The interplay between the Rab27A effectors Slp4-a and MyRIP controls hormone-evoked Weibel-Palade body exocytosis

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Weibel-Palade body (WPB) exocytosis underlies hormone-evoked VWF secretion from endothelial cells (ECs). We identify new endogenous components of the WPB: Rab3B, Rab3D, and the Rab27A/Rab3 effector Slp4-a (granuphilin), and determine their role in WPB exocytosis. We show that Rab3B, Rab3D, and Rab27A contribute to Slp4-a localization to WPBs. siRNA knockdown of Slp4-a, MyRIP, Rab3B, Rab3D, Rab27A, or Rab3B/Rab27A, or overexpression of EGFP-Slp4-a or EGFP-MyRIP showed that Slp4-a is a positive and MyRIP a negative regulator of WPB exocytosis and that Rab27A alone mediates these effects. We found that ECs maintain a constant amount of cellular Rab27A irrespective of the WPB pool size and that Rab27A (and Rab3s) cycle between WPBs and a cytosolic pool. The dynamic redistribution of Rab proteins markedly decreased the Rab27A concentration on individual WPBs with increasing WPB number per cell. Despite this, the probability of WPB release was independent of WPB pool size showing that WPB exocytosis is not determined simply by the absolute amount of Rab27A and its effectors on WPBs. Instead, we propose that the probability of release is determined by the fractional occupancy of WPB-Rab27A by Slp4-a and MyRIP, with the balance favoring exocytosis. (Blood. 2012;120(13):2757-2767)

Introduction

Hormone-evoked VWF secretion from endothelial cells (ECs) is mediated by exocytosis of specialized secretory granules (SGs) called Weibel-Palade bodies (WPBs).1 WPB exocytosis is triggered by increases in intracellular free Ca2+ or cAMP concentrations, and involves a number of molecular components, including the N-ethylmaleimide–sensitive factor, VAMP3, SNAP23, syntaxin 4, RaLA, the annexin A2/S100A10 complex, and phospholipase D.2-7 In addition, Rab proteins also regulate WPB exocytosis. A subset of Rab proteins, including Rab3A-3D, Rab27A/B, and Rab37, is associated with SGs in different cell types where they regulate SG biogenesis, trafficking, and exocytosis.8 Secretory cells often express a mixture of these “secretory” Rabs, which may have overlapping or distinct functions. Human ECs are reported to express mRNA for Rab3A, Rab3D, and Rab37,9,10 Rab3B protein,11 and Rab27A mRNA and protein.12,13 To date, Rab27A is the only endogenous EC Rab protein that has been detected on WPBs. Through its effector MyRIP and Myosin Va, Rab27A is proposed to negatively regulate WPB exocytosis.13,14 Rab27A can interact with different effector molecules, and many secretory cells express a mixture of these effectors.8 In these cases, SG exocytosis probably depends on the balance of Rab27A interactions with the complement of Rab effectors in the cell.

In addition to MyRIP, ECs contain mRNA for the Rab27A effector Slp4-a (granuphilin).13 Slp4-a links SGs to the plasma membrane (PM) through SG-associated Rab proteins (principally Rab27A), PM-associated syntaxins (1a, 2, or 3) and soluble Munc18 isoforms.15,16 Syntaxins exist in open and closed conformations that determine their participation in SNARE complex formation and membrane fusion. Slp4-a binds the closed conformation of syntaxin,16,19 which selectively interacts with Munc18 proteins to hold SGs in a fusion-incompetent state at the PM. Overexpression of Slp4-a increased numbers of PM-docked SGs but inhibited secretion, whereas knockout or knockdown (KD) studies showed the opposite.20 Unlike MyRIP, Slp4-a interacts with several different Rab proteins, including Rab3A-3D and Rab8,15,21 and with myosin Va,22 a point of convergence with MyRIP.

In this study, we identified Slp4-a, Rab3B, and Rab3D as new components of WPBs and investigated their roles in regulating hormone-evoked WPB exocytosis and VWF secretion. We found that, in contrast to other secretory cells, Slp4-a acts as a positive regulator of WPB exocytosis, and its interplay with the negative regulator MyRIP on Rab27A bound to WPBs (WPB-Rab27A) sets the probability of WPB exocytosis.

Methods

Tissue culture, transfection, secretion assays, antibodies, DNA constructs, and reagents

Primary HUVECs were purchased, cultured and transfected as previously described.23 Human aortic endothelial cells (HAECs) were cultured as previously described.24 Secreted VWF and EGFP were assayed by ELISA as previously described.25 Primary antibodies (Abs) along with the dilutions for immunofluorescence and Western blotting are given in supplemental Table 1A (available on the Blood Web site; see the Supplemental Materials

The online version of this article contains a data supplement.


