Weibel-Palade bodies recruit Rab27 by a content-driven, maturation-dependent mechanism that is independent of cell type

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Summary

The identification of organelles is crucial for efficient cellular function, yet the basic underlying mechanisms by which this might occur have not been established. One group of proteins likely to be central to organelle identity is the Rab family of small GTPases. We have thus investigated Rab recruitment to membranes using endothelial cells as a model system. We report that Weibel-Palade bodies, the Von Willebrand Factor storage compartment of human umbilical vein endothelial cells, contain Rab27a. We have also found that Weibel-Palade body-like structures induced in HEK-293 cells by the expression of von Willebrand factor can recruit endogenous Rab27a. In the absence of von Willebrand Factor, Rab27a is not lysosome associated, indicating that it can distinguish between the Weibel-Palade-body-like organelle and a classical lysosome. Finally, a time course of Weibel-Palade-body formation was established using a green-fluorescent version of von Willebrand factor. Newly formed Weibel-Palade bodies lack Rab27a, which is acquired some hours after initial appearance of the cigar-shaped organelle. We conclude that a lumenal cargo protein drives the recruitment of Rab27a to the organelle membrane by a novel mechanism that is indirect, maturation-dependent and cell-type independent.

Key words: Rab27, Weibel-Palade bodies, Endothelial cells, Lysosome-related organelles, Secretory granules

Introduction

Eukaryotic cells are divided into membrane-bounded compartments or organelles specialised for different biological processes. There are two major advantages to this compartmentalization: the separation of mutually incompatible processes and the efficiency gains made possible either by higher protein concentrations or by differentiation of the intraorganellar environment. However, a consequence of such segregation is the added complexity that this produces within the cell, where there are many different organelles carrying out different processes. The presence of many cytoplasmic organelles creates a challenge for the cell in correctly maintaining and controlling each compartment, which in turn requires their correct identification.

It is not known what features on an organelle confer its identity or, indeed, whether there is any single simple identification system (Pfeffer, 2001; Munro, 2002). However, one group of molecules that appear to be central to this process are the Rab family of small GTPases (Pfeffer, 2001; Segev, 2001; Stenmark and Olkkonen, 2001; Zerial and McBride, 2001; Goud, 2002; Seabra et al., 2002). This family of proteins show the requisite specificity of intracellular location and are also increasingly found to be at the centre of networks of protein-protein interactions leading from the organelle into the cytoplasm (Pfeffer, 2001; Zerial and McBride, 2001).

Proteins that can serve as markers of organelle identity must be capable of overcoming several problems, among which are those of distinguishing between closely related organelles and responding to organelle maturation. These two problems come together in the secretory organelles known as lysosome-related organelles (LROs). First, the relation between lysosomes and the LROs is close but complex and unresolved. LROs share marker proteins, low organellar pH and a high degree of accessibility from the endocytic pathway with true lysosomes. However, they differ from the latter in that they are usually primarily secretory organelles carrying different or additional cargo that reflects their varied functions (Dell'Angelica et al., 2000; Marks and Seabra, 2001; Blott and Griffiths, 2002; Cutler, 2002; Raposo and Marks, 2002). Whether LROs share their Rabs with lysosomes is not yet determined. Second, some LROs exhibit a complex maturation process, best exemplified by the four discrete stages of melanosome maturation (Marks and Seabra, 2001). Any organelle identification system must be capable of marking such changes differently, because certain functions of LROs (e.g. secretion) must be restricted to

the population of mature organelles and not allow the secretion of biosynthetic intermediates.

A key function of the identity system is to translate events within the interior of an organelle to the outside, and so we are examining the recruitment of Rabs by Weibel-Palade Bodies (WPBs). These secretory organelles of endothelial cells (Weibel and Palade, 1964; Wagner, 1990; Hannah et al., 2002; van Mourik et al., 2002) have been described as either LROs (Marks and Seabra, 2001; Cutler, 2002) or secretory granules (Daugherty et al., 2001) and thus appear to lie between three groupings: secretory granules, lysosomes and LROs. They are therefore good models to explore issues of organelle identity. They also have a further useful property: an organelle with indistinguishable properties from bone fide WPBs can be formed de novo by the heterologous expression of a single WPB content protein, von Willebrand Factor (VWF) (Wagner et al., 1991) (reviewed in Hannah et al., 2002). These WPB-like organelles can be secretagogue-responsive (Hop et al., 1997; Blagoveshchenskaya et al., 2002) and they selectively recruit an appropriate subset from the membrane proteins available (Blagoveshchenskaya et al., 2002). These organelles thus offer an optimal system for analysing how the interior of an organelle – and especially changes in that interior – can be monitored by the cell using the Rab identification system.

We chose to study Rab27a as a possible marker for Weibel-Palade bodies because it has previously been found on LROs in diverse cell types such as melanosomes, lytic granules of cytotoxic T cells and platelet α-granules and dense granules (Hume et al., 2001; Stinchcombe et al., 2001; Barral et al., 2002). We report here that WPBs efficiently recruit Rab27a in a time-dependent manner such that, when the nascent organelles first emerge from the Golgi, they are Rab27 negative, acquiring the Rab over the subsequent few hours. Interestingly, the WPB-like organelles that are induced in other cell types when VWF is expressed can also recruit this Rab. In the absence of VWF, the Rab27 does not get recruited by lysosomes but exhibits a diffuse intracellular distribution. Thus, Rab27a is recruited specifically to the VWF-containing organelles and not the lysosome in a maturation-dependent process that is independent of cell type.

Materials and Methods

Antibodies and reagents

Rabbit polyclonal anti-human VWF and horseradish peroxidase (HRP)-coupled anti-VWF were purchased from DAKO (Cambridgeshire, UK). Mouse monoclonal anti-human VWF was purchased from Serotec (Oxfordshire, UK). Sheep polyclonal antibody against green fluorescent protein (GFP) was purchased from Biogenesis (Poole, UK). Rabbit polyclonal anti-Rab27 was as previously described (Hume et al., 2001). Anti-human LAMP1 clone H4A3 was purchased from Pharmingen (Wiltshire, UK) Secondary antibodies coupled to fluorophores or HRP were purchased from Jackson (Luton, UK). Enhanced chemiluminescence (ECL) reagents and hyperfilm-ECL were purchased from Amersham (Buckinghamshire, UK). All other reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated.

