer FL fwd (5’-GGGCGCGCCCTGCTCTGCTCTGCTCGACTGCCAGGACC-3’ and
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RUFY LIC-primer FL rev (5’- GCGGCCGCCTGCTCGTCCACTAATGATGTTTTGGGA TCAGTTTATTTG-3’). The RUFY3-CC1 fragment containing amino acids 250-329 was amplified by PCR using RUFY LIC-primer C/CC1 fwd (5’-GGGCACCCTGTGGGGCCCATGGTAGAAGAACACGACATTTG-3’) and RUFY LIC-primer CC1 (5’- GCGGCCGCCTGCTCGTCCACTACTTCCGATTGGATTCC AGTATG-3’); the RUFY3-CC fragment containing amino acids 251-469 was amplified using RUFY LIC-primer C/CC1 fwd and RUFY LIC-primer FL rev; the RUFY3-RUN fragment containing amino acids 1-249 was amplified using RUFY LIC-primer FL fwd and RUFY LIC-primer RUN rev (5’-GCGGCCGCCTGCTCGTCCACTATTTCATACAGAAATTGGCATCAATG-3’). mCherry-Rab33A and mEGFP-Rab33B were kindly provided by Dr. T. Carter (Physical Biochemistry, MRC National Institute for Medical Research, London, United Kingdom)

Immunofluorescence
HUVECs were grown on gelatin coated coverslips, fixed with 3.7% formaldehyde for 15 minutes and permeabilized with 0.02% saponin in PBS containing 1% BSA. VWF was visualized using mouse monoclonal CLB-RAg-20 antibody [23] and Alexa 488- and Alexa 568-conjugated goat anti-mouse IgG. Cells were viewed by confocal microscopy using a Zeiss LSM 510.

Rap1 Activation Assays
The Ras binding domain (RBD) of RalGDS fused to a GST tag was expressed in isopropyl-β-D-galactopyranoside-induced bacteria as described previously (26). Purified GST-Ras binding domain (100 μg/sample) was pre-coupled to a 30 μl of glutathione-Sepharose 4B for 1 h at 4 ºC. The pre-coupled glutathione-Sepharose was then washed three times with lysis buffer containing 15% (v/v) glycerol, 1% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 2.5 mM MgCl₂, 10 mM benzamidine, 100 mM aprotinin, supplemented with 1 protease inhibitor tablet/50 ml (Roche Diagnostics, Mannheim, Germany). Following stimulation with 10 μM epinephrine and 100 μM IBMX cells grown in 6-well plates were lysed in 400 μl of lysis buffer. The activated GTP-bound form of Rap1 was then isolated from cell lysates by incubation of 300 μl of lysate with GST-RalGDS-RBD precoupled glutathione-Sepharose for 1 h at 4 ºC. Finally, the Sepharose beads were washed four times with lysis buffer, and bound proteins were resuspended in Laemmli sample buffer. Proteins were run on an SDS 12.5% polyacrylamide gel and analyzed by Western blotting employing an anti-Rap1 polyclonal antibody or monoclonal anti-EGFP antibody JL-8.
Chapter 4

Results

Localization of mCherry-tagged Rab33A and EGFP-Rab33B in endothelial cells.

We identified Rab33A as a putative downstream target of active Rap1 in endothelial cells [21]. Recently, transiently expressed GFP-Rab33A has been localized to WPBs [18,19]. To confirm these finding we overexpressed mCherry-Rab33A in endothelial cells. In the majority of transfected cells mCherry-Rab33A did not colocalize to WPBs but displayed a perinuclear staining that most likely overlaps with the Golgi apparatus (data not shown). In a minority of transfected cells mCherry-Rab33A did partially co-localize with WPBs (Figure 1A). Also in these cells a perinuclear staining was observed (Figure 1A). Detailed inspection of individual WPBs clearly revealed the presence of mCherry-Rab33A but also showed WPBs that did not contain detectable amounts of mCherry-Rab33A (Figure 1B). We also addressed whether EGFP-Rab33B was capable of localizing to WPBs. Again in the majority of transfected cells EGFP-Rab33B was not localized on WPBs; EGFP-Rab33B was primarily localized to a perinuclear region that most likely corresponds to the Golgi apparatus (Figure 1C). In a small subset of EGFP-Rab33B expressing cells EGFP-Rab33B was recruited to WPBs (Figure 1C). Also in this subset of EGFP-Rab33 cells concentrated perinuclear staining was observed which most likely corresponds to the Golgi-apparatus (Figure 1C). These results suggest that Rab33A and Rab33B can both localize to WPBs however in the majority of transfected cells exogenous Rab33A and Rab33B are not recruited to these organelles.