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link at the top of the online article) with sources of secondary Abs appended. Production of an anti-Rab27A Ab is described in supplemental Figure 1A. DNA constructs are described in the supplemental Appendix 1. Primers for amplification of full-length Rab3 isomers, MyRIP and Slp4-a, are listed in supplemental Table 1B. All reagents were from Sigma-Aldrich unless otherwise stated. Fura-2/AM was from Invitrogen.

Immunoblotting

Cells were rinsed with PBS and lysed in 2× sample buffer12 containing β-mercaptoethanol and protease inhibitors (Sigma-Aldrich: P8340) or in PBS containing 1% Triton X-114, 2mM EDTA, and protease inhibitors. To concentrate hydrophobic proteins, Triton X-114 partitioning of the lysate was performed as described.12 Protein in the detergent phase was precipitated with methanol-chloroform using 0.25 µg BSA as carrier. Proteins were separated on a 12% SDS-PAGE gel, transferred to 0.2 µm nitrocellulose membrane, and probed with primary Ab, followed by infrared dye-coupled secondaries. Membranes were scanned with the LI-COR Odyssey Infrared Imaging system (LI-COR Biosciences).

RNAi

Pools of 4 siRNA oligo duplexes (ON-TARGETplus SMARTpool, Dharmacon RNA Technologies) were used to deplete HUVECs of the mRNAs of Rab27A (#L004667), Rab3b (#L008825), Rab3d (#L010822), Slp4-a (#L007111), and MyRIP (#L013964). Oligo sequences are in supplemental Table 1C. A pool of 4 nontargeting siRNAs (ON-TARGETplus Nontargeting pool, #D001810) was used as a control (siCTRL). A total of 1000 pmol of siRNA duplexes was transfected by nucleofection into 7.5 × 10⁴ HUVECs. ECs were plated at confluent density (∼0.8 × 10⁶ cells/cm²) in gelatin-coated wells of 6- or 12-well plates or on gelatin-coated glass coverslips and cultured in HUVEC growth medium for 48 hours. Depletion of Rab27A, Rab3b, Rab3d, Slp4-a, and MyRIP was assessed by quantitative PCR, immunocytochemistry, or immunoblotting.

RT-PCR and quantitative PCR analysis

RNA was extracted using RNasy Mini Kit (QIAGEN) and integrity verified on a 2100 Bioanalyzer (Agilent Technologies). cDNA was synthesized using a QuantiTect Reverse Transcription kit (QIAGEN) from 500 ng of RNA. RT-PCR primers for Rab3A to Rab3D are in supplemental Table 1B. The probability of WPB exocytosis, ρ,, was determined as the mean percentage of degranulation of fluorescent WPBs after cell stimulation. The mean WPB-EGFP FI for EGFP-Rab27A was determined as described in “Immunocytochemistry and quantification of antigen immunoreactivity on WPBs.” Rab acquisition by immature WPBs was determined by time-lapse imaging of ECs expressing VWF-ttdTomato (VWF-tdT) and EGFP-Rab27A or mEGFP-Rab3B (16 hours after nucleofection). Microscope and experimental details are in the legend to supplemental Video 1. Mean WPB-EGFP and WPB-tdTomato FIs were determined from background-subtracted images as described in the previous section.

Whole-WPB fluorescence recovery analysis of Rabs and effectors cycling on WPBs

Leica TCS SP2 or Leica TCS SP5 confocal microscopes equipped with PL APO 100 × 0.7-1.4NA (SP2) or HCX PL APO CS 100 × 1.46NA (SP5) objectives were used to study fluorescence recovery after whole WPB bleaching (FRAP) at 37°C. Imaging and bleaching settings were as previously described.27 For FRAP, 1-2 pulses at full laser power were applied to a bleaching ROI encompassing the entire WPB, and the WPB FI followed to monitor recovery. For whole-cell inverse FRAP (iFRAP)28 a PL APO 63 × 0.6-1.4NA objective was used to image at least 2 fluorescent cells at a time. All but a small region of 1 cell (containing a few WPBs) was bleached using full laser power pulses at 1- to 2-second intervals over an ∼5-minute period. The decline in FI in the unbleached region of the test cell was followed at 1- to 10-minute intervals. For analysis, ROIs were defined by thresholding low-pass filtered images above the background to include just the bleached WPB (FRAP) or unbleached (iFRAP) WPBs in the cell. The average FI within the ROIs was scaled by the decline because of bleaching in adjacent unbleached control cells/WPBs in the image. Exponential fitting was performed in Origin Version 8.5 (OriginLab) without constraints on offset or final level.

Statistical analysis

Data were plotted in Origin Version 7.5 or greater or GraphPad Prism Version 5.0. Statistical analysis was by nonparametric t test (except where indicated) using GraphPad Prism Version 5.0. Significance values are shown on the figures or in figure legends. Data are shown as mean ± SEM.

