Formation and function of Weibel-Palade bodies

Daniel J. Metcalf*, Thomas D. Nightingale*, Helen L. Zenner*, Winnie W. Lui-Roberts* and Daniel F. Cutler‡
MRC Laboratory of Molecular Cell Biology, Cell Biology Unit and Department of Biochemistry and Molecular Biology, University College London, Gower Street, London, WC1E 6BT, UK
*These authors contributed equally to this work
‡Author for correspondence (e-mail: d.cutler@ucl.ac.uk)

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Summary
Weibel-Palade bodies (WPBs) are secretory organelles used for post-synthesis storage in endothelial cells that can, very rapidly, be triggered to release their contents. They carry a variety of bioactive molecules that are needed to mount a rapid response to the complex environment of cells that line blood vessels. They store factors that are essential to haemostasis and inflammation, as well as factors that modulate vascular tonicity and angiogenesis. The number of WPBs and their precise content vary between endothelial tissues, reflecting their differing physiological circumstances. The particular functional demands of the highly multimerised haemostatic protein von Willebrand Factor (VWF), which is stored in WPBs as tubules until release, are responsible for the cigar shape of these granules. How VWF tubules drive the formation of these uniquely shaped organelles, and how WPB density increases during maturation, has recently been revealed by EM analysis using high-pressure freezing and freeze substitution. In addition, an AP1/clathrin coat has been found to be essential to WPB formation. Following recruitment of cargo at the TGN, there is a second wave of recruitment that delivers integral and peripheral membrane proteins to WPBs, some of which is AP3 dependent.

Key words: Weibel-Palade bodies, von Willebrand Factor, Haemostasis, Endothelium, Lysosome-related organelles, Exocytosis, High-pressure freezing

Introduction
Endothelial cells play a crucial role in a variety of biologically important phenomena, including haemostasis, control of vascular tonicity, inflammation, angiogenesis and wound healing. Since so many of these processes require an acute response, it is no surprise that an exocytic compartment sensitive to external stimulation is where key bioactive molecules involved in these processes are stored. The Weibel-Palade bodies (WPBs) of endothelial cells provide this storage. First described 40 years ago (Weibel and Palade, 1964), and used as a hallmark of endothelial cells, WPBs are large (1-6 μm long) cigar-shaped granules. Viewed by TEM of chemically fixed samples, WPBs have a regular striated appearance, reflecting their content of tubules made of VWF (Wagner et al., 1982).

Similarly to the dense-cored secretory granules of endocrine and neuroendocrine cells, they initially form at the TGN and subsequently undergo maturation, before accumulating within the cytoplasm, ready to undergo exocytosis in response to secretagogue stimulation. However, the presence of the late-endosome/lysosome marker CD63 on their limiting membrane (Vischer and Wagner, 1993), and the significant role played by AP3 in delivery of components during their maturation (Harrison-Lavoie et al., 2006), has led to the view that they should be classified as lysosome-related organelles rather than secretory granules.

Below, we discuss recent research into WPB formation that has highlighted the importance of the unique shape of WPBs to their function (Michaux et al., 2006a) and revealed an increasing role for WPBs in a number of physiological processes by identifying new components of these structures (Table 1).

VWF and WPB formation
The main constituent protein of WPBs is the haemostatic protein von Willebrand Factor (VWF). The importance of this protein to WPB formation is illustrated by heterologous expression of VWF: this one protein can drive the formation of pseudo-WPBs indistinguishable from those of endothelia in several non-endothelial cells (Blagoveshchenskaya et al., 2002; Michaux et al., 2003; Wagner et al., 1991).

VWF is a large multi-domain (Fig. 1A) multimeric glycoprotein that binds to GpIIb/GpIIIa receptors on platelets, as well as collagen, heparin and factor VIII (reviewed by Ruggeri, 2007). It is therefore involved in both primary and secondary haemostasis*. VWF is synthesised as a 350 kDa monomer, that includes a signal sequence (22 residues), pro-peptide (741 residues) and the mature protein (2050 residues). Following translocation into the ER, it dimerises through formation of disulphide bonds within its C-terminal cysteine knot (CK) domain (Fig. 1B). Also within the ER, the pro-peptide forms a transient intrachain disulphide linkage with the D3 region (Purvis and Sadler, 2004). This interaction precedes the subsequent multimerisation that occurs in the TGN. On arrival at the TGN, the propeptide-D3 domain intrachain disulphide bonds are replaced by interchain disulphide bonds between the D3 domains. During these events, the pro-peptide is thought to act as chaperone in a low-pH-dependent oxidoreductase reaction to promote disulphide bond formation (Sadler, 2005). VWF multimerisation can lead to very large molecules, up to 20 MDa in size. After cleavage by furin, the pro-peptide continues to engage in a pH-dependent interaction with the D′-D3 domains of the mature protein and is needed for the final folding of the VWF multimers into the structures seen as tubules by EM (Ewenstein et

*Primary haemostasis serves to limit bleeding immediately through the formation of a loose platelet plug. VWF binds both to platelets and to the sub-endothelial matrix to help form this plug. Secondary haemostasis involves a ‘coagulation cascade’ of enzymatic reactions that leads to formation of fibrin monomers. The monomers are then cross-linked into insoluble strands that stabilise the loose platelet plug into a clot. VWF acts as co-factor to a member of the cascade (Factor VIII), preventing its inactivation by proteolysis.