Binding of the Rab33 effector RUFY3 to the Ras binding domain of RalGDS.

Previously, we have performed pulldown employing the Ras binding domain of RalGDS which has been shown to bind to active Rap1 [20,25]. Rab33A was identified as a putative downstream-effector of activated Rap1 in these studies [21]. The Rab33 effector RUFY3 also designated Rap2 interacting protein x (RPIPx) contains a RUN domain that binds to Rap2A [26]. We speculated that by means of its interaction with activated Rap1, RUFY3 provides a connection between Rap1 and Rab33A. To explore this possibility we expressed mEGFP-RUFY3 fusion and a control mEGFP protein in endothelial cells and tested its ability to interact with activated Rap1. Expression of mEGFP-RUFY3 was checked by immunoblot employing a
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Figure 1. Localization of mCherry-Rab33A and mEGFP-Rab33B in endothelial cells. HUVECs overexpressing mCherry-Rab33A or mEGFP-Rab33B were stained for VWF using mouse monoclonal antibody CLB-RAg-20. (A) mCherry-Rab33A localization in endothelial cells. Arrows indicate mCherry-Rab33A-positive WPBs. (B) mCherry-Rab33A localization in endothelial cells displaying a punctuate staining that does not overlap with WPBs. (C) mEGFP-Rab33B localization in endothelial cells. mEGFP-Rab33B displays predominantly a perinuclear localization; arrows indicate mEGFP-Rab33B positive WPBs. Scale bars indicate 10 μm.
monoclonal antibody directed towards GFP (Figure 2A). Monitoring of expression of mEGFP-RUFY3 by Western blot revealed that expression levels were lower than observed for mEGFP. In agreement with previous findings, an increase in the amount of active Rap1 was observed following stimulation with epinephrine for both mEGFP and mEGFP-RUFY3 expressing cells (Figure 2B). Strikingly, mEGFP-RUFY3 also interacted in an epinephrine-dependent manner with the Ras binding domain of RalGDS (Figure 2C). No binding of mEGFP to the Ras binding domain of RalGDS was observed (Figure 2C). These findings suggest that RUFY3 is part of a signaling complex that can interact with the Ras binding domain of RalGDS in a stimulus-dependent manner. These data potentially position RUFY3 as a putative downstream effector of Rap1-GTP in endothelial cells.

**Figure 2.** Epinephrine-dependent interaction between mEGFP-RUFY3 and the Ras binding domain of RalGDS. (A) Expression of mEGFP and mEGFP-RUFY3 was checked by immunoblot employing a mouse monoclonal antibody directed towards GFP. (B) Rap1 activation in HUVECs expressing mEGFP or mEGFP-RUFY3 respectively was monitored after stimulation with 10 µM epinephrine and 100 µM IBMX for 10 minutes. Activation of Rap1 was monitored as described in Material and Methods. Total amount of Rap1 is displayed in the bottom panel. (C) Binding of mEGFP and mEGFP-RUFY3 to Ras binding domain of RalGDS was assessed as described in Materials and Methods. Immunoblot was stained using mouse monoclonal antibody directed to GFP.
We next assessed which domain of RUFY3 mediates its direct or indirect binding to the Ras binding domain of RalGDS. To this end a series of RUFY3 variants was constructed that contained the RUN, CC1 and CC1 and CC2 domains (Figure 3A). Expression of these domain constructs was monitored by Western-blot (Figure 3B). No binding of the isolated CC1 domain and combined CC1 and CC2 domains to the Ras binding domain of RalGDS was observed (Figure 3C). Similar to mEGFP-RUFY3 full length, also the RUFY3 fragment the RUN domain was capable of binding in an epinephrine-dependent manner to the Ras binding domain of RalGDS (Figure 3C). These data are in agreement with the presence of a Rap-interacting site in the RUN domain of RUFY3 [26].
Overexpression of mEGFP-RUFY3 depletes Weibel-Palade bodies.