Expression vectors

Constructs allowing the expression of enhanced green fluorescent protein (EGFP)-Rab27a, EGFP-Rab1a and EGFP-Rab8 were as previously described (Hume et al., 2001). The full-length pre-pro-VWF (VWF-WT) was as previously described (Blagoveshchenskaya et al., 2002). Full-length pre-pro-VWF tagged at the C-terminus with EGFP (pre-pro-VWF-EGFP) was provided by P. Skehel (University of Edinburgh, UK) and will be described in detail elsewhere (M.J.H. et al., unpublished). pCMV7MYC-Rab1a was produced by PCR amplification of the canine *Rab1a* cDNA and cloned into the pCMV7 vector (Strom et al., 2002; Seabra et al., 1992).

Cell culture and transfection

Cryopreserved human-umbilical-vein endothelial cells (HUVECs) were obtained from TCS-Cellworks (Buckinghamshire, UK) and cultured as previously described (Arribas and Cutler, 2000). HEK-293 cells were obtained from BD Biosciences (Oxfordshire, UK) and cultured in α-MEM (Life Technologies, Paisley, UK), 10% foetal calf serum (FCS), 50 μg ml^{-1} gentamicin (Life Technologies). Cells were transiently transfected with either Transfast (Promega, Hampshire, UK; using a ratio of Transfast to DNA of 1:1), GeneJuice (Merck, Germany) or by Nucleofection™ (Amaxa, Germany) according to the manufacturers instructions.

Confocal immunofluorescence microscopy

Immunofluorescence labelling of cells on glass coverslips was carried out as previously described (Blagoveshchenskaya et al., 2002). Two- and three-colour fluorescence images were acquired sequentially using either a BioRad MRC 1024 or a Leica TCSNT confocal microscope.

Subcellular fractionation and VWF ELISA

HUVECs were grown to confluence on gelatine-coated 15 cm tissue culture dishes and harvested by trypsinization. Cell pellets were put onto ice and the following procedures performed on ice or in the cold until specified otherwise. Cells were suspended in homogenization buffer (HB: 250 mM sucrose, 10 mM HEPES pH 7.4, 1 mM $MgCl_2$, 800 U ml^{-1} DNase I) and homogenized using a ball-bearing homogenizer. The cell homogenate was spun at 800 ***g*** for 7 minutes to prepare a post-nuclear supernatant (PNS), and the PNS was mixed with isotonic Percoll and HB to give a final Percoll concentation of 50% (v/v). The PNS-HB-Percoll mixture was spun at 37,000 ***g*** in a fixed-angle (28°) rotor and the gradient fractionated from the top (i.e. low numbers are less dense). A small aliquot of each fraction was assayed for VWF using a solid-phase sandwich ELISA as described elsewhere (Blagoveshchenskaya et al., 2002) and half of the fraction stored at –20°C awaiting Triton X-114 (TX-114) partitioning. The remainder of the fractions, containing most of the WPB (in the experiment shown, fractions 7-9) were pooled together, made up to volume with 50% (v/v) isotonic Percoll in HB and respun as above except at 16,000 ***g***. The gradients were fractionated as above, a small aliquot assayed for VWF and the remainder used for the TX-114 partitioning and Rab27 immunoblot.

TX-114 partitioning and Rab27 immunoblot

TX-114 partitioning of the gradient fractions was used in order to concentrate the Rab27a and also to eliminate the Percoll, which interferes with SDS-PAGE sample buffer. Percoll-gradient fractions were made up to 1% (w/v) TX-114 using a 10% (w/v) solution of precondensed TX-114 (Bordier, 1981). Protease inhibitors were added and the samples incubated for 1 hour at 4°C with end-over-end mixing followed by centrifugation at 100,000 ***g*** for 2 hours at 4°C. The resultant Percoll-free, TX-114-soluble supernatants (preliminary experiments found that there was no detectable Rab27 immunoreactivity in the TX-114-insoluble material) were transferred to fresh tubes and partitioning achieved by warming to 37°C for 3 minutes followed by centrifugation at 12,000 ***g*** for 3 minutes at room

temperature. The proteins present in the detergent phase were precipitated with methanol-chloroform (Wessel and Flugge, 1984) using haemoglobin as a carrier protein, and the protein pellet redissolved in SDS-PAGE sample buffer. Discontinuous SDS-PAGE, transfer of electrophoresed proteins onto PVDF membranes and probing of the membranes with anti-Rab27 detected by ECL were performed according standard methods. Densitometry of unsaturated exposures of the ECL films were used to quantify the relative distribution of Rab27 immunoreactivity across the Percoll gradients.

Results

We hypothesized that Rab27 proteins could associate with WPBs, given their LRO-like characteristics (Marks and Seabra, 2001; Cutler, 2002). First, we determined whether the WPBs of cultured primary endothelial cells express Rab27, using cultured HUVECs as a model endothelial cell. Reverse-transcription PCR showed that mRNA encoding Rab27 was expressed in HUVECs, but that only Rab27a- and not Rab27b-encoding sequences were present, as confirmed by sequencing of the PCR product (data not shown). Immunofluorescent labelling of fixed HUVECs with antibodies against VWF and Rab27 suggested that the endogenous Rab27a immunoreactivity present in HUVECs was associated with the WPB (Fig. 1, arrows). In addition, it was apparent that not all WPBs (as defined by VWF-positive, elongated structures) were positive for Rab27a immunoreactivity (Fig. 1, curly brackets).