Results

Slp4-a is localized to WPBs

Identification of Slp4-a mRNA in HUVECs13 led us to examine whether this Rab27A effector was associated with WPBs and played a role in regulating WPB exocytosis. Endogenous Slp4-a-IR was readily detectable on WPBs in HUVECs (Figure 1A; supplemental Figure 2A) and adult HAECs (supplemental Figure 2B). Furthermore, expression of EGFP-Slp4-a specifically labeled WPBs (eg, see Figure 4C). However, several observations indicate that Rab27A is not solely responsible for recruiting Slp4-a to WPBs. First, HUVECs with few WPBs (eg, * in Figure 1B) consistently
had strong WPB-Rab27A-IR, whereas HUVECs with large numbers of WPBs (***) had almost undetectable WPB-Rab27A-IR (see also supplemental Figure 3A-B; quantified in Figure 6A). Control experiments showed the intensity of WPB-Rab27A-IR was proportional to WPB-Rab27A-antigen concentration (supplemental Figure 3C), so the reduction in WPB-Rab27A-IR reflects a reduction in WPB-Rab27A concentration. This pattern was mirrored by endogenous WPB-MyRIP-IR but not by WPB-Slp4-a-IR, which remained prominent irrespective of WPB number, and the same was seen in HAECs (Figure 1B; supplemental Figures 2 and 3B,D). Second, KD of Rab27A in HUVECs resulted in loss of both WPB-Rab27A- and WPB-MyRIP-IR, but not of WPB-Slp4-a-IR (supplemental Figure 3E). Finally, we observed a subpopulation of Rab27A-negative WPBs displaying Slp4-a-IR (Figure 2D). Because Slp4-a can also bind Rab8 and Rab3A-3D15,21 (and supplemental Figure 4A), we next looked for the expression and WPB localization of these Rab proteins. Although HUVECs express Rab8,11,29 we detected neither endogenous nor epitope-tagged Rab8...
on WPBs. We therefore focused on Rab3 isoforms (specificities of the Rab3 reagents are shown in supplemental Figure 4Bi-vi).

**Rab3B and Rab3D are expressed in ECs and localized to WPBs**

Expression of mEGFP-Rab3A-3D labeled WPBs in HUVECs (supplemental Figure 4Bi-vi); however, immunostaining showed that only endogenous Rab3B-IR was clearly detectable on WPBs in HUVECs (and HAECs), whereas WPB-Rab3D-IR was weakly detectable in only ~ 5% of cells (Figure 1C; supplemental Figure 4C-D). RT-PCR and Western blot analysis confirmed that only Rab3B and Rab3D were expressed in HUVECs (supplemental Figure 4E). No Rab3A mRNA was found despite using primers and conditions reported to detect this Rab in HUVECs (supplemental Figure 4E). Quantitative PCR quantification showed that Rab3B and Rab3D transcripts together were > 10 times more abundant than Rab27A transcripts.

Unlike WPB-Rab27A-IR, WPB-Rab3B-IR was clearly observed irrespective of WPB pool size and matched closely the pattern for endogenous Slp4-a-IR (Figure 2A). Similar to Rab27A, Rab3B-IR was absent from most newly formed WPBs at the trans-Golgi network (Figure 2B); however, we observed a subpopulation of immature Rab27A-negative WPBs that were Rab3B-positive (Figure 2C) and displayed clear Slp4-a-IR (Figure 2D). Together, the data show that Rab27A is not the sole Slp4-a-interacting Rab protein on WPBs and that the WPB localization of Rab3B matches closely that of Slp4-a.

**Rab3B is recruited slowly to immature WPBs but cycles rapidly on mature WPBs where it recruits Slp4-a**

Because differences in the kinetics of recruitment of Rab3B and Rab27A to immature WPBs might account for Rab3B-IR on perinuclear Rab27A-negative WPBs, we next determined the time constants of recruitment ($\tau_{rec}$) of mEGFP-Rab3B and EGFP-Rab27A to immature VWF-tdT-expressing WPBs in live cells (eg, supplemental Video 1). $\tau_{rec}$ for mEGFP-Rab3B and EGFP-Rab27A were not significantly different (Figure 3A), indicating that other factors may account for this phenomenon (see “Discussion”).

To investigate whether Rab3B or Rab3D might contribute to WPB recruitment of Slp4-a, we performed WPB bleaching experiments. The rationale for FRAP experiments was that Rab proteins and their effectors exchange (cycle) between their target membranes and the cytosol, and that this process, for both Rabs and effectors, is linked to the Rab GTP-GDP cycle. Where an effector (Slp4-a) has access to a mixture of different Rabs, as is potentially the case here, the cycling kinetics will reflect a composite function of the kinetics of the individual Rabs of the mixture. Thus, by determining the association time constants ($\tau_{ass}$) for individual WPB-Rabs and comparing $\tau_{ass}$ for Slp4-a cycling on WPBs expressing exogenous Rab27A, Rab3B, or Rab3D with Slp4-a cycling in the absence of expressed WPB-Rabs, we might identify with which WPB-Rab Slp4-a interacts.

