Gray Platelet Syndrome

Dissociation between Abnormal Sorting in Megakaryocyte α-Granules and Normal Sorting in Weibel-Palade Bodies of Endothelial Cells

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Abstract

The gray platelet syndrome (GPS) is a rare congenital bleeding disorder in which megakaryocytes and platelets are deficient in α-granule secretory proteins. Since the Weibel-Palade bodies (WPB) of endothelial cells as well as the α-granules contain the von Willebrand Factor (vWF) and P-selectin, we examined by transmission electron microscopy the dermis capillary network of two patients with GPS. Endothelial cells showed the presence of normal WPB with typical internal tubules. Using single and double immunogold labeling for vWF and P-selectin, we detected vWF within WPB, where it was codistributed with the tubules, whereas P-selectin delineated the outline of WPB. Therefore, the fundamental targeting defect in GPS is specific to the megakaryocytic cell line. (*J. Clin. Invest.* 1993. 92:3023–3028.) Key words: von Willebrand factor • P-selectin • standard electron microscopy • immuno-ultrastructural labeling

Introduction

The Gray platelet syndrome (GPS)[1] is a rare congenital bleeding disorder in which the platelets lack normal α-granule content (1, 2), and therefore appear gray after Romanovsky staining. Previous biochemical and ultrastructural studies demonstrated a deficiency in the α-granule secretory proteins, namely platelet factor 4, β-thromboglobulin, fibrinogen, von Willebrand factor (vWF), fibronectin, platelet-derived growth factor, and thrombospondin (3–7). However, the transmembrane protein P-selectin, also called CD62, PADGEM, or GMP.140 (8, 9), is expressed along the membranes of intracellular vesicles in gray platelets and fuse normally with the cell surface after activation (10), as in normal platelets (8, 9, 11).

Apart from megakaryocytes, which synthesize the vWF (12, 13), endothelial cells are also known to synthesize this large adhesive glycoprotein (14, 15). In endothelial cells, the vWF is stored in Weibel-Palade bodies (WPB), in its highest molecular weight form (16–19), whereas in megakaryocytes and platelets, vWF is localized in α-granules, along with many other hemostatic proteins (20, 21). Both WPB and α-granules are lined by a single membrane that contains P-selectin (8, 22, 23), a glycoprotein involved in monocyte and neutrophil recognition (24–27).

While studies performed on megakaryocytes and platelets of patients with GPS have shown that α-granule soluble proteins are normally synthesized but improperly targeted, endothelial-specific storage organelles for regulated vWF secretion so far have not been investigated. We have thus undertaken the following ultrastructural study of endothelial cells from two patients with GPS and describe the presence of normal WPB containing both vWF and P-selectin with a normal intracellular distribution.

Methods

Patients

The skin of two patients with GPS was biopsied from the forearm. The patients (M.S. and H.B.) are sister and brother and have been studied in previous reports (2, 4, 5, 10, 28). They volunteered for the present study. Control skin was also taken from the forearm of three patients when biopsied for myopathy, with their informed consent.

Standard electron microscopy

Samples of skin (1 mm^3) were immersed in fixative solution (2% [wt/vol] paraformaldehyde +2.5% [vol/vol] glutaraldehyde in 0.1 mol/liter [molar] sodium cacodylate buffer, pH 7.2) at +4°C. After 90 min, the fragments were washed in 0.1 mol/liter cacodylate buffer, postfixed in 1% (wt/vol) osmium tetroxide in the same buffer, block stained in 1% (wt/vol) aqueous uranyl acetate, dehydrated, and embedded in Epon.

Immuno-electron microscopy

The resected samples were minced into 0.5 mm^3 and prepared as previously described (21). Briefly, they were fixed in 1% glutaraldehyde in 0.1 mol/liter phosphate buffer, pH 7.2, for 2 h at 22°C, washed three times in the same buffer, and embedded in glycol metacrylate (GMA). Thin sections (60–90 nm) were collected on nickel grids for immunocytochemical reactions.

Antibodies. Rabbit polyclonal antibodies (IgG fraction) raised against human vWF were purchased from Dakopatts (Copenhagen, Denmark). Rabbit polyclonal antibodies (IgG fraction) raised against human P-selectin were kindly provided by Dr. M. Berndt (Victoria, Australia). Goat anti-rabbit (GAR) IgG coupled to 5 or 10 nm colloïdal gold (GAR G_5 or GAR G_{10}) were from Janssen Pharmaceutical (Belgium).

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1. *Abbreviations used in this paper:* GAR, goat anti-rabbit; GPS, gray platelet syndrome; vWF von Willebrand factor; WPB, Weibel-Palade bodies.