Subcellular fractionation of HUVECs PNSs on Percoll density gradients, combined with TX-114 partition and immunoblotting of the resulting fractions, confirmed that an anti-Rab27 antibody-reactive membrane protein of the appropriate molecular weight co-enriches through two gradients with VWF and is therefore associated with an organelle of the same buoyant density as WPBs (Fig. 2). This is in contrast to a control small GTPase of endothelial cells

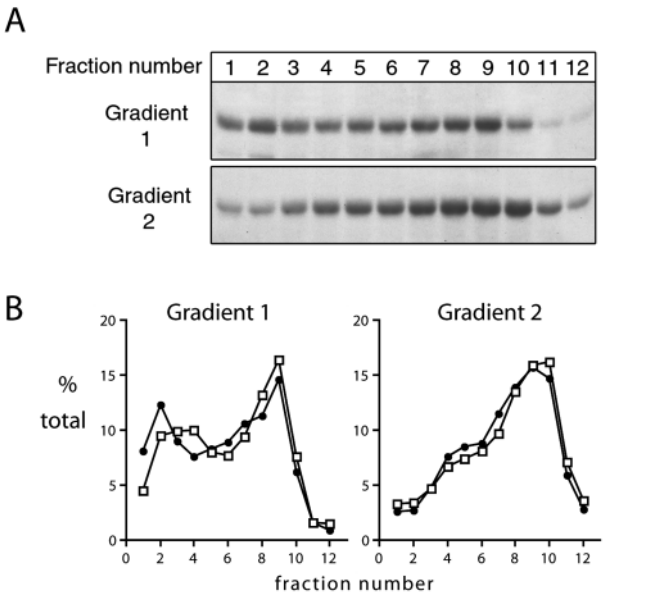

Fig. 2. Rab27 purifies with VWF on density gradients. A post-nuclear supernatant was prepared from HUVECs that had been grown to confluence and the organelles contained therein were separated according to density on two sequential self-forming Percoll gradients (aliquots of fractions 7-9 from gradient 1 were pooled and run on gradient 2). (A) The membrane proteins present in each fraction were enriched by TX-114 partitioning, separated according to size by SDS-PAGE and transferred onto a PVDF membrane. The membrane was probed with an antibody to Rab27 and detected with an enzymatically coupled secondary antibody and ECL. (B) The quantification of the Rab27 immunoblot data in (A) (filled black circles) plotted together with the VWF-ELISA values of the same fractions (open squares). Both these data are plotted as percentages of the sum of immunoreactivities across the whole gradient.

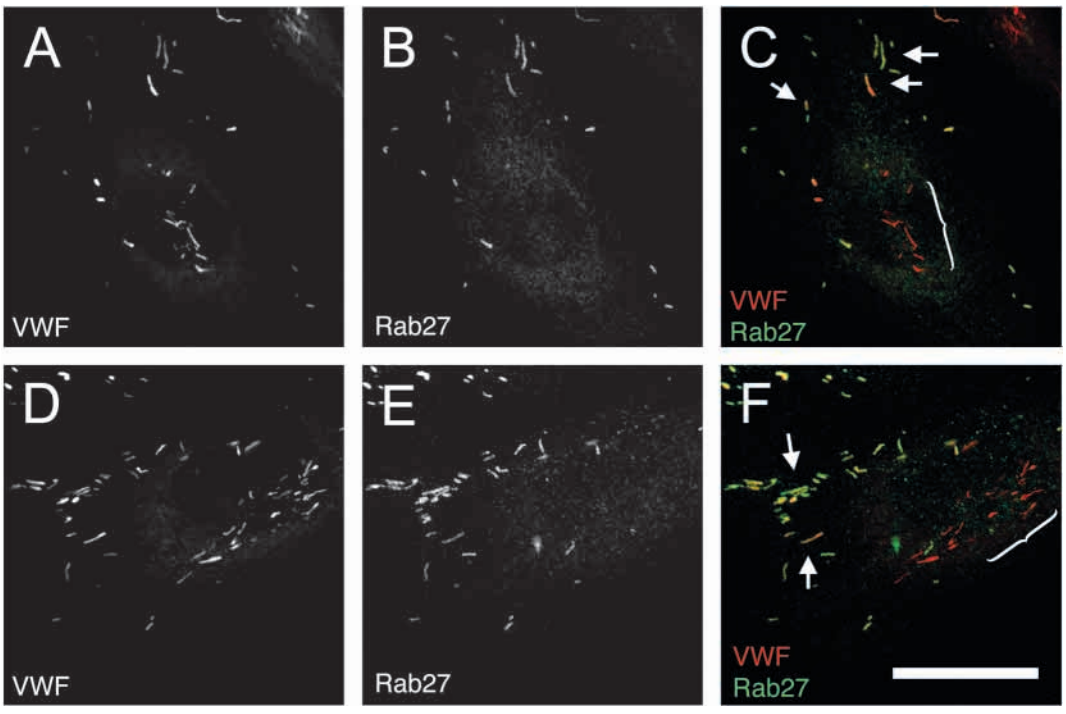

Fig. 1. Localization of Rab27 on WPBs in HUVECs by immunofluorescence microscopy. HUVECs grown on gelatine-coated glass coverslips were fixed, permeabilized and double immunolabelled for VWF (A,D and red in C,F) and endogenous Rab27 (B,E and green in C,F). Arrows in C and F point to WPBs that are positive for both VWF and Rab27, and the curly brackets indicate the population of VWF-positive organelles that do not stain for Rab27. Scale bar, 20 μm (F).

associated with the plasma membrane, which shows a radically different distribution on these gradients (data not shown).

One possible explanation for the failure of some WPBs to recruit Rab27a is that the level of synthesis of this protein might be too low to saturate all potential binding sites. The amount of material required to produce a quantifiable western blot (twelve 15-cm dishes of confluent HUVECs were used as starting material to obtain the data depicted in Fig. 2) strongly suggests that expression of Rab27a in HUVECs is low. We therefore overexpressed EGFP-Rab27a in HUVECs to discover whether all WPBs could be labelled. HUVECs were transfected with an expression vector encoding EGFP-Rab27a and, after 24 hours, the cells were fixed and stained for VWF to visualize the WPBs. Comparison of the VWF immunoreactivity with the EGFP-Rab27a fluorescence indicated that the Rab27a fusion protein was efficiently recruited to the VWF-positive WPB (Fig. 3C, arrows). Importantly, and like the endogenous Rab27 immunoreactivity, the EGFP-Rab27a appeared to be excluded from a population of WPB usually found in the pericentriolar region (Fig. 3A, curly bracket). A Myc-tagged Rab27a fusion protein and EGFP-Rab27b behaved like EGFP-Rab27a (data not shown), whereas other Rab proteins, MYC-Rab1a (Fig. 3E and green channel in Fig. 3F), EGFP-Rab1a and EGFP-Rab8 (data not shown) were not recruited to WPBs when overexpressed in HUVECs, confirming the specificity of Rab27 recruitment.

Rab27a is recruited to a late stage of maturation of WPB

WPBs are thought to form at the trans-Golgi network (Matsuda and Sugiura, 1970; Sengel and Stoebner, 1970). The association of Rab27a with a subset of WPBs raised the possibility that the Rab27-negative WPB are those that have only just been formed.