We quantified $\tau_{ass}$ for EGFP-tagged Rab27A, Rab3B, or Rab3D after whole WPB bleaching. EGFP-Rab27A recovered very slowly and often incompletely during the observation period (Figure 3B). mRFP-Rab27A behaved like EGFP-Rab27A. mEGFP-Rab3B and -Rab3D recovered significantly faster than EGFP-Rab27A (Figure 3B, D). Dissociation time constants ($\tau_{diss}$) for EGFP-Rabs, determined by iFRAP, were of similar magnitude to $\tau_{ass}$ (Figure 3B-C), both probably reflecting the off-rates of Rab unbinding from the WPB. For mEGFP-Rab3B, both $\tau_{ass}$ and $\tau_{diss}$ were significantly faster than for EGFP-Rab27A.

$\tau_{ass}$ for EGFP-Slp4-a to WPBs coexpressing exogenous mRFP-tagged Rab27A, Rab3B, or Rab3D were, in each case, similar to those of the Rab protein alone (Figure 3D-E). Notably, EGFP-Slp4-a when expressed alone recovered fast, with a $\tau_{ass}$ similar to that seen in the presence of exogenous Rab3B (or Rab3D; Figure

**Figure 2. Rab3B is detectable on WPBs before Rab27A and colocalizes with Slp4-a.** (A) HUVECs immunolabeled for endogenous Rab3B (mouse Ab, green), Slp4-a (rabbit Ab, red), and VWF (sheep Ab, blue). Here and in other figures, grayscale images are from regions indicated by white boxes. (B) HUVECs expressing Proregion-EGFP (Pro-EGFP, green, 24 hours after nucleofection) immunolabeled for endogenous Rab3B (mouse Ab, red), Rab27A (rabbit Ab, green), and VWF (sheep Ab, blue). (C) HUVECs immunolabeled for Rab3B (mouse Ab, red), Rab27A (rabbit Ab, green), and Slp4-a (rabbit Ab, blue). Inset: right, arrowheads point to Rab3B-positive, Rab27A-negative perinuclear immature WPBs; bottom, Rab3B- and Rab27A-positive mature WPBs. (D) HUVECs immunolabeled for Rab3B (goat Ab; red), Rab27A (mouse Ab, green), and Slp4-a (rabbit Ab, blue). Inset: right, Rab3B-positive, Rab27A-negative, Slp4-a-positive perinuclear immature WPBs; bottom, mature WPBs positive for all markers. Scale bars represent 10 µm.
3E). This indicates that Slp4-a can interact with endogenous Rab3B and/or Rab3D on WPBs.

Analysis of Slp4-a, MyRIP, and WPB-Rab function in WPB exocytosis

**Slp4-a is a positive and MyRIP a negative regulator of WPB exocytosis.** To assess the function of Slp4-a in hormone-evoked WPB exocytosis, we depleted endogenous Slp4-a by siRNA KD and for comparison analyzed the effect of MyRIP KD. Slp4-a KD (Figure 4A) substantially decreased while MyRIP KD (Figure 4B) increased histamine-evoked VWF secretion. To examine the effect of overexpression of EGFP-Slp4-a or EGFP-MyRIP on Ca\(^{2+}\)-driven WPB exocytosis, we performed live cell experiments. Both constructs labeled all WPBs, with the exception of immature organelles at the trans-Golgi network (Figure 4Ci). Figure 4Cii-iii compares the kinetics and extent of WPB exocytosis in control cells expressing the WPB-specific protein Proregion-EGFP3 (black) with that in EGFP-Slp4-a-expressing cells (red). Cumulative plots of WPB fusion events (Figure 4Cii bottom) are aligned to the increase in intracellular Ca\(^{2+}\) (top in Figure 4Ciii). The mean delay, maximal rate, and probability of WPB fusion (P), are summarized in Figure 4Ciii. In EGFP-Slp4-a-expressing cells, WPB exocytosis was initially slowed, although later the rate increased and reached control values in Proregion-EGFP cells. Surprisingly, P, was significantly increased (Figure 4Ciii). In marked contrast, expression of EGFP-MyRIP completely blocked Ca\(^{2+}\)-driven WPB exocytosis.