Table 1. Components of Weibel-Palade bodies

Component	Abbreviation	Protein	Site of recruitment	Expression regulated by	Function	References
von Willebrand factor	VWF	Secreted	TGN	–	Haemostasis	Wagner et al., 1982
P-selectin		Membrane	TGN	IL-4, Oncostatin M	Leukocyte binding, haemostasis	Bonfanti et al., 1989; Harrison-Lavoie et al., 2006
Osteoprotegerin	OPG	Secreted	TGN	TNFα, IL-1b	Leukocyte binding	Zannettino et al., 2005; Zauli et al., 2007
a1.3-Fucosyltransferase VI	a1.3 FucT VI	Secreted	TGN*	–	Selectin ligand modification – adhesion effects?	Schnyder-Candrian et al., 2000
Angiopoietin-2	Ang-2	Secreted	TGN*	–	Autocrine regulator enhancing TNFα sensitisation	Fiedler et al., 2006; Fiedler et al., 2004
Calcitonin gene-related peptide	CGRP	Secreted	TGN*	–	Possible vasodilator and upregulator of adhesion molecules	Doi et al., 2001; Ozaka et al., 1997; Smith et al., 1993
Endothelin-1 and endothelin-converting enzyme	ET-1 & ECE	Secreted	TGN*	–	Vasoconstriction	Doi et al., 2004; Doi et al., 2002; Nomiyama et al., 1998; Ozaka et al., 1997; Russell and Davenport, 1999; Russell et al., 1998a; Russell et al., 1998b; Sakamoto et al., 1993
Interleukin-8	IL-8	Secreted	TGN*	IL-1b, IL-1a, TNFα, LPS	Activates integrin-mediated adhesion of leukocytes	Oynebraten et al., 2004; Romani de Wit et al., 2003a; Utgaard et al., 1998; Wolff et al., 1998
Eotaxin-3		Secreted	TGN*	IL-4	Leukocyte recruitment	Oynebraten et al., 2004
CD63		Membrane	Post budding	–	Membrane organisation/ integrin interaction	Harrison-Lavoie et al., 2006; Vischer and Wagner, 1993
Rab3D		Membrane-associated	Post budding	–	WPBs size regulation	Knop et al., 2004
Rab27A		Membrane-associated	Post budding	–	General LRO maturation or exocytosis role?	Hannah et al., 2003

*These components are presumed to be recruited at the TGN as they colocalise with all populations of WPBs; however, direct evidence remains to be established.

al., 1987; Vischer and Wagner, 1994; Wagner et al., 1987; Wagner et al., 1986; Wise et al., 1991). The free pro-peptide can be found as a dimer, and it does bind in a pH-dependent manner to the N-terminal region of mature VWF. This leads us to hypothesise that it might bring the N-termini of the VWF multimer together to form loops in a coiled structure that could appear as tubules (Fig. 1B).

The most highly multimerised forms of VWF are stored within WPB, whereas lower molecular weight variants are released constitutively (Sporn et al., 1986). Regulated exocytosis of WPBs in endothelial cells activated, for example, by vascular injury, leads to release of VWF into the bloodstream as strings up to several millimetres long (Dong et al., 2002). These platelet-binding strings are likely to play a significant role in forming a haemostatic plug. The most highly pro-thrombotic ultra-large VWF is subsequently and rapidly reduced to its normal plasma molecular weight range through cleavage by the plasma metalloprotease ADAMTS13 (Dong et al., 2002; Plaimauer et al., 2002). The lower molecular weight material released constitutively may be secreted apically into plasma to assist in thrombus formation and to bind factor VIII [although see arguments in Haberichter et al. (Haberichter et al., 2006)], thereby protecting it from degradation by plasma proteases. Alternatively, it may also be secreted basally to bind the subendothelial matrix and thereby assist in forming the haemostatic plug.

The formation of interchain disulphide bonds is critical to the function of VWF because a failure to multimerise causes the bleeding disorder von Willebrand disease (VWD) type 2A. However, multimerisation does not correlate with the ability of VWF to drive the formation of the cigar-shaped storage organelle, which instead depends on its folding into tubules (see below). In HEK293 cells, AtT20 or canine VWD aortic endothelial cells, expression of full-length VWF leads to formation of cigar-shaped storage organelles, as does expression of the pro-peptide plus the mature protein together *in trans*. Expression of either the pro-peptide or the mature protein (with a signal sequence for co-translational translocation added at the N-terminus) alone does not result in the formation of elongated WPBs (Voorberg et al., 1993). However, very surprisingly, expression of the pro-peptide plus a truncated mature protein that lacks the CK domain and therefore cannot undergo C-terminal dimerisation, can still lead to the formation of cigar-shaped organelles with tubules. This is a surprise because the protein can dimerise via N-terminal interactions but cannot undergo C-terminal dimersation, and will thus form dimers but nothing larger (Haberichter et al., 2000; Michaux et al., 2006a; Voorberg et al., 1993; Wagner et al., 1991). Thus the presence of both the pro-peptide and the mature protein, in a low pH environment where they can interact, plus N-terminal