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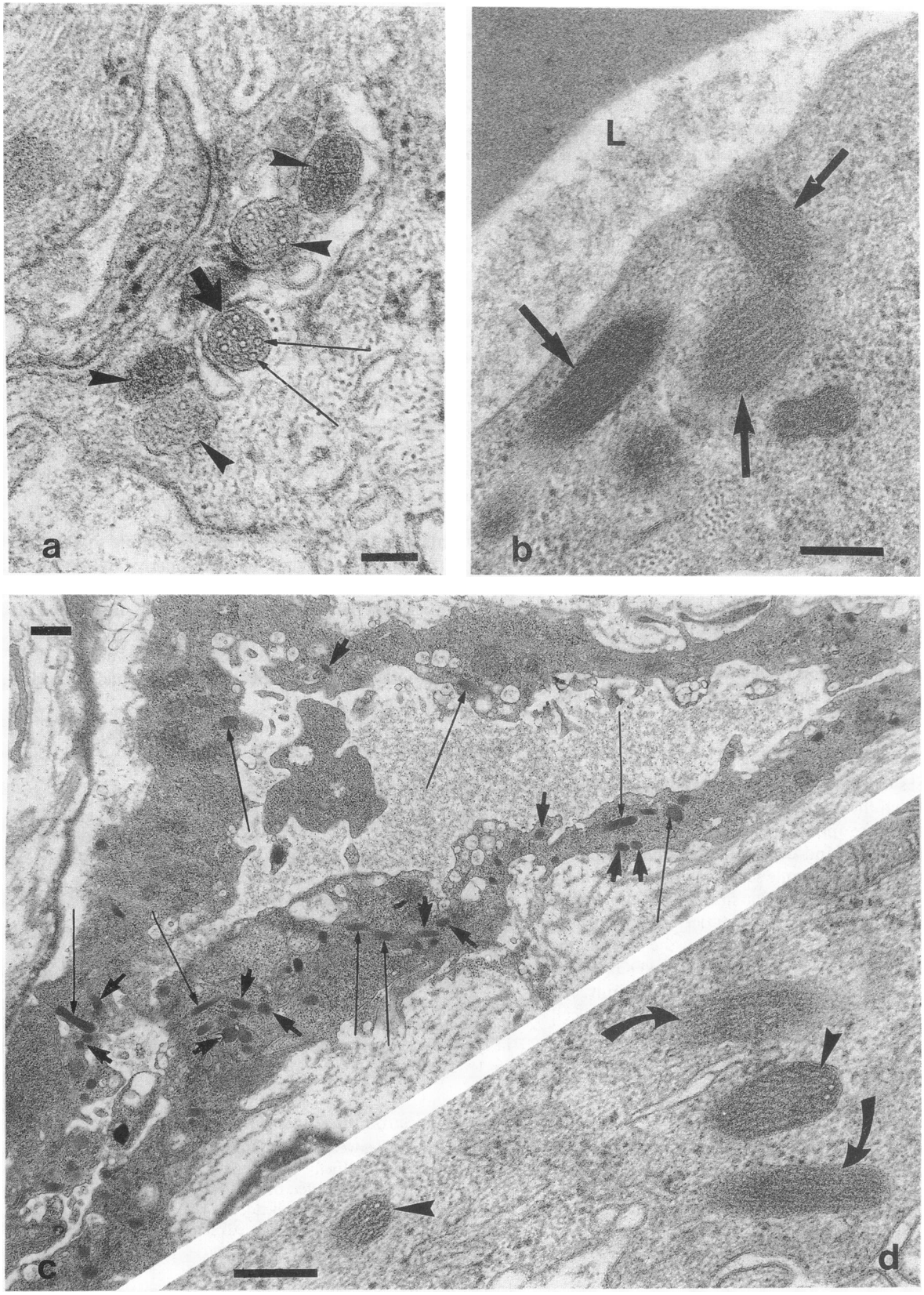

Figure 1. Uranyl acetate and lead citrate (Ur-Pb). (*a*) Endothelial cell from control skin showing transversally cut WPB. In these organelles, the tubules are helicoidally arranged (*arrowheads*) or longitudinally disposed in the matrix (*thick arrow*). Some tubules in this granule include a central dense dot (*thin arrows*) (×89,000; bar = 0.1 mm). (*b*) Endothelial cell from control skin. In the WPB seen, the tubules generally pursue a straight parallel course along the long axis of the rod (*arrows*); lumen (*L*) (×70,400; bar = 0.2 mm). (*c*) Dermal capillary from a patient with GPS. As in the control skin, the endothelium contains numerous WPB (*arrows*) (×14,300; bar = 0.5 mm). (*d*) Endothelial cell from a patient with GPS. WPBs appear to be identical to those in control skin, with internal tubules exhibiting a straight course (*curved arrows*) or a twisted course (*arrowheads*) (×70,400; bar = 0.2 mm).