To test this possibility we investigated the timing of Rab27a recruitment by studying the fate of a transiently expressed pre-pro-VWF-EGFP fusion protein. This chimera is effectively incorporated into newly synthesized WPBs in HUVECs (M.J.H. et al., unpublished) and therefore provides a morphological means of studying the maturation of WPBs over time. In order to express pre-pro-VWF-EGFP in HUVECs, we used the relatively new technology of Nucleofection™, which electroporates DNA directly into the nucleus of cells. For the purposes of this experiment, a major advantage of this procedure is the short lag period between Nucleofection and protein expression, leading to a more synchronous expression of the transgene within the transfected cells.

The first appearance of green-fluorescent structures with the typical morphology of WPBs occurs between 4 and 5 hours after pre-pro-VWF-EGFP Nucleofection, in the pericentriolar region of the cells (Fig. 4B and green structures in Fig. 4C). At this early time after transfection, there may be many other (older) WPBs present within these cells [as shown by the number of Rab27-positive EGFP-negative structures seen in Fig. 4A,B (see asterisks for good examples)], but those WPBs containing the VWF-EGFP transgene must be newly formed (they did not exist at earlier time points). These newly formed WPBs are negative for endogenous Rab27a immunoreactivity (arrowheads in Fig. 4A,B). It is important to realise that the many Rab27-positive, EGFP-negative WPB that can clearly be seen in this example demonstrate that the absence of Rab27a on the newly formed (i.e. EGFP-positive) WPBs is not due to a lack of expression of Rab27a in this cell.

At the 24 hour time-point after Nucleofection with pre-pro-VWF-EGFP, the situation is markedly different (Fig. 4D-F). Just as for the steady-state labelling of endogenous WPB (Fig. 1), most of the EGFP-containing WPBs are now Rab27a immunoreactive (Fig. 4D-F; examples of double-labelled structures are indicated by arrows in Fig. 4D,E). There are still a few EGFP-positive structures that are negative for Rab27 (Fig. 4D,E, arrowheads), presumably reflecting the ongoing synthesis of the pre-pro-VWF-EGFP expression vector.

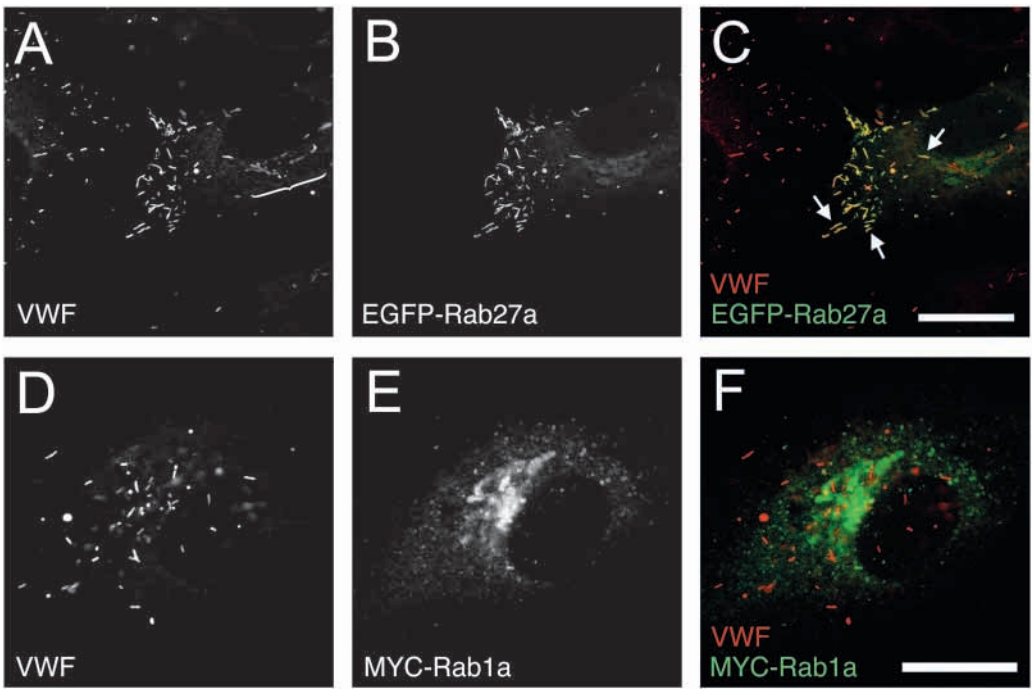

Fig. 3. Exogenously expressed EGFP-Rab27a localizes with WPB in HUVECs. HUVECs were transfected with expression vectors for either EGFP-Rab27a (A-C) or MYC-Rab1a (D-F) and, 24 hours later, fixed, permeabilized and immunolabelled for VWF (A-C) or VWF and the MYC epitope (D-F). VWF immunoreactivity is shown in (A,D) and is red in (C,F). EGFP fluorescence and MYC immunoreactivity are shown in (B) and (E), respectively, and are green in (C) and (F), respectively. A single EGFP-Rab27a-expressing cell can be seen in (B) and, in green, in (C). The arrows in (C) point to WPBs that are positive for both VWF and EGFP-Rab27a, and the curly bracket in (A) delineates a group of juxtanuclear WPBs that are EGFP-Rab27a negative. Scale bars, 20 μm (C,F).