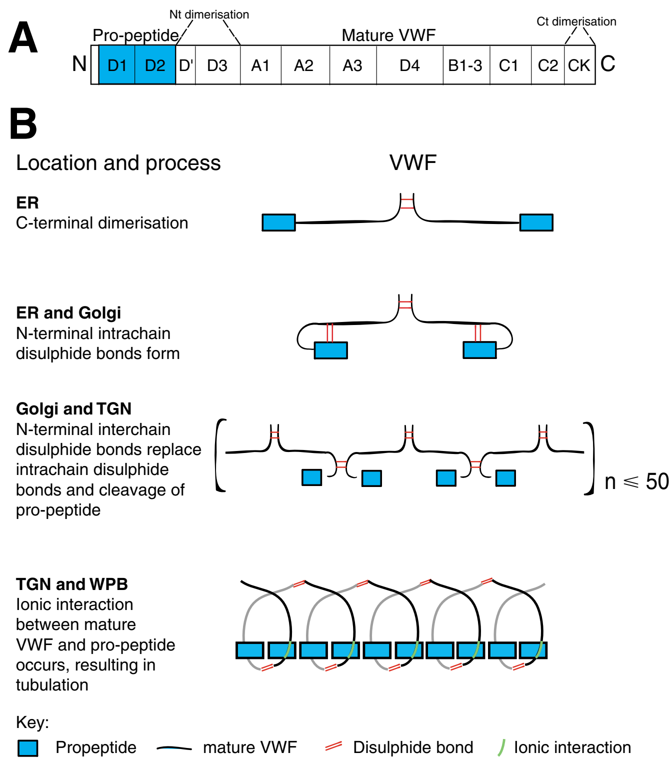

Fig. 1. Biosynthesis of VWF. (A) Block diagram showing the domains of VWF (D1-C), and the regions of the mature protein (D′-CK) involved in dimerisation. Nt, N-terminus; Ct, C-terminus. (B) Working model describing main events occurring during formation of VWF tubules and multimers, with intracellular locations.

dimerisation, appears sufficient to drive the formation of a compartment that is still recognisable as a WPB.

Four lines of evidence support a requirement for tubulation but not multimerisation in elongation of WPBs. First, treatment of endothelial cells with monensin [which will disrupt the pH-dependent interaction between the pro-peptide and mature VWF (Mayadas and Wagner, 1989) but should have no effect on the preformed disulfide bonds] causes disruption of tubular structures and dramatic rounding of post-Golgi WPBs. Second, the converse experiment – disruption of disulfide bonds using DTT – has no effect on the shape of the WPBs (Michaux et al., 2006a). Third, heterologous expression of two human variants of VWF – R273W and C788R – that have similar multimerisation defects results in an almost total abolition of elongation in the case of the R273W variant but has no significant effect on WPB shape in the case of the C788R variant (Michaux et al., 2003). Finally, truncations of VWF that lead it to form only dimers (see above) do not abolish tubule formation and generation of cigar-shaped WPBs (Michaux et al., 2006a). The intimate relationship between multimerisation and tubulation, both requiring an interaction of the pro-peptide with the mature protein, may explain why mutations affecting multimerisation can also affect tubulation. For example the clinically significant point mutation within the pro-peptide Y87S, which reduces multimerisation, also reduces WPB elongation when overexpressed against a background of wild-type VWF in HUVECs (Haberichter et al., 2005; Michaux et al., 2006a; Rosenberg et al., 2002). Although it is the storage of VWF as proteinacious tubules within the WPBs, rather than multimerisation, that is responsible for the characteristic elongated shape of the VWF organelle (Michaux et al., 2006a; Wagner et al., 1991), it is very hard to see how such tubules can be assembled from dimers, and much further work in this area is needed.

Both tubulation and multimerisation are functionally important. Too low a degree of multimerisation or extra-high multimerisation both cause haemostatic problems: in the former case a failure to form clots causes bleeding (von Willebrand Disease); in the latter case, VWF can be too pro-thrombotic. Indeed, mutations in ADAMTS13 or acquired autoimmunity to this VWF-cleaving protease can lead to thrombotic thrombocytopenic purpura (TTP) (reviewed by Plaimauer et al., 2002; Porta et al., 1999). TTP is a lethal disorder in which platelets are caught up into thrombi that need to be removed by plasmaphoresis; otherwise death ensues from the consequences of multiple microvascular occlusions (Moake, 2007). Multimerisation must also be required for the formation of the long platelet-decorated VWF strings that can be seen attached to the surface of endothelial cells immediately after WPB exocytosis (Michaux et al., 2006a). Note, however, that this has not yet been directly tested.