Procedure of on-grid immunomarking. A simple two-step reaction was used as previously described (17). The sections were first incubated in 0.02 mol/liter Tris-HCl buffer, pH 8.2, mixed with 1% gelatin for 40 min. The grids were then transferred to a drop of the first antibodies (in concentrations of 1:60 for the anti-vWF and 1:80 for the anti-P-selectin) and left for 1 h at 22°C. After washing in Tris-HCl buffer, the sections were exposed to a drop of GAR G_5 or GAR G_{10} at a dilution of 1:100, for 1 h at room temperature. The grids were again washed with Tris-HCl buffer, and contrasted with uranyl acetate and lead citrate before being examined on a transmission electron microscope (EM 10;

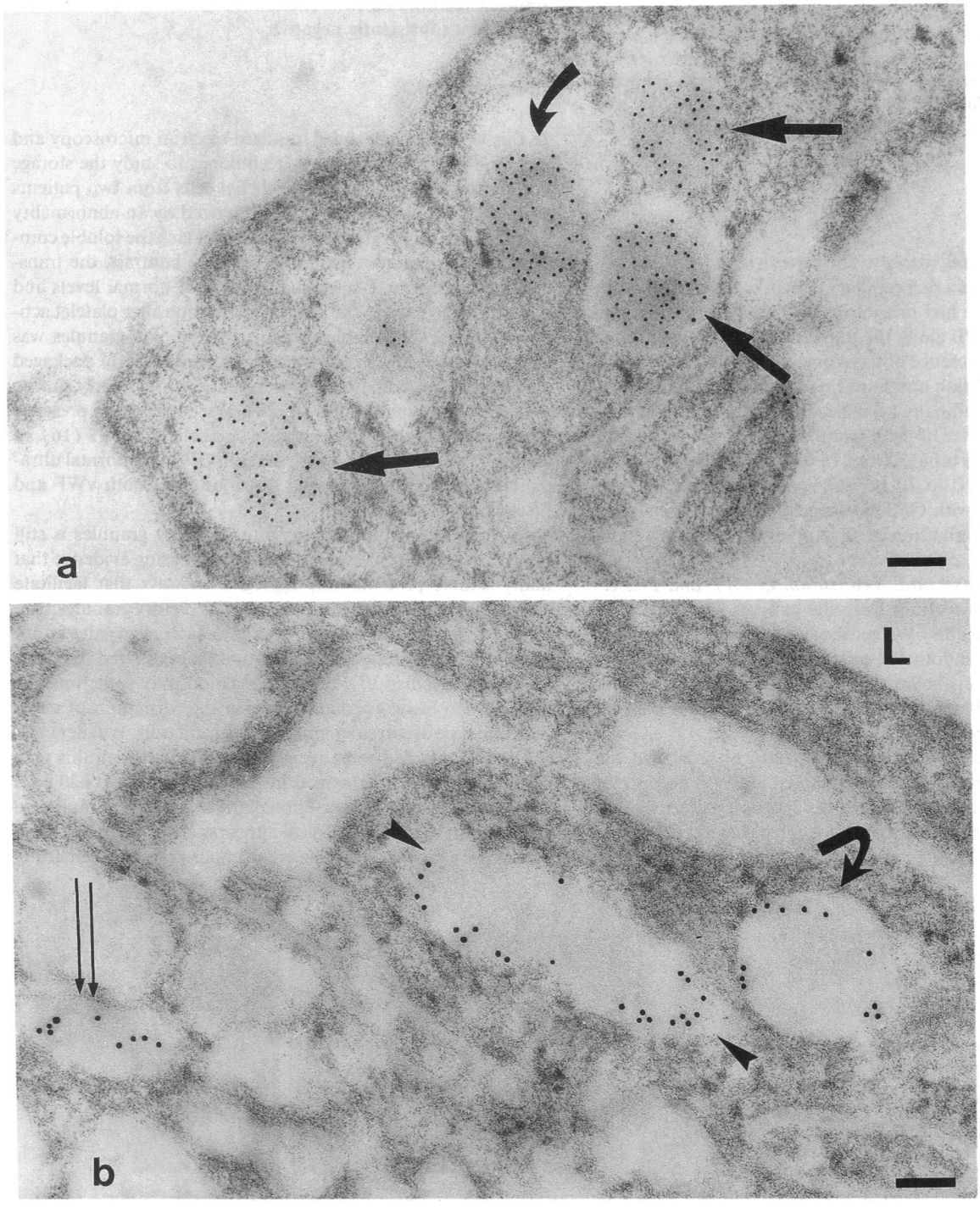

Figure 2. (*a*) Endothelial cell from a patient with GPS exposed to 1:100 polyclonal antibodies to vWF, followed by 1:125 GAR G_5 (Ur-Pb). WPB are heavily labeled for vWF, the 5-nm gold particles being distributed over the entire matrix of the granules (*arrows*) (×89,000; bar = 0.1 mm). (*b*) Dermal capillary from a patient with GPS. Endothelial section stained with 1:80 polyclonal antibodies to P-Selectin, followed by 1:100 GAR G_{10} (Ur-Pb). The 10-nm gold particles outline the rounded shape (*curved arrow*) or elongated shape (*arrowheads*) of WPB, so designated even though the preparation procedure does not provide the optimal preservation required to allow morphological classification of these granules as WPB. The double arrow shows a tangentially cut WPB; lumen (*L*) (×89,000; bar = 0.1 mm).

Zeiss, Oberkochen, Germany). To demonstrate the specificity of the staining, primary antibodies were replaced by nonimmune rabbit IgG. The reaction was otherwise performed in identical conditions.

For double immunolabeling, we used the principle of the technique described by Bendayan (29) but replaced the protein A–gold by GAR coupled to colloidal gold particles of different sizes. Briefly, ultrathin tissue sections were mounted on uncoated nickel grids, such that both faces of the sections were available for labeling. Based on the protocol described above, we first labeled one face of the tissue section for the vWF using GAR-G_5. Then, the grids were dried and turned over. The second face of the sections was then incubated with the antibodies against P-selectin followed by GAR-G_{10}. The tissue sections were stained with uranyl acetate and lead citrate before examination in the electron microscope.

Results