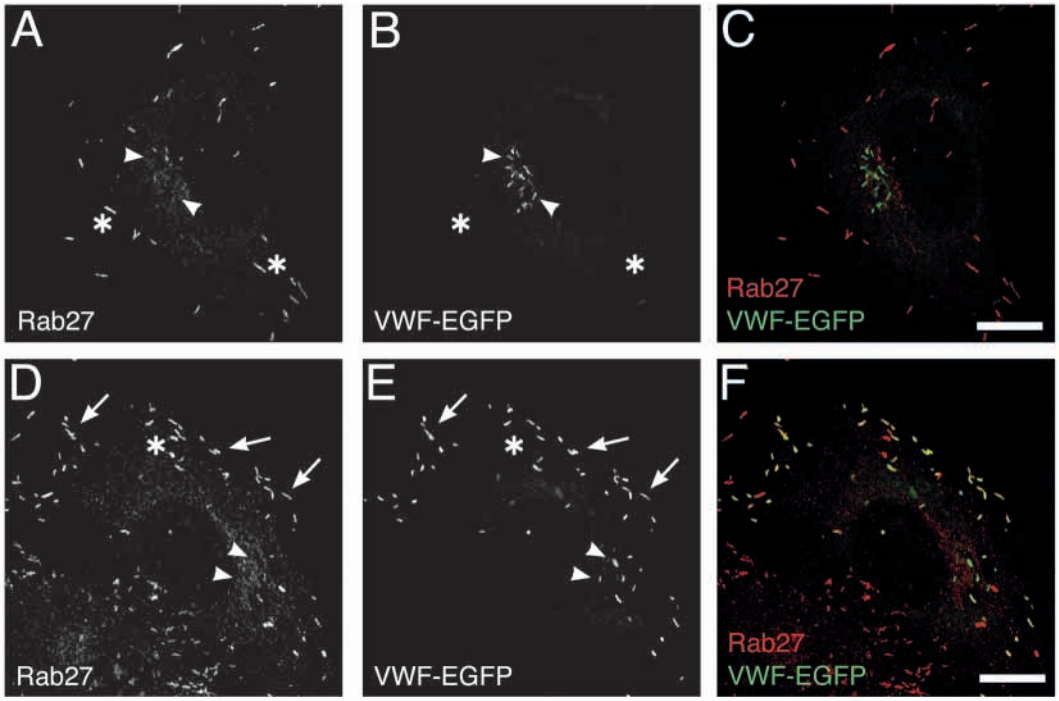

Fig. 4. Expression of a VWF-EGFP fusion protein in HUVECs reveals that newly formed WPBs are Rab27-negative. HUVECs were nucleofected with pre-pro-VWF-EGFP, replated onto gelatine-coated glass coverslips and allowed to recover for either 5 hours (A-C) or 24 hours (D-F), after which they were fixed and processed for immunocytochemistry as described in Materials and Methods. The images shown are single confocal sections of endogenous Rab27 immunoreactivity (A,D and red in C,F) and EGFP fluorescence/immunoreactivity (B,E and green in C,F). Arrowheads indicate examples of green-fluorescent WPBs that are not immunoreactive for Rab27, asterisks indicate examples of Rab27-immunoreactive WPBs that do not contain EGFP and arrows indicate examples of WPBs that are positive for both EGFP and Rab27 immunoreactivity. Scale bars, 10 μm.

Attempts to completely deplete the cell of these (VWF-EGFP positive, Rab27 negative) structures using the protein synthesis inhibitor cycloheximide were prevented by the acute toxicity of the drug on nucleofected HUVECs (data not shown).

A quantitative estimate of this delayed recruitment phenomenon was made by careful examination of two-colour confocal images such as those shown in Fig. 4 with the addition of another time point of 7 hours after nucleofection. EGFP-positive WPBs (WPBs were defined as appearing at least twice as long as they were wide) were counted and scored for Rab27a immunoreactivity. For each individual cell analysed, the number of Rab27a-positive, EGFP-positive WPBs was expressed as a percentage of the total number of EGFP-positive WPBs in that cell. The mean percentages (±s.e.m. and number of cells analysed) of EGFP-positive WPBs that were also Rab27a positive were 4.4±1.3% (n=29), 39.2±4.3% (n=22) and 80.8±2.9% (n=18) at the 5, 7 and 24 hour time points, respectively.

The simplest interpretation of these data is that WPBs emerge from the trans-Golgi in an immature form lacking Rab27a, and then undergo a maturation process that involves the acquisition of Rab27a. Because most WPBs appear to be Rab27a positive at steady state, we predict that Rab27a then remains associated with the organelle until it is consumed by exocytosis.

Recruitment of Rab27 is defined by the organelle and not by the cell type

As discussed above, VWF has the unusual and striking property that, when it is heterologously expressed in some cultured cell lines (such as HEK-293), it causes the formation of structures similar to bona fide WPBs (Wagner et al., 1991; Hannah et al., 2002). The extent to which Rab27a recruitment is driven by the organelle content and not by any other endothelial specific factors can thereby be studied by heterologous expression of VWF. Further, examination of the relative recruitment of Rab27a by the LRO-like WPB-like organelles versus true lysosomes will produce information about the relation between these two organelles and the ability of Rab27a to distinguish between LROs and lysosomes.

The localization of endogenous Rab27 immunoreactivity was assessed in HEK-293 cells with and without expression of VWF (Fig. 5). In mock transfected cells (Fig. 5A-D), we observed low but clearly detectable Rab27 immunoreactivity, which is often found enriched in a compact pericentriolar region probably corresponding to the microtubule organizing centre (Fig. 5A, asterisk). Staining of these cells with an antibody to a late endosomal/lysosomal protein, LAMP1 (Fig. 5B and red in Fig. 5D) demonstrates that although there is an occasional low level of association (arrows in Fig. 5A,B), there is little or no enrichment of Rab27 on lysosomes.

However, in HEK-293 cells expressing wild-type VWF (VWF-WT) (Fig. 5E-L) significant enrichment of endogenous Rab27 immunoreactivity can be found on the VWF-containing (Fig. 5G), WPB-like organelles that have been formed (see, for good examples of co-localization between VWF and Rab27 on elongated structures, Fig. 5E,G,I,K, arrows). Electron microscopy of cells treated in a parallel experiment confirmed

