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Phenotypic Heterogeneity in the Gray Platelet Syndrome Extends to the Expression of TREM Family Member, TLT-1

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Abstract

The Gray platelet syndrome (GPS) is a rare inherited disorder linked to undefined molecular abnormalities that prevent the formation and maturation of α-granules. Here, we report studies on two patients from unrelated families that confirm phenotypic heterogeneity in the disease. First we used immunoelectron microscopy (I-EM) to confirm that TREM-like transcript-1 (TLT-1) is mostly localized to α-granule membranes of normal platelets. Then we performed Western blotting (WB) and flow cytometry with permeabilized platelets to show that TLT-1 is selectively reduced in the platelets of patient 1, previously noted to be deficient in glycoprotein (GP)VI (Nurden et al, Blood 2004; 104:107−114). Yet both TLT-1 and GPVI were normally expressed in platelets of patient 2. Usual levels of JAM-C and claudin-5, also members of the immunoglobulin receptor family, were detected in platelets of both patients. In contrast, P-selectin was markedly decreased for patient 1 but not patient 2. Two metalloproteases, MMP-2 and MMP-9 were normally present. As predicted, platelets of patient 1 showed little labelling for TLT-1 in I-EM, whereas residual Fg was seen in small vesicular structures and P-selectin lining vacuoles or channels of what may be elements of the surface-connected canalicular system. Our results identify TLT-1 as a glycoprotein potentially targeted in platelets of GPS patients, while decreases in at least three membrane glycoproteins suggest that an unidentified proteolytic activity may contribute to the phenotype in some patients with this rare disease.

Introduction

The triggering receptors expressed on myeloid cells (TREMs) contain a single V-set immunoglobulin (Ig) domain, and are involved in cell activation within the innate immune system (1). A glycoprotein with significant homology to the TREMs, TREM-like transcript-1 (TLT-1), has been exclusively found in the mouse and human megakaryocyte (MK) lineages (2-4). TLT-1 was tentatively localized to the α-granule membrane, a conclusion made from its colocalization with P-selectin in confocal microscopy. Two isoforms of TLT-1 have been described; the first has a cytoplasmic domain with two consensus immunoreceptor tyrosine-based inhibition motifs (ITIMs), the second has a cytoplasmic domain lacking ITIMs (3). Interestingly, a Src homology domain-containing tyrosine phosphatase (SHP) is recruited to the ITIM (at Y281) of TLT-1 after activation, although there is a lack of consensus on its identity for it has been identified as SHP-1 or SHP-2 according to the publication (2,3). At the

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present time, the counter ligand for platelet TLT-1 remains to be identified although a role for this membrane glycoprotein in thrombin-induced platelet aggregation has recently been proposed (5).

The hypothesis of Washington et al (2), that TLT-1 is not simply cargo of α-granules but may instead regulate granule construction or dispersal led us to examine its expression in the platelets of two patients with the Gray platelet syndrome (GPS), a rare inherited bleeding disorder characterized by the absence or severe decrease of α-granules and the platelet storage pool of proteins (6). We compared TLT-1 expression in GPS platelets with that of P-selectin, a membrane marker of both platelet α-granules and dense granules (7) and also with that of two other members of the Ig receptor family, junctional adhesion molecule-C (JAM-C) and claudin-5. JAM-C is a 43-kD membrane glycoprotein that acts as a counter receptor on platelets for the leukocyte β2-integrin (αMβ2, CD11b/CD18) (8,9). Claudin-5 belongs to the claudin family, glycoproteins that play a role in cell contact interactions and which, for example, assemble into endothelial cell tight junctions (10). GPS is a heterogeneous but moderate bleeding disorder with patients showing variable defects in collagen or thrombin-induced platelet aggregation; while autosomal recessive inheritance is seen for the majority of patients, autosomal dominant inheritance is apparent in a large Japanese family (data reviewed in 6, 11). We have examined two GPS patients with different modes of inheritance, patient 1 has platelets that respond poorly to collagen and express little or no GPVI, another member of the Ig receptor family and a major platelet receptor for collagen (12). Patient 2 has a relatively normal collagen-induced platelet aggregation (6). Decreased expression of TLT-1 and P-selectin paralleled that of GPVI in platelets of patient 1 but both were normal in patient 2, confirming that in some GPS patients the storage pool defect is aggravated by a selective loss of membrane glycoproteins. They also show that TLT-1 is another target protein susceptible to be modified in GPS.