Standard electron microscopy. The superficial layer of the dermis, which contains a rich capillary network, was preferentially observed. Since we had previously described a heterogeneous distribution of WPB along the porcine vascular tree (17), we first checked the presence of these organelles in the endothelial cells from control skin and found typical WPB with the internal tubules longitudinally or helicoidally arranged (Fig. 1, *a* and *b*). The diameter of these granules ranged from 0.1 to 0.2 μm, and that of the tubules from 14 to 17 nm. The number of WPB varied from 0 to 20 per cell section. Endothelial cells from the patients with GPS displayed WPB having the same size, shape, and distribution as those observed in control endothelial cells (Fig. 1, *c* and *d*).

Immuno-ultrastructural localization of vWF and P-selectin. When the skin sections from the patients with GPS were incubated with the anti-vWF antibodies followed by immunogold labeling, the endothelial cells showed intense labeling of the WPB. These organelles sometimes appeared to fuse with the plasma membrane. vWF was not detected in any other cytoplasmic organelle (Fig. 2 *a*).

The polyclonal antibodies to P-selectin only labeled the membrane of granules that we identified as WPB because of their typical size and number. The distribution pattern of gold particles outlined the rounded or elongated shapes of these organelles (Fig. 2 *b*).

To confirm that vWF and P-selectin are colocalized in WPB, we performed double immunogold labeling of both proteins on the same tissue section. Fig. 3, *a–c,* demonstrates the colocalization of vWF (5-nm gold particles) with P-selectin (10-nm gold particles) in the same organelle. vWF was found in the matrix of WPB, while P-selectin was located along their limiting membrane. In sections incubated with control IgG, WPB were consistently negative.

Discussion

In this work, we have used standard electron microscopy and immuno-electron microscopy techniques to study the storage compartment for vWF of endothelial cells from two patients with GPS. This syndrome is characterized by an abnormality of both megakaryocytes and platelets that lack the soluble components of α-granule, including vWF. In contrast, the transmembrane protein P-selectin is present at normal levels and can be translocated to the plasma membrane after platelet activation (10). The soluble protein content of α-granules was shown to be synthesized normally but improperly packaged and lost in the demarcation membrane system early during megakaryocyte maturation (4, 28). We describe the presence of typical WPB, the specific storage organelles for vWF (16), in endothelial cells from two patients with GPS with normal ultrastructure and normal internal distribution of both vWF and P-selectin.