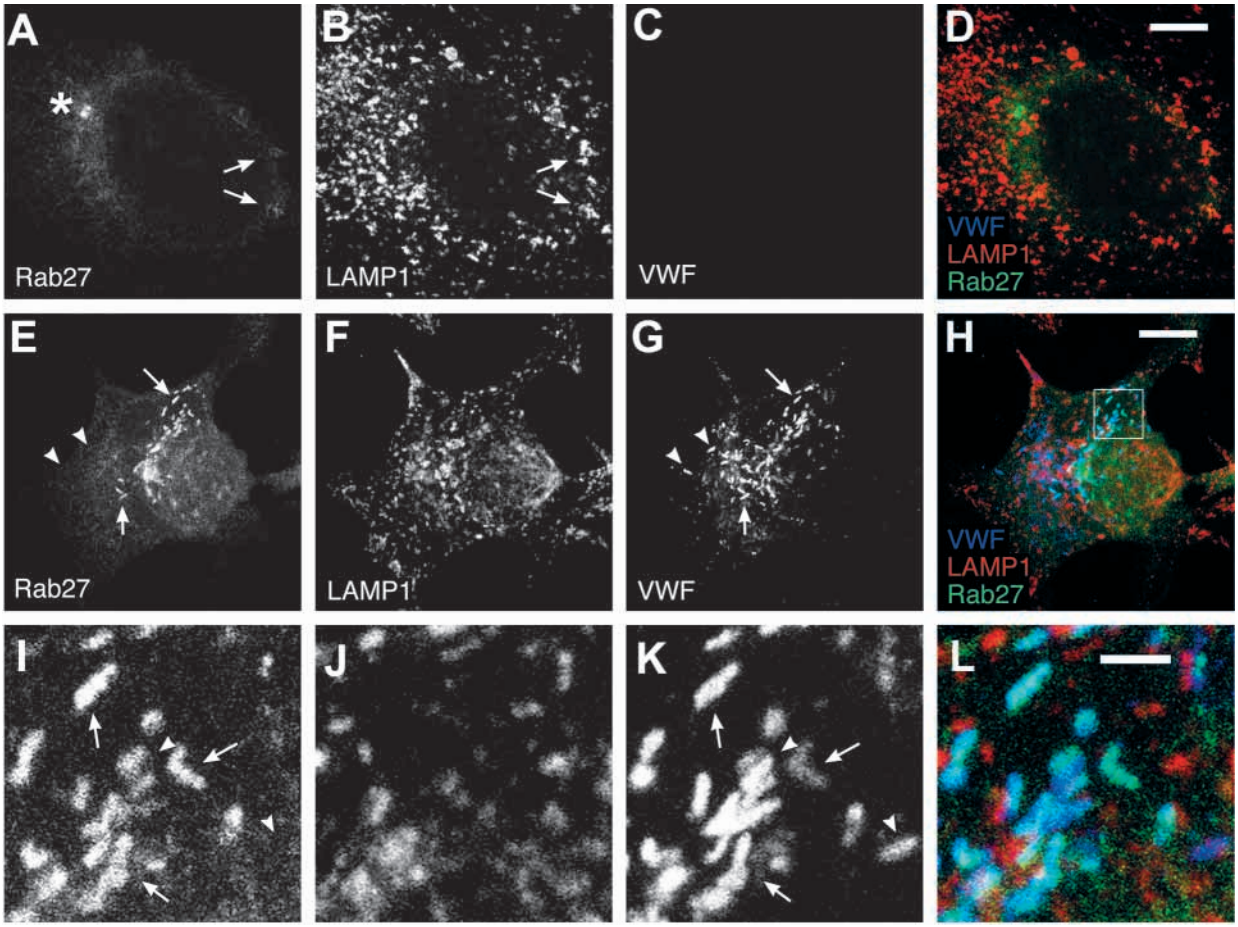

Fig. 5. In HEK-293 cells endogenous Rab27 immunoreactivity is not enriched on lysosomes, but it is recruited to the WPB-like organelles induced by expression of VWF. HEK-293 cells were either mock transfected (A-D) or transfected with VWF (E-L); after 48 hours, they were fixed and processed for immunocytochemistry as described in Materials and Methods. The images shown are composite confocal images of Rab27 (A,E,I and green in D,H,L), LAMP1 (B,F,J and red in D,H,L) and VWF (C,G,K and blue in D,H,L) immunoreactivities. The same confocal parameters were used to collect the data for both groups (transfected and untransfected). (I-L) An enlarged area from (H) (outlined by the white box). Asterisk in (A) indicates immunoreactivity associated with the microtubule organizing centre. Arrows in (A,B) indicate the slight association of Rab27 immunoreactivity with a minority of the LAMP1-positive structures. Arrows in (E,G,I,K) indicate examples of co-localization between the VWF-positive structures and Rab27, and the arrowheads point to examples of VWF-positive structures that are negative for Rab27 immunoreactivity. Scale bars, 10 μm (D,H), 2 μm (L).

that these structures induced by VWF-WT expression in HEK-293 cells had the characteristic WPB ultrastructure (Hannah et al., 2002). Interestingly, as seen above in HUVECS, it is also possible to find VWF-positive WPB-like structures that appear to be negative for Rab27 immunoreactivity (Fig. 5E,G,I,K, arrowheads).

We have already shown that, when overexpressed in HUVECs, EGFP-Rab27a is efficiently recruited by WPB (Fig. 3). However, what happens when the Rab is overexpressed in a cell type that doesn't have WPBs or any other known LRO to which it can be recruited? Under these conditions, is Rab27a recruited onto lysosomal membranes? When EGFP-Rab27a [or MYC-Rab27a (data not shown)] is expressed in HEK-293 cells, it is not enriched on LAMP1-positive organelles (Fig. 6A-D). Instead, the widespread fine reticulum and prominent nuclear envelope localization (Fig. 6A) indicates endoplasmic reticulum rather than late endosomes or lysosomes. Co-staining of EGFP-Rab27a expressing HEK-293 cells with a marker for endoplasmic reticulum (calnexin) confirmed that some of the Rab27a fusion protein was associated with the endoplasmic reticulum (data not shown). When HEK-293 cells are transfected with EGFP-Rab27a together with VWF-WT (Fig. 6E-L), the exogenous EGFP-Rab27a is, as expected, targeted to the membranes of the WPB-like organelles induced by the expression of VWF (Fig. 6E-L; see arrows in Fig. 6I,K for good examples of co-localization between VWF and EGFP). In addition to these examples of co-localization, there were also (as seen above in Fig. 3) a few VWF-positive structures that appeared to remain EGFP-Rab27a negative (Fig. 6I,K, arrowheads). Together, the HEK-293 data suggest that Rab27 is recruited by a cell-type-independent process driven by an LRO cargo protein, in this case VWF.