Methods

Patients

Patient 1 is an elderly woman born in a consanguinous marriage and with a moderate bleeding tendency. She is diabetic (type II) and overweight; she has suffered from phlebitis. Superficial ulcers on her legs failed to heal spontaneously. Her current platelet count is around 30,000/μL. Her platelets show the typical morphological abnormalities of GPS in electron microscopy. They are mostly enlarged, round, with absent α-granules and show marked vacuolization (illustrated in 6,12). They are severely deficient in α-granule proteins and are refractory to collagen as a result of what appears to be an acquired deficiency of GPVI (12). Neither of her parents nor her two adult children has shown signs of GPS. Patient 2 also has a moderate bleeding tendency; her current platelet count is about 50,000/μL. She is a middle-aged woman from the Manouche Gypsy tribe in France and her father (now deceased but previously studied by us) also had GPS while several members of her large family have reported mild bleeding or thrombocytopenia suggesting autosomal dominant inheritance (6). The bulk of her platelets also have the typical GPS appearance when studied by electron microscopy. Nevertheless, in some platelets occasional α-granules are seen. The platelets of both patients gave the typical "gray" appearance on Wright-Giemsa stained blood smears and both have a normal storage pool of dense granule contents.

Materials

The polyclonal human anti-TLT-1 antibody was prepared in the McVicar laboratory by immunizing rabbits with a peptide derived from the membrane proximal region of human TLT-1 (amino acids 228−241) as detailed elsewhere (2). P-selectin was studied using VH10, a monoclonal antibody (MoAb) prepared by us (12) or using a rabbit polyclonal antibody

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(purified IgG) purchased from Pharmingen (Montrouge, France). The rabbit polyclonal antibody to fibrinogen (purified IgG) was from Dakopatts (Glostrup, Denmark). Dr. Sentot Santoro kindly provided the MoAb Gi11 to JAM-C (8). The mouse anti-claudin-5 MoAb (clone 4C3C2) was purchased from ZYMED laboratories (San Francisco, CA). Matrix metalloproteinase 2 (MMP-2) in human platelets was detected using OPA1−08022 (Affinity BioReagents™,Golden, CO, USA), a rabbit antibody directed against a peptide sequence found in the hinge region of human MMP-2. It does not react with other MMP family members. For MMP-9, we used a specific rabbit antibody (Lab Vision, CA, USA) directed against a peptide sequence located near the C-terminal domain of human MMP-9. We have previously given the sources of MoAbs to GPIbα (Bx-1), αIIb (SZ22) and β3 (Y2/51) (12).

Flow cytometry

Platelets in citrated PRP from control donors or the GPS patients were fixed in 1% paraformaldehyde (PFA) as described (12). Briefly, to allow antibody access to the internal compartment, platelets were then treated with 0.1% Triton X-100 for 30 min at 4°C, washed, and then incubated 1h at 4°C with the following antibodies: Gi11 (anti-JAM-C) (0.4 μg/ml); rabbit anti-MMP-2 (4.5 μg/ml); rabbit anti-MMP-9 (5.0 μg/ml), Bx-1 (anti-GPIbα) (3 μg/ml), VH10 (anti-P-selectin) (5 μg/ml), anti-claudin-5 (1/100) and rabbit anti-TLT-1 serum (dil 1:500). After further washing, platelets were incubated for 30 min at room temperature in the dark with FITC-labelled $F(ab')_2$ fragments of sheep anti-rabbit or anti-mouse IgG (Eurobio, Les Ulis, France) at predetermined optimal concentrations. Platelets were analyzed in a Cytomics FC500 (Beckman Coulter, France) or a Becton Dickinson FACScan (San José, CA) flow cytometer. Gating to select the majority of platelets was based on preliminary determinations of forward and wide-angle light scatter. Mean fluorescence intensity (MFI) was measured after passage through a 530 nm long pass interference filter. Histograms were generated from measurements of 10,000 cells and data were analyzed using CXP (Beckman-Coulter) or LYSIS II (Becton-Dickinson) software.

Western blotting

Washed platelets were solubilized with sodium dodecyl sulphate (SDS) under nonreducing conditions and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (12). After transfer to nitrocellulose membrane, nonspecific protein binding was blocked by incubating in 20 mM Tris buffer, pH 8.2, containing 0.15 M NaCl, 0.05% Tween 20 and 5% fat-free milk and individual strips incubated with mouse MoAb to JAM-C (Gi11, 2.5 μg/ml), P-selectin (VH10, 1 μg/ml) or a mixture of MoAbs to GPIbα (Bx-1, 1 μg/ml), αIIb (SZ22, 0.5 μg/ml) and β3 (Y2/51, 0.5 μg/ml). The rabbit antibody to TLT-1 was used as serum diluted 1/500. After washing, bands were incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) and bound antibody detected by chemiluminescence (Amersham, Cortaboeuf, France) (12).