Sorting of soluble proteins into secretory granules is still poorly understood. However, there is increasing evidence that many soluble proteins may aggregate in ways that facilitate association with membranes of nascent secretory granules. The sorting signal that directs proteins into such aggregates is not known but it is thought to be a signal that is shared by many secretory proteins (30). Such aggregation may sometimes be mutually exclusive, perhaps allowing the formation of more than one type of secretory granule in certain cells. Wagner et al. (31) have provided some insights into the nature of this process. When vWF is expressed in mouse pituitary AtT20 cells that contain a regulated secretion pathway, storage granules are formed with the morphological appearance of WPB, but these granules are distinct from the endogenous granules containing

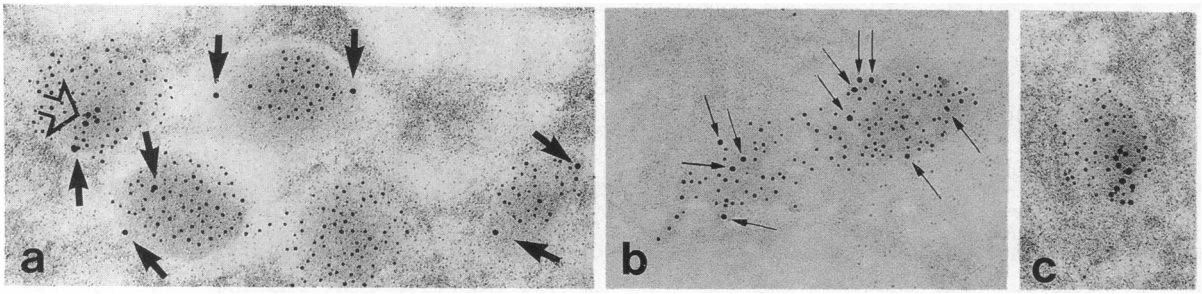

Figure 3. Double labeling of endothelial cells from a patient with GPS, with antibodies against vWF and P-selectin (×82,000). (*a*) The label for vWF in the matrix of WPB is detected by small gold particles (5 nm), and the label for P-selectin near the membrane (*arrows*) is detected by the large gold particles (10 nm). The large open arrow indicates the immunogold labeling of P-selectin over a granule tangentially sectioned (see also *c*). (*b*) Longitudinal section of WPB showing the distribution of anti-P-selectin (10 nm gold) at the periphery (*arrows*), whereas the vWF fills the matrix of the granule. (*c*) The WPB shows the colocalization of both proteins: the 5-nm gold labels the vWF, and the 10-nm gold for P-selectin seems to cover the matrix. This image may reflect a tangential section, since both faces of the tissue section were labeled and since only antigens exposed at the surface of the section were available for the antibodies.

ACTH. This property appears to be preserved in endothelial cells from GPS, but in megakaryocytes an unknown factor prevents sorting (or aggregation) of vWF as well as other proteins normally delivered to α-granules. Protein condensation is thought to involve unique ionic conditions and, in the trans-Golgi network, a mildly acidic pH (partly insured by certain H^+ ATPases) and millimolar calcium ion concentrations. Whether the pH or calcium ion concentration in the trans-Golgi network of GPS megakaryocytes might be affected, such that this defect is restricted to this particular cell type, should be investigated. However, it is of importance to note that in GPS platelets P-selectin is normally present, and that the defect does not extend to sorting of P-selectin into secretory granules as well as other membrane proteins, such as GPIIb-IIIa (32). This must imply a different mechanism, since a recent report (33) has shown that the cytoplasmic domain of P-selectin is both necessary and sufficient for sorting of membrane proteins into secretory granules in AtT20 cells. Since soluble proteins do not have a component facing the cytoplasm, their sorting system might be different.

Finally, although normal endothelial cells and megakaryocytes share common features (34), such as the processing steps in biosynthesis and storage of vWF, the presence of P-selectin in their granule membrane, and the release of the protein content from WPB and α-granules, they nevertheless present some differences. One of these differences concerns adhesive proteins such as fibronectin, thrombospondin, and vWF, which are all retained in α-granules (22), whereas in endothelial cells only the vWF is stored in WPB, while thrombospondin and fibronectin are secreted constitutively (35). Likewise, WPB usually originate from a single biosynthetic pathway (36), whereas α-granules are formed by two general routes: endogenous synthesis and an endocytic pathway (37, 38), which provides yet another difference between these organelles. We can conclude that the anomaly observed in megakaryocytes and platelets of patients with the GPS is specific to this cell type. Further investigations will be needed to understand the different conditions of protein sorting in normal and GPS megakaryocytes.

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