Discussion

In this paper, we show that WPBs are can recruit Rab27a efficiently. Hitherto, only P-selectin and CD63 were well-established membrane components of this poorly characterized

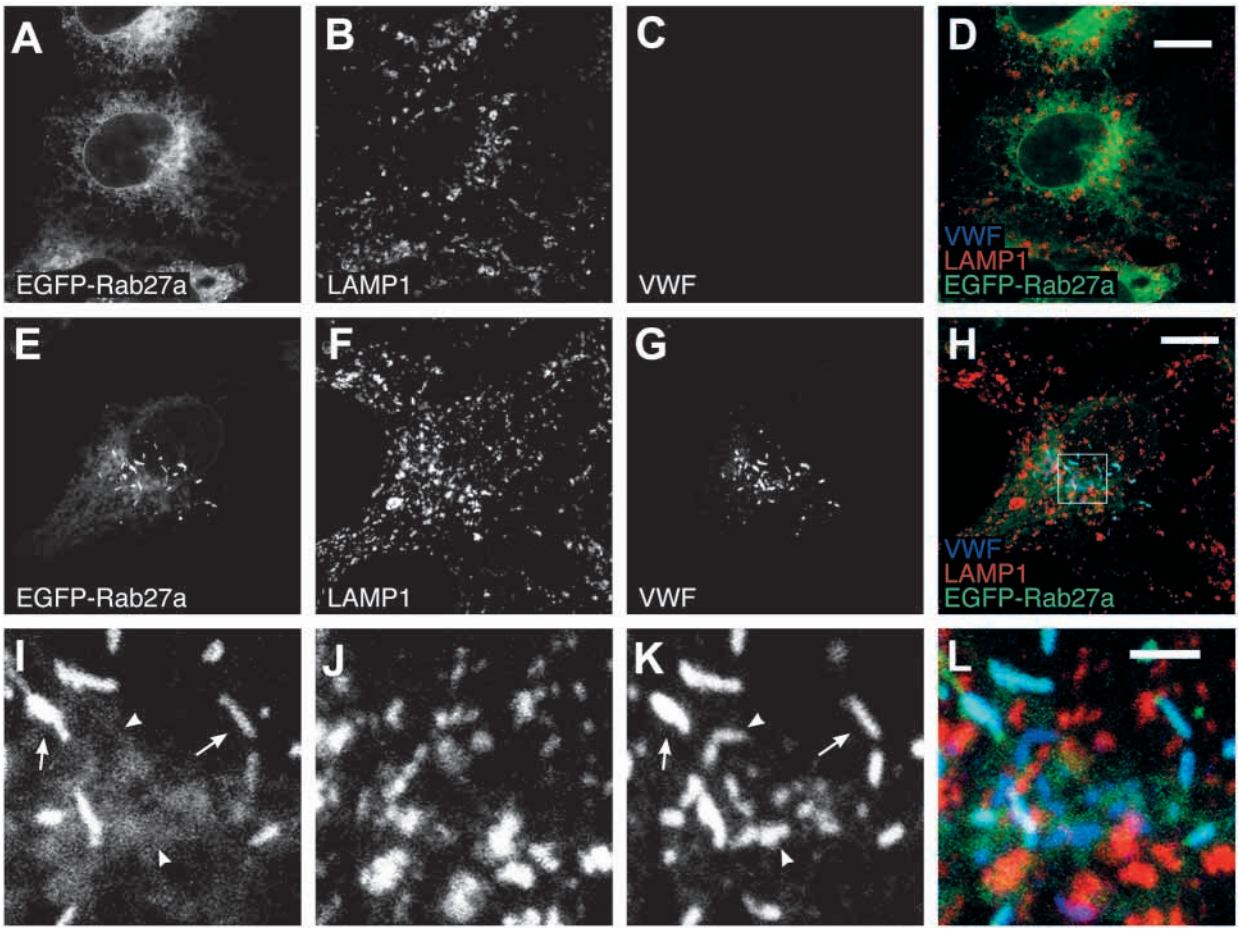

Fig. 6. Exogenously expressed EGFP-Rab27a localizes with WPB-like organelles and not lysosomes in HEK-293 cells. HEK-293 cells were transfected with either EGFP-Rab27a (A-D) or EGFP-Rab27a together with VWF-WT (E-L) and, after 48 hours, they were fixed and processed for immunocytochemistry as described in Materials and Methods. The images shown are composite confocal images of EGFP (Rab27a) (A,E,I and green in D,H,L), LAMP1 (B,F,J and red in D,H,L) and VWF (C,G,K and blue in D,H,L). The same confocal parameters were used to collect the data for both groups (plus and minus VWF-WT). (I-L) An enlarged area from (H) (outlined by the white box). Arrows in (I,K) indicate examples of co-localization between the VWF-positive structures and EGFP, and the arrowheads indicate examples of VWF-positive structures that are negative for EGFP. Scale bars, 10 μm (D,H), 2 μm (L).

organelle (Bonfanti et al., 1989; McEver et al., 1989; Vischer and Wagner, 1993). Rab27a thus provides a third membrane marker for these granules. We have exploited the ability of VWF to induce the formation of WPB-like organelles in non-endothelial cells to show that this content protein drives the recruitment of Rab27a in a process that is independent of cell type. We also find that the recruitment of Rab27a by WPBs is a delayed, time-dependent process that parallels the post-Golgi maturation of this secretory organelle. Finally, in the presence or absence of WPBs, Rab27a is not recruited by classical lysosomes.

Recruitment of Rab27

The recruitment of Rabs to organelles and, in particular, the mechanism that ensures the exquisite specificity of this phenomenon are poorly understood (Seabra, 1998; Pfeffer, 2001; Munro, 2002). Although the high specificity of recruitment points to protein-protein interactions, few examples of membrane protein-Rab interactions have been described.

Recently, an ability of cargo molecules to bind Rab proteins thereby affecting their own trafficking has been discovered (Smythe, 2002). However, a simple interaction between an itinerant membrane protein travelling between multiple organelles and a Rab seems unlikely to lead to the highly specific location seen for many Rabs. Perhaps combinatorial binding sites for interaction between a Rab and multiple membrane proteins, possibly along with organelle-specific post-translational modifications could provide sufficient increased specificity, as suggested by Pfeffer for other elements of the cytoplasmic trafficking machinery (Carroll et al., 2001; Pfeffer, 2001; Zerial and McBride, 2001).

The indirect recruitment driven by the lumenal VWF appears to be a new phenomenon. How it might occur is still unclear but the ability of VWF to influence its surrounding membrane has been previously documented.