Immunoelectron Microscopy (I-EM)

Blood was taken into tubes containing acid-citrate-dextrose-A (ACD-A) anticoagulant (12). PRP prepared by centrifugation for 10 min at 120g was incubated for 10 min at 37°C prior to fixation in 1.25 % glutaraldehyde (Fluka Buchs, Switzerland) diluted in 0.1 M phosphate buffer (pH 7.2) for 1 h at room temperature. After platelet sedimentation, pellets were infused with 2.3 M sucrose before being frozen in propane and then in liquid nitrogen using a Reichert KF 80 freezing system (Leica, Vienna, Austria). Ultrathin sections were prepared as described (12) and incubated for 10 min on drops of washing buffer consisting of phosphate-buffered saline (PBS) supplemented with 0.5 % bovine serum albumin (BSA). Sections were then floated on washing buffer containing rabbit anti-TLT-1 antibody (dil 1/500), the rabbit

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antibody to P-selectin (dil 1/500) and a rabbit antibody to fibrinogen (Fg) (dil 1/100). After washing, bound IgG was detected using goat anti-rabbit IgG conjugated to 5 or 10 nm gold particles (Amersham, Orsay, France) (12). Sections were stained with uranyl acetate and osmium according to standard procedures. After embedding in methylcellulose, sections were observed using a Jeol JEM-1010 transmission electron microscope (Jeol, Croissy-sur-Seine, France). Controls were performed in the absence of primary antibody.

Results

Immunolocalization of TLT-1 in control platelets

Washington et al (2) previously showed by confocal microscopy that TLT-1 colocalized with P-selectin in unstimulated human platelets suggesting that it was largely found in the membrane of α-granules. We have now confirmed this result by I-EM. Figure 1 shows a gallery of frozen-thin sections of normal human platelets incubated with the rabbit antibody to TLT-1. As can be seen (Fig 1a-d), much of the labelling is confined to α-granule membranes or to membranous structures in the close vicinity of the storage organelles. Notwithstanding, occasional labelling was seen on the platelet surface (Figure 1d), a result also seen by Washington et al (2) who observed an approximate 10 fold increase in surface expression after thrombin-induced platelet activation.

Flow cytometric detection of TLT-1 and other selected antigens in normal and GPS platelets

Studies were performed using PFA-fixed unstimulated platelets permeabilized or not with Triton X-100. Results for patient 1 are shown in Fig. 2 (panel A). Histograms for TLT-1 are compared with those obtained with MoAbs to P-selectin and two other members of the immunoglobulin gene family JAM-C, and claudin-5 as well as GPIbα. For each antibody, the results for normal platelets and for patient 1 are superimposed. In agreement with the results of the I-EM, only small amounts of TLT-1 were expressed on intact normal platelets, the bulk only becoming available to the antibody after permeabilization. In this way, the distribution of TLT-1 continued to resemble that of P-selectin. Expression of TLT-1 was markedly decreased in the platelets of patient 1, a result that mimicked our previous results for GPVI (12). Significantly, platelet levels of P-selectin were also decreased and while small amounts of P-selectin were seen on the non-permeabilized fixed control platelets these were not seen for the patient. As expected, GPIbα was abundant on PFA-fixed platelets of the patient and pretreatment with 0.1% Triton X-100 gave only a small increase in antibody binding, confirming its largely surface localization. Expression of JAM-C was normal for the patient with the glycoprotein having both surface and internal pools. Claudin-5 was expressed in low amounts in control platelets and was only surface-localized on a subpopulation; results were identical for the patient. Studies were extended to the metalloproteases MMP-2 and MMP-9; both were normally present in the platelets of patient 1. MMP-2 was the most abundant and, as expected, the bulk was intracellular. MMP-9 was also primarily made available after permeabilization although some MMP-9 was found on the surface of the platelets of the patient.

Platelets from patient 2 contrasted with those presented above in that they contained relatively normal amounts of TLT-1 and P-selectin after permeabilisation (Fig. 2, panel B). Levels of GPIb, claudin-5 and MMP-9 were also unchanged; JAM-C and MMP-2 were not studied for this patient due to insufficient material. Small differences in TLT-1 and P-selectin expression on non-permeabilized platelets of different control donors (and patient 2) probably reflect donor variation and/or small amounts of platelet activation during bleeding and PRP preparation.