Tubulation allows a 100-fold compaction of VWF, without which intracellular storage of VWF would be impossible. It avoids tangles forming within the VWF strings. When VWF tubules are disrupted before exocytosis by neutralisation of the acidic intra-WPB pH by treatment with monensin, chloroquine or NH_4Cl, shortened tangled strings that have reduced platelet-binding capacity are released from endothelial cells (Michaux et al., 2006a). This phenomenon represents a kind of premature unfolding: during normal exocytosis, the shift in pH from pH 5.5 within the WPBs (Erent et al., 2007) to the neutral pH of plasma leads to the unfolding of VWF tubules to generate strings; unfolding within the confines of the WPB leads to tangling. Michaux et al. (Michaux et al., 2006a) suggest that the rise in pH, causing the pro-peptide to be released from the mature protein, allows the chain of VWF multimers to unfold in an orderly manner.

New insights into the compaction of VWF have come through the use of high-pressure freezing and freeze substitution (HPF/FS) followed by electron microscopy (Fig. 2). The earliest stage in the secretory pathway that VWF tubules are observed is in the TGN, where cleavage of VWF occurs and the environment is increasingly acidic (Zenner et al., 2007). Thus, tubulation precedes formation of the independent WPB and so may play a role in membrane deformation early in biogenesis, as well as in maintenance of the elongated shape of the WPBs. Previous EM analyses of samples chemically fixed to crosslink proteins made the VWF tubules appear orderly, but the use of HPF/FS indicates that the relationship between the tubules and the membrane is complex. In immature WPBs, the membrane is not directly wrapped around the tubules, such that the tubules do not contact the membrane except at the tips (Fig. 2B,C). However, the consistent relationship between the width of the WPBs and the number of internal tubules suggests that some kind of interaction (positive or negative) between the membrane and tubules and between the tubules themselves occurs. There seems to be no requirement for individual tubules to extend from end to end of the WPBs, which indicates that the tubules must either interact with each other or with additional components in the organelle to maintain the shape of the WPBs.

HPF/FS has also made very clear that mature WPBs (Fig. 2D) show a dramatic increase in electron density compared with the immature WPBs, partly because of a much tighter, more highly

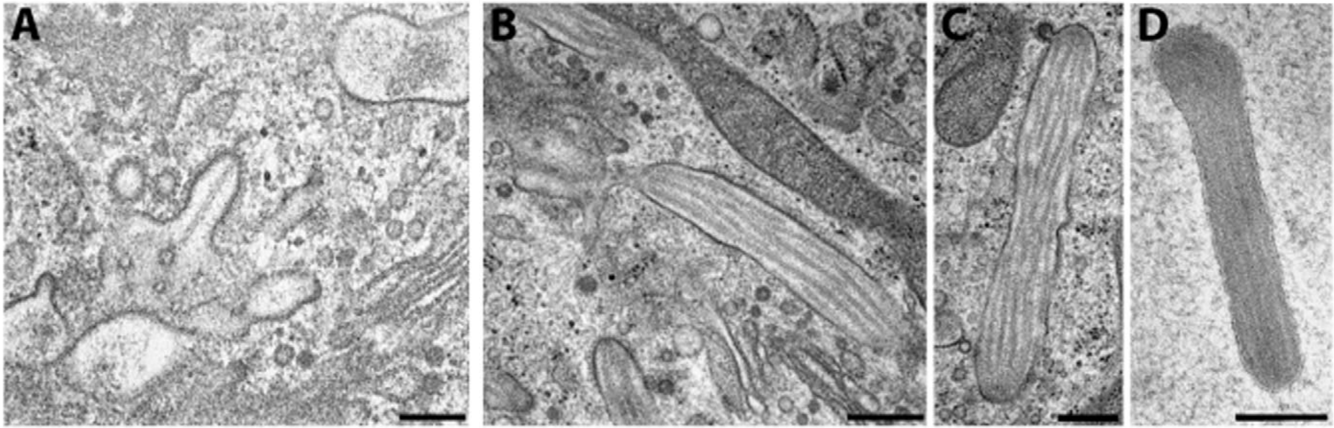

Fig. 2. Biogenesis of WPBs in cultured human endothelial cells seen by HPF/FS. (A) Tubule formation in the TGN and the cooperation of AP1 and clathrin in initial formation. (B) Immature WPB with electron-lucent interior, but with a membranous stalk still attaching it to the TGN. (C) Immature WPB with clathrin-coated bud, which is presumably involved in retrieval of material not required in the mature WPBs. (D) Mature WPB showing the remarkable increase in electron density that occurs during maturation. Bars, 200 nm.

organised packing of VWF tubules (Zenner et al., 2007). The high degree of organisation in the WPBs competent for release is likely to be important for forming long strings rather than tangles of VWF. Compaction and organisation of VWF thus seems to be an ongoing process, beginning with tubulation and multimerisation in the TGN, followed by further multimerisation in WPBs, and culminating as the tubules themselves become compacted as the organelle matures. The compression of the space between the tubules may not fully explain the increase in electron density; a search for new components recruited to the peripheral WPBs may thus be warranted.