We overexpressed mEGFP-RUFY3 in endothelial cells and monitored its subcellular localization. Overexpressed mEGFP-RUFY3 displayed a cytoplasmic staining; no vesicular structures were observed in endothelial cells overexpressing RUFY3 (Figure 4A). Remarkably, no WPBs were observed in cells that expressed mEGFP-RUFY3; whereas in non-transfected cells considerable numbers of these organelles were observed (Figure 4A). These findings suggest that overexpression of mEGFP-RUFY3 results in depletion of WPBs. We also addressed whether the individual RUN and CC1 and CC1/CC2 domain were capable of inducing depletion of WPBs. In contrast to full length RUFY3 the number of WPBs was not reduced in cells expressing these individual domains (Figure 4B). Altogether these data show that only full-length RUFY3 can deplete WPBs upon its overexpression in endothelial cells.

Discussion

In this study we show that mCherry-Rab33A and eGFP-Rab33B can be recruited by WPBs. Previous, over-expressed GFP-Rab33A has been localized to WPBs [18,19]. Interestingly, Zografou and co-workers observed that GFP-Rab33A localized to most WPBs (74 ± 3%) whereas our analysis revealed only a limited number of cells (<5%) in which mCherry-Rab33A was localized to WPBs [19]. Under our experimental conditions also eGFP-Rab33B was localized to WPBs in a small percentage of transfected HUVEC. Zografou and coworkers reported that eGFP-Rab33B localized to the Golgi-apparatus but not WPBs [19]. It should be noted that we were unable to address whether endogenous Rab33A or Rab33B were also localized to WPBs. Rab33A is mainly expressed in the brain, lymphocytes and melanocytes [27-29]. In the same study it was reported that Rab33A was not expressed in endothelial cells [27]. In hippocampal neurons Rab33A is located to the Golgi apparatus and synaptophysin-positive vesicles [30]. Rab33A depletion using siRNA revealed that Rab33A contributes to anterograde axonal transport of synaptophysin-positive vesicles and thereby participates in axon outgrowth [30]. These findings raise the possibility that Rab33A also controls the kinesin-mediated anterograde transport of WPBs along microtubules. The low level of colocalization of mCherry-Rab33A and WPBs may indicate that only relatively immature WPBs that are not yet stably anchored to the actin cytoskeleton are targeted by Rab33A. In contrast to Rab33A, Rab33B is ubiquitously expressed [29].
Both Rab33A and Rab33B have been implicated in the formation of autophagosomes through their binding to Atg16L, which is part of a protein complex involved in the generation of the isolation membrane or phagophore, [31,32]. Binding of Rab33A and Rab33B to Atg16L is mediated by the coiled coiled domain [32]. Autophagosomes arise as a consequence of energy needs or cellular stress and are involved in the engulfment and subsequent degradation of cytoplasm or subcellular organelles [33]. Endogenous Rab33B has been localized to the Golgi while autophagosomes are considered to be generated from endoplasmic reticulum. Interestingly, Rab33B depletion had little effect on the formation of autophagosomes.
raising the possibility that the observed effects of GTPase deficient, active variant of Rab33B on autophagosome formation might be due to blocking or depletion of AtgL16 rather than through a direct effect of Rab33B itself [32]. Recently, autophagy has been implicated in regulation of WPB turnover and/or release [34]. Whether dysregulation of autophagy specifically affect WPB exocytosis or causes a general dysfunction of vesicular trafficking remains to be determined.