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Immunoblotting of TLT-1 and other selected antigens in normal and GPS platelets

The rabbit antibody to TLT-1 gave two bands in the 38–40 kDa range for normal platelets. The upper band is thought to be native TLT-1 and the lower band a degradation product or an alternatively spliced isoform. Significantly, both bands representing TLT-1 were severely reduced on the profile obtained for patient 1 (Fig. 3A). Interestingly, a weak but much faster migrating band representing a possible degradation product is now to be seen (asterisk on Fig 3A). This was not observed when using the second antibody alone. Also reduced for the patient was P-selectin (130 kDa) while JAM-C remained unchanged. These changes are significant and were seen on 3 separate gels, the normal presence of GPIb and β3 in platelets of patient 1 are shown in the insert on Fig. 3A (see also ref 12) while protein staining confirmed equivalent sample loading. No abnormalities in the density or migration of blots corresponding to GPIb, αIIb, β3, TLT-1, JAM-C or P-selectin were seen for the platelets of patient 2 (Fig. 3B). Figure 3B additionally confirms a normal presence of GPVI in the platelets of patient 2, a parallel blot confirmed the previously reported deficit of GPVI platelets of patient 1 (data not shown).

I-EM studies on platelets of the patients

When ultrathin sections of platelets from patient 1 were incubated with anti-TLT-1 antibody there was little labelling (not illustrated). Immunogold labelling was seen for patient 2 (Fig 4 panels a-c), a finding consistent with the normal TLT-1 content of the platelets. Gold beads were observed both on the platelet surface and intracellularly primarily lining the vacuoles or elements of the OCS (open canalicular system) that typify GPS platelets. Occasional residual α-granules or small granules was seen in the platelets of patient 2 and the anti-TLT antibody continued to label the α-granule membrane (panel c). In parallel, we localized Fg and P-selectin in the platelets of the patients. Typical results for patient 1 are compared to those of a control donor in Fig. 5. Whereas α-granules of control platelets were distinctively labelled for Fg (panel a), the labelling of GPS platelets was confined to small granules or associated membranous structures within the cytoplasm (panel b). No evidence for Fg-containing structures resembling mature α-granules was obtained for patient 1, mitochondria were present but not labelled. While a polyclonal antibody to P-selectin primarily localized to the luminal side of the membrane of α-granules of control platelets (panel c), it was confined to the inner surface of vacuoles or elements of the OCS as well as other small vesicular structures in the platelets of patient 1 (panel d). Labelling of P-selectin was fairly heterogeneous in the GPS platelets, appreciable areas of some platelets showed no labelling.

Discussion

Our results clearly confirm molecular heterogeneity in GPS. Previously, we have shown that patient 1 associates decreased platelet aggregation to collagen with an absence of GPVI (12). Now we show that platelets of this patient are also severely deficient in another membrane glycoprotein, TLT-1. TLT-1 is a relatively little-studied membrane glycoprotein of human platelets, and belongs to the TREM receptor family (1). Expression of TLT-1 is restricted to the megakaryocyte lineage and, typical of the TREM family contains an Ig superfamily V-type domain. TLT-1 is present as two isoforms that differ in their cytoplasmic domain. The larger form of TLT-1 contains two concensus ITIMs, potential sites for the recruitment of SH-domain-containing tyrosine phosphatases (3). It is this isoform that is primarily recognized by our Western blot assay.

Previous confocal microscopy studies in two laboratories have shown that in normal platelets TLT-1 is colocalized with P-selectin, suggesting that this newly identified intrinsic membrane glycoprotein of platelets also has a granular localization (2,3). Early I-EM studies by Israels et al showed that while P-selectin is primarily found in the membranes of α-granules, it also occurs in those of dense granules (13). Our results clearly show that TLT-1 is principally

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associated with the α-granule membrane. Notwithstanding, some labelling was also found associated with smaller vesicular structures and occasionally on the platelet surface. As dense granules are not readily identifiable by the procedure used, the question as to whether the localization of TLT-1 extends to the dense granule membrane must remain open.

GPVI is a major platelet receptor for collagen (14). Its virtual absence from the platelets of patient 1 is associated with a much decreased platelet response both to collagen and the GPVI-specific ligand, convulxin (12). In contrast, platelets from patient 2 with a normal presence of GPVI, respond well to collagen (6). Washington and his coworkers (2,5) have shown that TLT-1 is a potential candidate to participate in the platelet aggregation response. This conclusion was based on the selective inhibition of thrombin-induced platelet aggregation using single-chain Fv antibodies specific for TLT-1. The same antibodies failed to inhibit collagen- and ADP-induced platelet aggregation, but surprisingly did inhibit that induced by the TXA_2 analogue, U46619. One possibility is that these antibodies block the interaction between newly exposed TLT-1 and its purported (but non-identified) counter-ligand on adjacent platelets. An alternative theory put forward by Giomarelli et al (5) is that TLT-1, through its ITIM domains, may regulate signalling pathways during platelet aggregation and SHP-sensitive Rho A-dependent activation was put forward as a candidate.