Moving images of WPBs (Romani de Wit et al., 2003b; Zenner et al., 2007) suggest that although the WPBs are unable to curl they can bend at hinge points. These hinge points coincide with an interruption to the VWF tubules; tubules do not turn the corner in parallel with the limiting membrane but stop at the bend. Each piece of the bending cigar has its own set of tubules that roughly parallel the long axis of that individual section. The hinges may allow WPBs to move around the cell effectively (Zenner et al., 2007). Many of these observations have recently been confirmed by a tomographic analysis of WPBs (Valentijn et al., 2007).

Role of AP1 in WPB formation

The ability of VWF to drive the formation of pseudo-WPBs by heterologous expression is consistent with the formation of WPBs being driven by a selective aggregation of contents. This supports the model of secretory granule biogenesis that relies on sorting by selective retention (reviewed by Arvan and Castle, 1998). However, cytoplasmic machinery is also crucial for WPB formation. Clathrin and the heterotetrameric adaptor protein (AP) complex AP1 help form vesicles that transport proteins between post-Golgi compartments (reviewed by Edeling et al., 2006; Traub, 2005) and recently were also implicated in WPB biogenesis (Lui-Roberts et al., 2005). Immature WPBs in the perinuclear region of the cell are extensively coated with clathrin and AP1. The extent to which the immature WPB surface is covered with clathrin suggests a structural role for this coat. By disrupting clathrin function using a dominant negative AP180 construct (Ford et al., 2001) or interfering with AP1 function using an siRNA (Hirst et al., 2003), one can inhibit WPB formation and regulated secretion of VWF (Lui-Roberts et al., 2005). Interference with AP1/clathrin function prevents the formation of the large cigar-shaped structures. Instead, small rounded VWF-positive punctae are seen within the cytoplasm by light microscopy, and secretion through the constitutive secretory pathway is increased (Lui-Roberts et al., 2005). Since there is a very clear correlation between the cigar shape of the WPB and the presence of tubules, the implication of these data is that folding VWF into tubules requires AP1/clathrin. Once a WPB is properly formed, it does not require the coat to maintain its cigar shape, because brefeldin A treatment, which releases all clathrin coats (Robinson and Kreis, 1992), does not deform preformed WPBs (Lui-Roberts et al., 2005).

The AP1/clathrin coat might provide a stabilising scaffold for the initial folding of VWF into the tubules that drives the formation of cigar-shaped WPBs (Lui-Roberts et al., 2005). The extensive coating on forming WPBs is consistent with this hypothesis, as are images such as that in Fig. 2A showing that the elongated bud surrounding a newly forming tubule is coated. In this latter case, it almost looks as though the tubule is preventing scission, allowing the extension of a rounded bud into the elongated shape seen here – could this be the very beginning of WPB formation? This role for AP1/clathrin, as with the shape of the WPB, may be endothelial specific, since a dominant negative clathrin hub construct expressed in pancreatic β-cells affects neither the storage nor the stimulated secretion of insulin (Molinete et al., 2001). WPBs may well be different from conventional secretory granules.

The way in which clathrin is used in formation of WPBs – both in the scale and the curvature of the coating – is different from its role in formation of small 'conventional' transport vesicles. Whether any of the known AP1 effectors [e.g. aftiphilin (Mattera et al., 2004), γ-synergin (Page et al., 1999), p200 (Lui et al., 2003), epsinR (Hirst et al., 2003; Kalthoff et al., 2002; Mills et al., 2003; Wasiak et al., 2002) and PACS1 (Crump et al., 2003)] are involved in the adaptor complex used to form WPBs is not yet established. Indeed, WPBs may be a fruitful context for screens for novel AP1-interacting proteins. In addition, since heterologous expression of VWF can drive WPB formation, which is in turn dependent on AP1, it will be interesting to discover how a soluble cargo protein might drive the recruitment of a protein complex to the other side of the membrane that surrounds it. Finally, HPF/FS analyses

(Zenner et al., 2007) have revealed that clathrin-coated buds are present on immature WPBs. These (Fig. 1C) are likely to be involved in removal of material during maturation as in endocrine/neuroendocrine granule maturation (Dittie et al., 1999; Klumperman et al., 1998). However, which adaptor complex is involved and what the cargo might be are as yet not known.

Recruitment of WPB components at the TGN

Some components of WPBs, such as the secretory proteins angiopoietin-2, eotaxin, osteoprotegerin, but also the integral membrane protein leukocyte receptor P-selectin, are recruited during initial organellar formation at the TGN (Fig. 3). CD63, Rab27A and Rab3D, however, are recruited after budding from the TGN, possibly when the transition from an electron-lucent to an electron-dense internal structure occurs (see Table 1 and Fig. 3). Although the biosynthetic targeting of soluble secretory proteins to forming WPBs has not been studied in depth, if sorting-by-retention occurs (Arvan and Castle, 1998) then direct interactions between these bioactive molecules and VWF would be predicted. For osteoprotegerin, this has proven to be the case (Shahbazi et al., 2007; Zannettino et al., 2005).