Previously we identified Rab33A as a possible downstream interaction of Rap1-GTP [21]. Rab33A and to a lesser extent also Rab33B have been shown to interact to the RUN domain containing protein RUFY3 [35]. The RUN domain RUFY3 has also been shown to bind to Rap2B-GTP but not Rap1A-GTP [26]. These observations raise the possibility that RUFY3 provides a link between activated Rap1/2 and Rab33A. Indeed we observed that mEGFP-RUFY3 can interact with components of a signaling complex that binds in a stimulation-dependent manner to the Ras binding domain of RalGDS (Figure 2 and 3). As expected binding of RUFY3 to the Rap binding domain of RalGDS is mediated by its RUN domain (Figure 3). These data indeed suggest that RUFY3 provides a link between active Rap1/2 and Rab33A. It should be noted that our data do not specify how RUFY3 is recruited to the Ras binding domain of RalGDS. Both Rap1A, Rap1b, Rap2A, Rap2B and Rap2C are expressed in endothelial cells [36]. The RBD domain of RalGDS can bind to Rap1 and Rap2B [25,37,38]. The RUN domain of RUFY3 has been shown to bind Rap2A but not Rap1A [26]. Future studies need to show whether Rap1/2 is needed for binding to the Ras binding domain of RalGDS. Subsequently, specific Rap1/2 isoforms involved in this process will need to be identified. RUFY3 has been shown to interact with the p85 and P110α subunit of phosphatidylinositol 3-kinase (PI3K) [39]. The p85 subunit of PI3K was also identified as a potential downstream target of Rap1 in endothelial cells [21].

Overexpression of RUFY3 but not its domain variants results in depletion of WPBs (Figure 4). The molecular mechanism underlying this observation remains elusive. The lack of WPBs in mEGFP-RUFY3 expressing cells can potentially be attributed to an effect on the biogenesis of WPBs, spontaneous release of newly formed WPBs or autophagocytic degradation of WPBs. RUFY3 and other RUN domain containing proteins have been suggested to functionally bridge Rap and Rab proteins [40,41]. The identification of Rab33A and RUFY3 as potential downstream targets of active Rap1/2 suggests that RUFY3 may provide a link between active Rap1/2 and Rab33A. RUFY3 (or single axon related 1 singar1) was previously shown to
be upregulated during single axon formation in hippocampal neurons [39].
Depletion of RUFY3 employing siRNA resulted in loss of polarity resulting in
the induction of multiple axons [39]. Morphological analysis revealed that
RUFY3 was localized in the growth cones of axons [30]. In a separate study
a similar distribution was observed for Rab33A raising the possibility that
Rab33A/RUFY3 complexes are both involved in the anterograde transport
of synaptophysin positive vesicles in extending axons [30]. The RUN domain
containing protein UNC-14 indirectly associated with the motor protein
kinesin-1 in Caenorhabditis elegans [41]. Current data suggest that RUFY3
promotes anterograde transport; as yet it is not known whether the RUFY3
is also part of a scaffolding complex that is linked to kinesins. The observed
depletion of WPBs in HUVECs overexpressing mEGFP-RUFY3 may be caused
by enhanced anterograde transport of WPBs resulting in a dramatic increase
in their basal release. In this respect it is interesting to note that knockdown
of Rab33B also resulted in an increased basal release of WPBs [19].
Alternatively, overexpression of RUFY3 may promote recruitment of Rab33B
to WPBs thereby marking them for autophagosomal degradation. Dissection
of the precise molecular mechanism underlying WPB depletion by RUFY3
requires further study.

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References
cofactor to leukocyte recruitment by endothelial P-selectin. Blood 118: 4265-4273.
secretion of tPA and cytokines from cultured endothelial cells. Blood 116: 2183-2191.
sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of
angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell
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