Quantification of the intensity of endogenous WPB-MyRIP-IR as a function of the FI of WPB-EGFP-Slp4-a showed that overexpression of EGFP-Slp4-a reduced endogenous WPB-MyRIP-IR (Figure 4Civ black symbols). In contrast, EGFP-MyRIP overexpression had only a small effect on endogenous WPB-Slp4-a-IR (Figure 4Civ gray symbols), presumably because Slp4-a, unlike MyRIP, interacts with GTP-Rab3B, GTP-Rab3D, and GDP-Rab27A on WPBs. The data show that Slp4-a and MyRIP exert opposing effects on WPB exocytosis and VWF secretion and that Slp4-a competes with MyRIP for its WPB-specific binding partner Rab27A.

**Rab27A, but not Rab3B or Rab3D, regulates VWF secretion.** Having established that Slp4-a acts as a positive regulator of histamine-evoked VWF secretion and that it can interact with WPB-Rab3s, we next determined the relative contributions of Rab3B, Rab3D, and Rab27A to hormone-evoked VWF secretion. To do this, we performed KDs of each Rab individually and of Rab3B and Rab3D together (Figure 5). KDs profoundly depleted each Rab, with endogenous WPB-Rab3B- and WPB-Rab27A-IR
greatly diminished in both single and double KDs (Figure 5Ai-iii). KD of Rab27A also depleted WPB-MyRIP- but not WPB-Slp4-a-IR (supplemental Figure 3E). Substantial depletion of WPB-Slp4-a-IR was observed only after Rab3B/Rab27A double KD (Figure 5Ai-iv), and not after Rab3B or Rab27A KDS (supplemental Figures 3Eii and 5Aiv), and not after Rab3B or Rab27A KDs (supplemental Figures 3Ei and 5).

KD of Rab27A, but not of Rab3B or Rab3D, significantly reduced histamine-evoked VWF secretion (Figure 5Bi-ii), and no further significant decrease was seen after Rab3B/Rab27A double KD. Together, the data show that Rab27A and not Rab3B or Rab3D regulate histamine-evoked VWF secretion and suggest that the influence on exocytosis of their common effector, Slp4-a, is mediated via Rab27A alone.

The probability of WPB exocytosis is unaffected by reductions in WPB-Rab27A concentration with increasing WPB pool size. The inhibition of histamine-evoked VWF secretion by Rab27A KD led us to ask whether the natural reduction in WPB-Rab27A concentration with increasing WPB pool size (Figure 1B; supplemental Figure 3) was mirrored by a decrease in \( P_e \). The reduction in WPB-Rab27A concentration with increasing WPB pool size is summarized in Figure 6A (along with the corresponding variation in WPB-MyRIP and WPB-Slp4-a levels). As the same relationship was seen in Proregion-EGFP–expressing HUVECs (supplemental Figure 6A), we could assess directly how \( P_e \) varied with WPB pool size, normalized by their total number, in Proregion-EGFP- (black, 46 cells, 2151 fusion events), EGFP-Slp4-a– (red, 15 cells, 849 fusion events), and EGFP-MyRIP–expressing cells (blue, 21 cells, total absence of fusion events).

Inset: The initial period after stimulation on an expanded time scale. (Ciii) Mean delays (seconds), maximal rates of exocytosis (WPBs/second), and probabilities of WPB exocytosis, \( P_e \), (percent, note broken Y scale) for Proregion-EGFP (black) or EGFP-Slp4-a–expressing cells (red). (Civ) Relationship between the average intensity of EGFP-Fusion (\( P_e \)) and Slp4-a or MyRIP, respectively, after nucleofection. Data were pooled from 3 independent experiments. (Aii,Bii) Immunoblots for Slp4-a (Ai) and MyRIP (Bi) after nucleofection with control, Slp4-a KD, and MyRIP KD oligos as indicated. mRNA was depleted by 65% ± 3% (Ai) or 67% ± 3% (Bi) in KD compared with control. Data were pooled from 3 independent experiments. (Aii,Bii) Immunoblots for Slp4-a or MyRIP, respectively, after nucleofection with control (siCTRL) or specific KD oligos as indicated. Data are representative of 3 independent experiments. Position of molecular weight markers (kDa) are shown to the left and \( \alpha \)-tubulin used as loading control. (Ai,Bii) Immunostaining of HUVECs for Slp4-a (rabbit Ab, green) and VWF (sheep Ab, red; Aii) or MyRIP (goat Ab; green) and VWF (rabbit Ab, red; Bii) in control or after KD as indicated. Scale bars represent 10 μm. (Aiii,Biii) Histogramme-activated VWF secretion, quantified by ELISA, from HUVECs nucleofected with control, Slp4-a (Aiv) or MyRIP (Biv) siRNA oligos as indicated. White and gray bars represent unstimulated (vehicle alone) and histamine-stimulated conditions, respectively. Data were pooled from 3 independent experiments. A small but significant decrease in basal release was seen with Slp4-a KD (siCTRL, 0.8% ± 0.08% vs siSlp4-a, 0.57% ± 0.1% of total VWF, \( P = 0.01 \)). and a trend to an increase in basal secretion for MyRIP KD (siCTRL, 0.27% ± 0.07% vs siMyRIP, 0.43% ± 0.15% of total VWF), although the latter did not reach significance (\( P = 0.09 \)). (C) HUVECs expressing EGFP-Slp4-a (top, green) or EGFP-MyRIP (bottom, green) 24 hours after nucleofection and immunolabeled with VWF (sheep Ab; red). Grayscale images are from regions indicated by white boxes. Scale bars represent 20 μm. (Cii) Top: Fura-2 fluorescence ratio showing intracellular Ca2+ rise. White boxes. Scale bars represent 20 μm. (Cii) Bottom: Cumulative plot of WPB fusion event times, normalized by their total number, in Proregion-EGFP- (black, 46 cells, 2151 fusion events), EGFP-Slp4-a– (red, 15 cells, 849 fusion events), and EGFP-MyRIP–expressing cells (blue, 21 cells, total absence of fusion events).