P-selectin targeting is more complex, since both newly synthesised and recycled protein are recruited via the TGN (Arribas and Cutler, 2000; Subramaniam et al., 1993). The cytoplasmic tail of P-selectin has two motifs that are critical for recycling: KCPL, which directs movement from early to late endosomes; and YGVF, which targets it from endosomes to the TGN (Harrison-Lavoie et al., 2006). Once P-selectin arrives in the TGN, it can be recruited by a direct physical interaction with VWF. The lumenal domain of P-selectin binds to the D′-D3 domains of VWF (Michaux et al., 2006b) and expressing the lumenal domain of P-selectin alone in HUVECs is sufficient for targeting to WPBs (Harrison-Lavoie et al., 2006). However, a P-selectin chimera in which the lumenal domain has been replaced by the enzymatic reporter horseradish peroxidase is also recruited to WPBs. This may be because the cytoplasmic tail can direct the chimera (via the recycling route) to the TGN, from where it may be incorporated by an indirect mechanism. Loss of the lumenal domain, and hence complete dependence on the cytoplasmic motifs, makes recruitment of the chimera to WPBs slower than when direct binding of VWF is possible. One additional feature of P-selectin recruitment is that it is AP1 dependent. The VWF-positive punctae seen in AP1-deficient endothelial cells no longer recruit P-selectin (Lui-Roberts et al., 2005). This change in shape from large cigar-shaped granules to small secretory vesicles also correlates with a loss of the ability to fold VWF into tubules. We speculate that folding VWF into tubules in the TGN also forms a P-selectin-binding site within this protein.

A second wave of recruitment to mature WPBs

Several components are delivered to mature WPBs. At least one, the tetraspanin CD63, is dependent on AP3 – in contrast to the AP1-dependent recruitment of P-selectin to newly forming WPBs (Fig. 3). AP3 is an adaptor involved in targeting of proteins from early endosomes to lysosomes and lysosome-related organelles (reviewed by Raposo et al., 2007; Robinson and Bonifacino, 2001) as well as synaptic vesicles (Newell-Litwa et al., 2007). The presence of CD63 within the membrane of WPBs and their ability to recruit components after budding from the TGN suggest that WPBs may be regarded as lysosome-related secretory organelles, although the primary role of VWF in driving their formation at the TGN might justify classifying them as also being close to classical

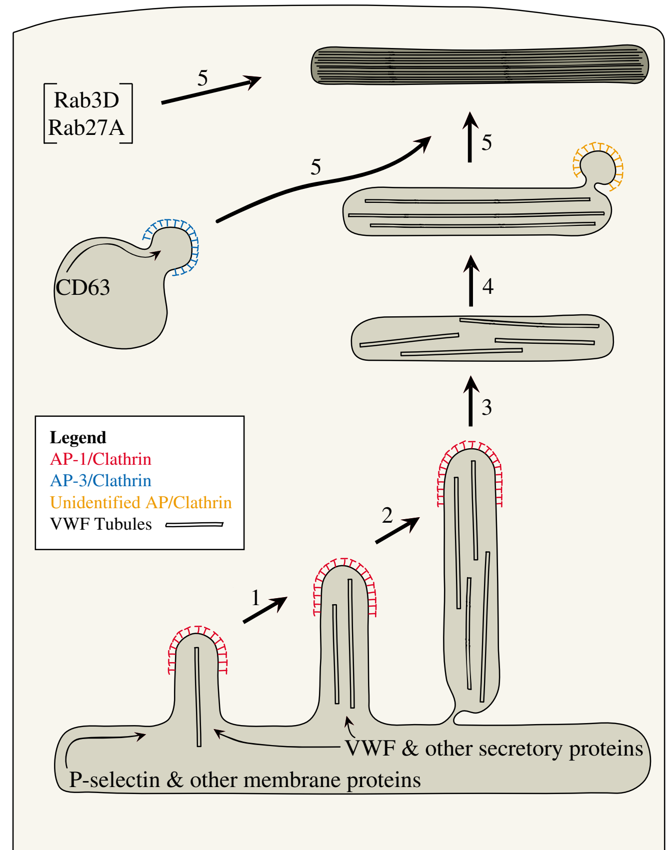

Fig. 3. Diagrammatic representation of the biogenesis of WPBs. (1) VWF tubulation and AP1/clathrin coats are necessary for WPB formation at the TGN. P-selectin is incorporated into WPBs by a direct interaction with VWF. Some other integral membrane proteins may also be recruited at this point. (2) Further extension of the WPBs from the TGN occurs. Tubulation of VWF continues while the WPB remains attached to the TGN by a 'stalk-like' connection. (3) The immature WPB is in the perinuclear region of the cell. Uncoating of clathrin occurs, although the tubules remain disorganised. (4) VWF tubules appear longer and more ordered. Clathrin-coated buds can be seen, often towards the tips of WPBs; this is presumably a sorting step to remove misdirected proteins. (5) Mature WPBs are identified at the ultrastructural level by their electron density and tight packing of VWF tubules, whereas at the light-microscopic level the recruitment of Rab27a, Rab3D, as well as the AP3-dependent recruitment of CD63 are the distinguishing features in this final maturation step. Although in both cases the WPBs are now in the periphery of the cell, whether these steps precisely coincide is not yet known.