Rab27a recruitment is not cell-type dependent

Our results in HEK-293 cells show that VWF-driven

recruitment of Rab27a is not dependent on endothelial-specific factors; not even haematopoietic cell-specific factors are required. The lack of dependence of LRO biogenesis and function on cell-type-specific machinery but rather on adapting widely expressed components has been seen previously. For example, the universally expressed adaptor protein complex AP3 (Robinson and Bonifacino, 2001) has been shown to play a major role in the biogenesis of many LROs (Dell'Angelica et al., 2000; Starcevic et al., 2002). Rab27 itself is widely expressed (Seabra et al., 1995; Chen et al., 1997) and is involved in the functioning of several LROs (Stinchcombe et al., 2001; Griffiths, 2002; Seabra et al., 2002) as well as secretory granules (Fukuda et al., 2002; Yi et al., 2002; Zhao et al., 2002).

Maturation-dependent recruitment of Rab27a

Our experiments with HUVECs show that a minor pericentriolar population of WPBs is Rab27a negative, whereas the more disperse major population of WPB is Rab27a positive. By following a wave of newly synthesized EGFP-tagged VWF through the secretory pathway of HUVECs into WPBs, we could show that the first cigar-shaped structures containing VWF-EGFP that emerge between 4 and 5 hours after nucleofection do so in the perinuclear region. These WPB are newly formed (they did not exist previously) and they are almost entirely Rab27 negative (<5% of the VWF-EGFP-containing WPBs are positive for Rab27 immunoreactivity). Only some time after their biogenesis do these EGFP-containing WPBs become Rab27 positive (about 40% are positive after a further 2 hours and 80% are positive the next day). A simple explanation for these data is that the WPBs are first formed in an immature, Rab27a-negative form, with the subsequent time-dependent Rab27a recruitment reflecting maturation of the organelle.

That WPBs take a considerable time to mature fully is already established, because Wagner and co-workers have shown that many hours are required for metabolically labelled VWF to reach a compartment that has the buoyant density characteristic of mature (i.e. secretagogue-responsive) WPBs (Reinders et al., 1984; Vischer and Wagner, 1994). However, because we have found the recruitment of Rab27a to be an explicitly VWF-dependent process, there must be some intra-WPB VWF-processing event that occurs during maturation of the organelle, triggering a change in the WPB membrane surface and consequently leading to the recruitment of the Rab. The processing of VWF is a complex process, beginning with dimerization of this very large protein in the ER and then subsequent cleavage of the pro region in the Golgi, probably by Furin, accompanied by oligomerization of the molecules to form entities with molecular weights in the millions of Daltons (for reviews, see Wagner, 1990; de Wit and van Mourik, 2001; Hannah et al., 2002). At present, we can only speculate that conformational changes caused by VWF oligomerization somehow lead to an altered interaction with the surrounding membrane that in turn mediates this indirect process. Irrespective of the mechanism, we now have a molecular basis for dividing WPBs into two populations (immature and mature).

VWF can organize its surrounding membrane

Our data provide further evidence of the ability of VWF to influence its local environment. The initial observation that VWF expressed in regulated secretory cells forms its own distinctive organelles first demonstrated this remarkable property (Wagner et al., 1991), which was extended to cells thought to lack a regulated secretory pathway (Voorberg et al., 1993; Haberichter et al., 2000; Hannah et al., 2002). Thus, specialized machinery required to assemble secretory granules is unnecessary for the formation of WPB-like organelles. More recently, we have shown not only that the VWF-like organelles only recruit appropriate membrane proteins but also that the recruitment of P-selectin is dependent on a cytoplasmic tyrosine motif (Blagoveshchenskaya et al., 2002). VWF can thus influence events on the other side (i.e. the cytoplasmic side) of its surrounding lipid bilayer, in a phenomenon akin to the recruitment of Rab27a demonstrated in this paper. These data are consistent with VWF interacting directly with lipid molecules in the WPB membrane to create a protein/lipid environment leading indirectly to Rab recruitment, similar to the way that the granins of neuroendocrine secretory granules are thought to operate (Pimplikar and Huttner, 1992; Thiele and Huttner, 1998).

LROs versus other organelles

We observed that Rab27a present within HEK-293 cells that lack WPB-like organelles does not co-localize with LAMP1, unlike many components of LRO membranes, which are targeted to the lysosome or other organelles of the late endocytic system when heterologously expressed (Vijayasaradhi et al., 1991; Green et al., 1994; Honing et al., 1998; Rous et al., 2002). VWF thus drives the formation of an organelle that is distinguished from lysosomes by the acquisition of Rab27a, despite sharing some lysosomal characteristics. These include the presence of the endosomal/lysosomal marker protein CD63 (Vischer and Wagner, 1993) and access to the organelle directly from the endocytic pathway (Kobayashi et al., 2000). In addition, the VWF-induced organelles are secretagogue responsive under conditions that do not allow the classical lysosomes to respond (Blagoveshchenskaya et al., 2002). Thus the VWF-containing structures are clearly not lysosomes and Rab27a, by specifically recognizing them, is actually identifying a different class of organelle.

If WPBs are not lysosomes, how should they be classified? Secretory granules are thought to form at the trans-Golgi, to be heavily influenced in their biogenesis by their content and to mature after budding, as well as to be regulated secretory organelles (Kelly, 1991; Tooze and Stinchcombe, 1992; Huttner et al., 1995; Arvan and Castle, 1998). These three characteristics appear also to be true of WPBs and WPB-like organelles. It has long been thought that WPBs form at the trans-Golgi (Matsuda and Sugiura, 1970; Sengel and Stoebner, 1970), they are clearly secretory organelles (Loesberg et al., 1983; McNiff and Gil, 1983; Reinders et al., 1984) and (as discussed above) VWF provides the most extreme example of a content protein controlling secretory organelle formation. WPB maturation involves a slow increase in density and secretagogue responsiveness (Reinders et al., 1984; Vischer and Wagner, 1994). We have demonstrated the post-budding acquisition of Rab27a to divide the WPBs into two populations. However, this phenomenon is different to that

of neuroendocrine granule maturation where the direct introduction of new components after budding from the Golgi has not yet been reported (Tooze and Stinchcombe, 1992). By contrast, LROs can acquire new components in post-Golgi trafficking, such as in melanocytes where membrane proteins can be delivered to late stage melanosomes (Raposo et al., 2001). The acquisition of Rab27a falls into this pattern of behaviour. WPBs and the WPB-like organelles thus seem to fall between the classical lysosomes and the secretory granules, and to be best classified as LROs.

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