WPB-Rab27A levels with increased WPB pool size. We next addressed the cellular mechanism for the reduction in WPB-Rab27A levels with increased WPB pool size.

We hypothesized that, if total cellular Rab27A levels remain unchanged with increasing WPB numbers, then Rab27A cycling between WPBs would account for the dilution in WPB-Rab27A concentration. To test this hypothesis, we took advantage of the fact that VWF content and WPB numbers in HUVECs increase with time in culture.52 We determined how cellular levels of the WPB-specific marker Proregion and of Rab27A change with time
in culture. HUVEC numbers and total protein content increased with time, reaching a plateau by 96 hours (supplemental Figure 6B). As expected, levels of Proregion, adjusted for cell number, increased progressively, whereas those of Rab27A and housekeeping proteins remained unchanged (Figures 6C). Thus, HUVECs maintain a relatively constant intracellular pool of Rab27A in the face of large increases in the WPB pool size, consistent with Rab27A cycling underlying the dilution of individual WPB-Rab27A levels.

Finally, we examined how \( P_r \) changed with time after WPB formation. We performed a morphologic pulse-chase using Proregion-EGFP, and at specific times after nucleofection cells were either fixed and stained for endogenous Slp4-a or MyRIP, or used in live secretion experiments. Figure 6Di-iii shows the increase in mean number of fluorescent WPBs per cell with time after nucleofection (Figure 6Di), the mean percentage of these fluorescent WPBs that were positive for Slp4-a (○) or MyRIP-IR (○; Figure 6Diii), and \( P_r \) (Figure 6Diii; ●). The fraction of WPBs positive for Slp4-a or MyRIP-IR was similar at each time, consistent with the time-dependent recruitment of Rab27A. At early times, when most WPBs lacked Slp4-a or MyRIP-IR, \( P_r \) was low but increased to close to maximal levels as the fraction of WPBs positive for both effectors increased. Figure 6Diii also shows \( P_r \) values recalculated for the fraction of Slp4-a-positive WPBs at each approximate time point (○). The data suggest that \( P_r \) is closely linked to the WPB recruitment of both Slp4-a and MyRIP.

Discussion

Rab proteins on WPBs: composition and recruitment

WPBs in HUVECs and HAECs recruit Rab3B and Rab3D in addition to Rab27A. In a small number of perinuclear Rab27A-negative (immature) WPBs, we observed detectable Rab3B-IR (Figure 2C), Rabs are delivered to their target membranes by 1 of 2 routes: as newly synthesized Rabs in association with Rab proteins on WPBs: composition and recruitment WPBs in HUVECs and HAECs recruit Rab3B and Rab3D in addition to Rab27A. In a small number of perinuclear Rab27A-negative (immature) WPBs, we observed detectable Rab3B-IR (Figure 2C), Rabs are delivered to their target membranes by 1 of 2 routes: as newly synthesized Rabs in association with Rab
Escort Protein (REP), or during subsequent Rab cycling, in association with guanine-nucleotide dissociation inhibitor (GDI). The key step for Rab delivery by either route is membrane insertion that requires Rab GDP-GTP exchange catalyzed by a guanine-nucleotide exchange factor (RabGEP), and possibly other as yet unidentified molecules. Comparison of Rab3B $\tau_{\text{ex}}$ with Rab3B $\tau_{\text{ass}}$ or $\tau_{\text{ass}}$ (Figure 3) showed that the initial recruitment is much slower than Rab cycling on immature WPBs, suggesting that immature WPBs may preferentially recruit newly synthesized Rabs. Because newly synthesized Rab3B, Rab3D, and Rab27A share many common molecular components for delivery to target membranes including a RabGEP (RabGEP), it is not surprising that $\tau_{\text{ex}}$ for mEGFP-Rab3B and EGFP-Rab27A to immature WPBs were similar. However, if endogenous Rab3B were more abundant than Rab27A at the protein level, as seen at the transcript level, then competition between Rab3B and Rab27A for shared molecular components could account for Rab3B-positive Rab27A-negative WPBs. Indeed, a very low GTPase activity for Rab3B may lend an additional competitive advantage to Rab3B in accessing REP.7,38 Direct evidence for competition between secretory Rabs in HUVECs came from observations that EGFP-Rab27A or mEGFP-Rab3s’ expression depleted endogenous WPB-Rab3B- or Rab27A-IR, respectively (supplemental Figure 7A-B). The primary point of competition between these Rabs is probably membrane insertion because neither cytosolic EGFP nor YFP-Rab1A, which uses distinct GEFs (and GTPase activating proteins), displaced Rab3B from WPBs (supplemental Figure 7C).