secretory granules. Harrison-Lavoie et al. (Harrison-Lavoie et al., 2006) found that AP3 depletion interferes with the ability of WPBs to recruit CD63. CD63 is ubiquitously expressed (Hunziker and Geuze, 1996) and is found in late endosomes/lysosomes and also within secretory lysosomes of haemopoietic cells (Stinchcombe and Griffiths, 1999). In HUVECs it shows a universal late endosome/lysosome distribution as well as being seen on the limiting membrane of WPBs (Vischer and Wagner, 1993); loss of AP3 leads to it being found exclusively on the former.

The function of CD63 is unclear, but it is a member of the tetraspanin family. These proteins have been shown to associate with integrins, various Ig superfamily members and other tetraspanins, organising themselves and their interacting proteins into membrane microdomains (reviewed in Hemler, 2005). Integrins play a key role in leukocyte recruitment thus the presence

of an integrin-binding tetraspanin within WPB suggests that it may play a role in early stages of the endothelial inflammatory response. However, the functional consequences of losing CD63 from WPBs are, as yet, unclear.

The recruitment of the small GTPase Rab27A is also maturation dependent (Fig. 3). Rab27A has been found on many lysosome-related organelles [for an overview of LROs see Dell'Angelica et al. (Dell'Angelica et al., 2000) and Stinchcombe et al. (Stinchcombe et al., 2004)] – organelles that are essentially secretory endosomes or lysosomes modified by the cell-type-specific expression of certain cargo (Raposo et al., 2007). Rab27A has also been found on classical secretory granules and may be involved in most secretory events (Tolmachova et al., 2004). It operates through many different effectors to control the behaviour of organelles, including their movement and exocytosis. The function of Rab27A on WPB has not yet been determined.

Immunofluorescence microscopy shows that Rab27A is on all WPBs in HUVECs except for a population of newly formed perinuclear WPBs (Hannah et al., 2003). At steady state, most WPBs are Rab27A positive, which indicates that Rab27A probably stays on WPBs until they undergo exocytosis. Although the mechanism of recruitment of Rab27A is unknown, it is interesting to note that recruitment is cargo driven, and cell-type independent (Hannah et al., 2003). Rab3D has also been reported to be a WPB component (Knop et al., 2004) but may be present on mature WPBs only (D.F.C., unpublished data). Rab3D has been implicated in regulated secretion in other systems, such as adrenocorticotrophic hormone secretion in AtT-20 cells and calcium-triggered exocytosis in stimulated PC12 cells (Baldini et al., 1998; Schluter et al., 2002). Overexpression of Rab3D mutants in HUVECs (Knop et al., 2004) affects WPB formation and exocytosis of VWF by as-yet-unknown mechanism(s).

Exocytosis

As WPBs mature, they become responsive to secretagogues such as thrombin and histamine [see Rondaij et al. (Rondaij et al., 2006) for a comprehensive list of secretagogues]. Generally, these act by increasing intracellular Ca^{2+} levels or stimulating production of cAMP (reviewed by Rondaij et al., 2006). This release can occur very rapidly, the time between free [Ca^{2+}] increase and WPB release being as little as 1.6±0.2 seconds (Erent et al., 2007). Recent research has highlighted additional WPB secretagogues. The activation of Toll-like receptor 2 by lipoteichoic acid causes the release of WPB, which suggests a potential link between innate recognition of bacteria and vascular inflammation (Into et al., 2007). Also antibodies against human leukocyte antigen, present during organ transplantation, can trigger WPB release and this may upregulate an inflammatory response by causing the appearance of P-selectin at the endothelial cell surface (Yamakuchi et al., 2007).

The exocytic machinery involved in WPB release is currently poorly characterised (reviewed by Lowenstein et al., 2005). Two GTPases have been shown to have a role in WPB release: Rab3D and RalA. When wild-type or activated Rab3D is overexpressed it causes an inhibition of WPB release, which suggests a negative regulatory role of Rab3D in exocytosis (Knop et al., 2004). By contrast, the exocyst-associated GTPase RalA (Wang et al., 2004) has a positive regulatory role in exocytosis, since overexpression of a constitutively active RalA causes the release of WPBs (de Leeuw et al., 1999; Rondaij et al., 2004). Three SNARE proteins have been implicated in WPB exocytosis: syntaxin 4, VAMP3 and SNAP23 (Fu et al., 2005; Matsushita et al., 2003). Antibodies against syntaxin 4 reduce WPBS exocytosis by 75% whereas an antibody against VAMP1, VAMP2 and VAMP3 (only the latter is expressed in endothelial cells) reduces exocytosis by 25% (Matsushita et al., 2003).