Slp4-a recruitment to WPBs

We show that WPB recruitment of Slp4-a involves not only Rab27A but also Rab3B (and potentially Rab3D; Figures 3E and 5Aiv), accounting for several otherwise unexpected observations: the failure of Rab27A KD to remove Slp4-a from WPBs (supplemental Figure 3Eiii), the presence of Slp4-a-IR on a population of endogenous Rab27A-negative WPBs, and retention of Slp4-a-IR on WPBs after displacement of endogenous Rab27A-IR and MyRIP-IR by overexpressed EGFP-Rab3s (eg, supplemental Figure 7D).

Photobleaching studies showed fast Rab3s cycling on individual WPBs, consistent with previous bulk granule bleaching studies in PC12 cells. Because Slp4-a binds to Rab3s only in the GTP form (supplemental Figure 4A), the fast $\tau_{\text{ass}}$ of EGFP-Slp4-a with mRFP-Rab3-expressing WPBs (Figure 3E) indicates that Slp4-a cycling is strongly influenced by the underlying cycling of Rab3s. Rab27A also cycled, but its $\tau_{\text{ass}}$ was very slow, consistent with studies in PC12 cells. Slow Rab27A cycling could reflect the fast constitutive GDP-GTP exchange and slow GTP hydrolysis rates reported to hold Rab27A predominantly as GTP-Rab27A on SGs. Unlike for Rab3s, conversion to GDP-Rab27A is not associated with loss from membranes. Whether this arises because Rab27A is a poor substrate for GDI or because access to GDI is hindered by binding to effectors in the GDP-state (eg, Slp4-a, coronin-3), remains to be established. The cycling of EGFP-Slp4-a on mRFP-Rab27A-expressing WPBs mirrored the very slow cycling of Rab27A itself, presumably because Slp4-a,
could involve the aforementioned processes, but this remains to be established. The latter is also consistent with the underlying kinetics of exocytosis remaining unchanged. In contrast, Rab3B and Rab3D depletion had no effect (Figure 5B). A reduction in unstimulated VWF secretion caused by Rab27A KD. This raises the intriguing possibility that the increase in P, observed on EGFP-Slp4-a expression might arise simply from the competitive displacement of endogenous MyRIP from GTP-Rab27A on WPBs. Indeed, we observed a complete inhibition of WPB exocytosis, although depletion of endogenous Slp4-a-IR from WPBs was masked by the capacity of WPB-Rab3s to recruit Slp4-a to WPBs. Compared with the relatively small effect of MyRIP depletion from WPBs, it indicates that endogenous WPB-MyRIP levels exert a relatively weak brake on WPB exocytosis.

Slp4-a and MyRIP have opposite effects on WPB exocytosis

Our data show that Slp4-a acts as a positive regulator of hormone-evoked WPB exocytosis and confirm that MyRIP is a negative regulator of this process. The latter is also consistent with MyRIP’s reported role in phorbol 12-myristate 13-acetate (PMA)–stimulated secretion of VWF. The results for Slp4-a are particularly interesting because this molecule is reported to be inhibitory for SG exocytosis in other secretory cells. Overexpression of epitope-tagged Rab3D has also been reported to inhibit hormone-evoked VWF secretion, whereas Rab3B or Rab3D depletion had no effect (Figure 5B). A reduction in unstimulated secretion in other KD conditions (eg, Slp4-a) also mirrored the effect on stimulated secretion. Because the majority of unstimulated VWF secretion arises from basal release of WPBs, this indicates that the same molecular machinery influences stimulated and basal VWF secretion. Our Rab27A KD data differ from a previous study reporting a substantial increase in PMA-stimulated VWF secretion, presumably reflecting differences in the mechanisms by which PMA and hormone trigger VWF secretion. An increase in histamine-evoked VWF secretion was also reported in that study; however, those results were equivocal because of a 3-fold increase in unstimulated VWF release caused by Rab27A KD.

Overexpression of epitope-tagged Rab3D has also been reported to inhibit hormone-evoked VWF secretion, and we observed the same result (supplemental Figure 7E). However, we found that Rab3D overexpression was accompanied by loss of endogenous WPB-Rab27A-IR and MyRIP-IR (supplemental Figure 7Bii), although WPB-Slp4-a-IR was still observed, consistent with Slp4-a binding to WPB-Rab3s (supplemental Figure 7D). The data suggest that the effect of Rab3D overexpression arises through
competing displacement of Rab27A from WPBs and that, despite recruiting Slp4-a to WPBs, Rab3s do not support the positive action of Slp4-a on VWF secretion. The latter idea is consistent with previous data in PC12 cells.\textsuperscript{21} Slp4-a binds to GTP- and GDP-Rab27A states,\textsuperscript{41} whereas MyRIP binds to GTP-Rab27A only.\textsuperscript{45} If Slp4-a and MyRIP compete for WPB-Rab27A, as suggested by data in Figure 4Civ, then the capacity of Slp4-a to bind GDP-Rab27A could confer a competitive advantage over MyRIP in a positive drive toward exocytosis.

KD of Rab3B (and Rab3D) suggests little or no role for these Rabs in controlling WPB exocytosis, raising the intriguing question as to their function on the WPBs. Rab3B and Rab3D are implicated in processes not directly related to control of exocytosis, including the regulation of catecholamine uptake into SGs,\textsuperscript{46} receptor transcytosis,\textsuperscript{47} SG-microtubule interactions,\textsuperscript{48} and SG maturation.\textsuperscript{49} The function of Rab3s on WPBs remains to be established.

The probability of WPB exocytosis does not depend solely on the absolute WPB-Rab27A concentration over the endogenous range

Despite marked intercellular variations in the amount of endogenous Rab27A (and MyRIP) on WPBs because of increasing WPB pool size (Figures 1B and 6A; supplemental Figure 3A-B), \( P_r \) was constant for a given stimulus strength (Figure 6B). This suggests that \( P_r \) is not determined solely by the absolute amount of Rab27A and its effectors on WPBs, although some finite amount of each must be required. Instead, some other parameter(s) must be important in determining \( P_r \). We found that the total cellular content of Rab27A remains essentially constant with time in culture, and our preliminary results indicate that the same is true for MyRIP and Slp4-a (and Rab3B). In this case, the steady-state fractional occupancy of Rab27A by its effectors should also remain constant, irrespective of the absolute amounts of Rab27A on any individual organelle. This raises the possibility that the fractions of WPB-Rab27A molecules occupied by MyRIP and Slp4-a are the crucial factor in determining \( P_r \). Most newly formed WPBs lack endogenous Slp4-a-IR, MyRIP-IR (Figure 6Di), Rab27A-IR\textsuperscript{12} (and Rab3B-IR, Figure 2B), and for these organelles \( P_r \) was very low (Figure 6Di). The close correlation between the increase in \( P_r \) (Figure 6Di) and increase in the fraction of WPBs with detectable Slp4-a- and MyRIP-IR (Figure 6Di) shows that the capacity to undergo exocytosis is closely linked to acquisition of both effector molecules. By assuming that WPBs completely lacking these components are not fusion-competent, we found that newly formed WPBs (7 hours after nucleofection) with detectable Slp4-a-IR and MyRIP-IR had a \( P_r \) no much lower than that seen for much older organelles (Figure 6Diii, ○). The slightly higher \( P_r \) seen for older WPBs may reflect their predominantly peripheral localization and closer apposition to the PM. Manipulations that alter the steady-state fractional occupancy of Rab27A (eg, overexpression or KD of Slp4-a or MyRIP) change \( P_r \), and VWF secretion. Substantial reductions in WPB-Rab27A levels after Rab27A KD simulate a return toward the “immature state” where Rab27A, MyRIP, and Slp4-a are missing, rendering WPBs less responsive to stimulation.

In conclusion, WPBs recruit multiple Rabs; however, our data suggest that Slp4-a and MyRIP acting via Rab27A regulate WPB exocytosis and VWF secretion. Our data indicate that the fractional occupancy of WPB-Rab27A by its effectors rather than just the absolute amount of these molecules on WPBs is an important factor in determining \( P_r \). Thus, by simply maintaining the cellular content of Rab27A and its effectors at approximately constant levels, ECs maintain \( P_r \) constant irrespective of WPB pool size (Figure 7). This provides a simple mechanism to ensure a coordinated and proportionate response of the EC population to external stimuli of varying intensity.

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The interplay between the Rab27A effectors Slp4-a and MyRIP controls hormone-evoked Weibel-Palade body exocytosis

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