Variations in WPBs

Endothelial cells are not a homogenous cell type; marked heterogeneity between cells from the lymphatic and blood vascular lineages and at different vessel calibre in the vascular tree is apparent both from microarray analysis (Chi et al., 2003; Hendrickx et al., 2004; Hirakawa et al., 2003) and from immunohistochemical staining (Pusztaszeri et al., 2006) (reviewed by Aird, 2007a). Organ-specific variation (Aird, 2007b) also occurs and certain specialised endothelia, such as alveolar capillary endothelial cells, lack VWF and therefore possess no WPBs (Pusztaszeri et al., 2006). Finally VWF expression levels (and thus presumably WPB number) vary with vessel type, veins exhibiting higher message levels than arteries (Chi et al., 2003; Yamamoto, 1998).

The release of VWF from WPBs is controlled by multiple factors in addition to transcription. Factors involved in WPB biogenesis and protein sorting, in the secretagogue-stimulated signal for exocytosis and in the exocytic machinery will all affect VWF release. These may vary from vascular bed to vascular bed and with vessel diameter. One example is the $Ca_v3.1$ T-Type Ca^{2+} channel that is expressed in microvascular but not macrovascular endothelial cells of the lung (Zhou et al., 2007). Thrombin-induced activation and pro-coagulant effects thus occur only in microvascular cells (Wu et al., 2003). The release of VWF from WPBs is also governed by the activation status of endothelia; VWF levels in plasma are markedly higher in chronically activated endothelium such as in patients infected with malaria (de Mast et al., 2007; Hollestelle et al., 2006). The trigger for this activation is unclear and may be due to inflammatory cytokines or due to a direct stimulation from parasitized red blood cells (de Mast et al., 2007).

The endothelium receives multiple stimuli: from surrounding cell types; from molecules and cells in the blood stream (Aird, 2005); and from shear force (Fujiwara, 2006). Such signals may be pro-inflammatory, pro-angiogenic, pro-thrombotic and fibrinolytic. Exocytosis of WPBs is stimulated by both pro-angiogenic stimuli (Bhatia et al., 2004; Matsushita et al., 2003; Matsushita et al., 2005) and pro-thrombotic stimuli (Hamilton and Sims, 1987; Levine et al., 1982) and additionally they have been reported to contain both fibrinolytic and pro-coagulant, and vasodilatory and vasoconstrictory cargo. This suggests either that formation and release of WPBs with specific contents must be tightly controlled or that the endothelial or surrounding cells have a means to counteract/control the inappropriate constituents.

Interestingly, endothelial cells appear to tailor the contents of their WPBs in response to various stimuli. Certain cargo is constitutively carried in WPBs whereas other constituents appear within WPBs only following an inflammatory stimulus such as TNFα or IL1 (see Table 1). The presence of these constituents in WPBs indicates the necessity for a rapidly releasable pool of that protein for an endothelial response. In the case of P-selectin, for example, the requirement for an immediate releasable pool is for the rapid recruitment of leukocytes.

A further complication is that more than one WPB population may be present in a single cell, each having different constituents, different secretagogue responsiveness and therefore a different

function. For example, Cleator et al. have reported that P-selectin and VWF can be differentially released from HUVECs (Cleator et al., 2006). HUVECs stimulated with cAMP or an agonist peptide that binds to protease-activated receptor 2 (PAR2) display a delayed release of VWF and a reduced release of P-selectin compared with HUVECs stimulated with histamine, thrombin, or agonist peptides that bind to PAR1. In addition, co-storage of P-selectin and angiopoietin 2 in the same WPB does not occur (Fiedler et al., 2004) even though both P-selectin and angiopoietin 2 are essential for a normal inflammatory response (Fiedler et al., 2006), are stored in WPB and are expressed in endothelial cells (in the case of angiopoietin 2 following an inflammatory stimulus) (Fiedler et al., 2004). Such findings suggest a further level of complexity in the control of WPB biogenesis, perhaps similar to that in other cells that have complex populations of granules, such as the bag cell neurons of aplysia or hormone-secreting pituicytes. To generate different populations within a cell might require sequential formation of the varied organelles through control of transcription of the non-VWF cargo; this seems simpler than making different organelles in parallel.

Concluding remarks

While progress is being made towards understanding the formation and function of these uniquely shaped lysosome-related secretory organelles, several areas stand out as requiring more attention. Firstly, the structural basis for how VWF synthesis leads to the formation of the protein tubules responsible for WPB shape is unclear. Working models such as that in Fig. 1 are neither detailed nor even able to deal with all the current data. Until questions such as whether each tubule is really a coiled-up string of VWF are answered, we will be unable to build accurate models. Secondly, a proteomic analysis of the proteins that comprise this organelle, and in particular its membrane composition, would lead more rapidly to an understanding of WPB biogenesis and exocytosis. Finally, the remarkable plasticity between WPBs presumably reflects the multiple regulatory roles played by endothelial cells. Unravelling the differences between WPBs from different cells should help us to understand which physiological responses are tied together by co-storage of bioactive molecules. Altogether, these issues should keep these relatively mysterious organelles under investigation for some time yet.

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