While for some GPS patients collagen-induced aggregation is the most severely affected, in others the defect primarily concerns the thrombin response (data reviewed in 6). The basis for the altered thrombin response has never been explained. In a new case of GPS in Italy, a markedly reduced aggregation and Ca^{2+}-mobilization by thrombin was mimicked by peptides activating platelets through the PAR-1 and PAR-4 receptors (15). An abnormal thrombin-induced Ca^{2+}-mobilization had been earlier reported for two French (16) and an American patient (17). It is interesting then that TLT-1 ligation has been reported to induce Ca^{2+}-mobilization (3). Platelets from patient 1 also respond poorly to TRAP peptides acting through PAR-1 (see ref 12); however, the decrease is not as severe as with collagen. As TLT-1 may play a role in secretion-dependent platelet aggregation together with secreted soluble α-granule proteins such as thrombospondin-1 (18), it will be important to compare TLT-1 expression in a more extensive series of GPS patients.

GPVI deficiency in platelets is most often acquired and due to antibody-driven proteolysis (19,20). Receptor loss through proteolytic activity by the so-called “sheddases” (a name given to a group of MMPs) is a natural way of down-regulating platelet function following ligand binding (21,22). GPIbα, GPV, GPVI and P-selectin are among reported substrates in platelets. The fact that each of GPVI, TLT-1 and P-selectin were decreased in platelets of patient 1 reinforces the hypothesis that rogue MMP activity may be responsible and suggests that TLT-1 is also a potential target for cleavage. The fact that a soluble fragment of TLT-1 is found in human sera and in the supernatant after thrombin-induced normal platelet activation is compatible with it's being a target for MMPs (4).

Platelets contain many MMPs including MMP-1, MMP-2 (gelatinase A), MMP-3, MMP-9 (gelatinase B), membrane-bound MT1-MMP, the VWF-cleaving protease ADAMTS-13, ADAM10 and tumor necrosis factor-α-converting enzyme (ADAM17) (21-30). Our initial results showed the normal presence of MMP-2 and MMP-9 in platelets in GPS and although their proteolytic activity has not been measured, the α-granule deficiency was not accompanied by their loss suggesting other storage sites for these proteases. Principle candidates for cleaving GPVI are ADAM17 and especially ADAM10 (reviewed in 21,28). The fact that GPIbα was normally present in the platelets of patient 1 means that the proteolysis is not generalized and further work is now required to establish the mechanism leading to selective GPVI, TLT-1 and P-selectin loss in the platelets of patient 1 and to establish the identity of the responsible

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protease(s) and the site where the cleavage occurs. In particular, the localization and activity of ADAM10 will need to be assessed.

In normal megakaryocytes, α-granule maturation passes by an intermediate step with the production of multivesicular bodies (31). P-selectin is selectively sorted into the regulated secretory pathway by way of a recognition signal in the cytoplasmic domain (32). Whether TLT-1 is likewise sorted will require further study. Some proteins are synthesized and packaged directly into the maturing α-granules in the megakaryocytes; PF4 is a well-studied example and, like P-selectin, has a granule targeting sequence (33). In a second mechanism, proteins are captured by endocytosis and this is the case for Fg (34). The presence of Fg in endocytic vesicles and/or precursor α-granules was clearly seen for platelets of patient 1 thereby confirming an earlier study (35). When not cleaved, P-selectin and TLT-1 were mostly found lining vacuoles or elements of the OCS or even on the surface confirming that predestined α-granule membrane glycoproteins are redistributed in GPS platelets (7,35).

Two other members of the Ig receptor family, JAM-C and claudin-5 were normally present in the platelets of both GPS patients. Unlike P-selectin and TLT-1, JAM-C was abundant on the surface of unstimulated platelets (see also refs 8,9). To the best of our knowledge, claudin-5 has not been reported in platelets before. Neither JAM-C nor claudin-5 was identified as a target for proteolytic degradation in the GPS again confirming that receptor loss is selective. Whether the observed receptor loss occurs in the circulating blood or in the bone marrow during megakaryocyte maturation and/or platelet release remains to be shown. If the protease can be identified, then possible measures could be taken to inhibit this activity. The molecular defect (s) responsible for GPS remains elusive. Whether the phenotypic heterogeneity observed here is related to the different mode of inheritance in the two families is unknown. But it is tempting to suggest that more than one genetic defect can lie at the basis of the disease as is seen, for example, in the Hermansky-Pudlak syndrome, where multiple gene defects have been reported (36).

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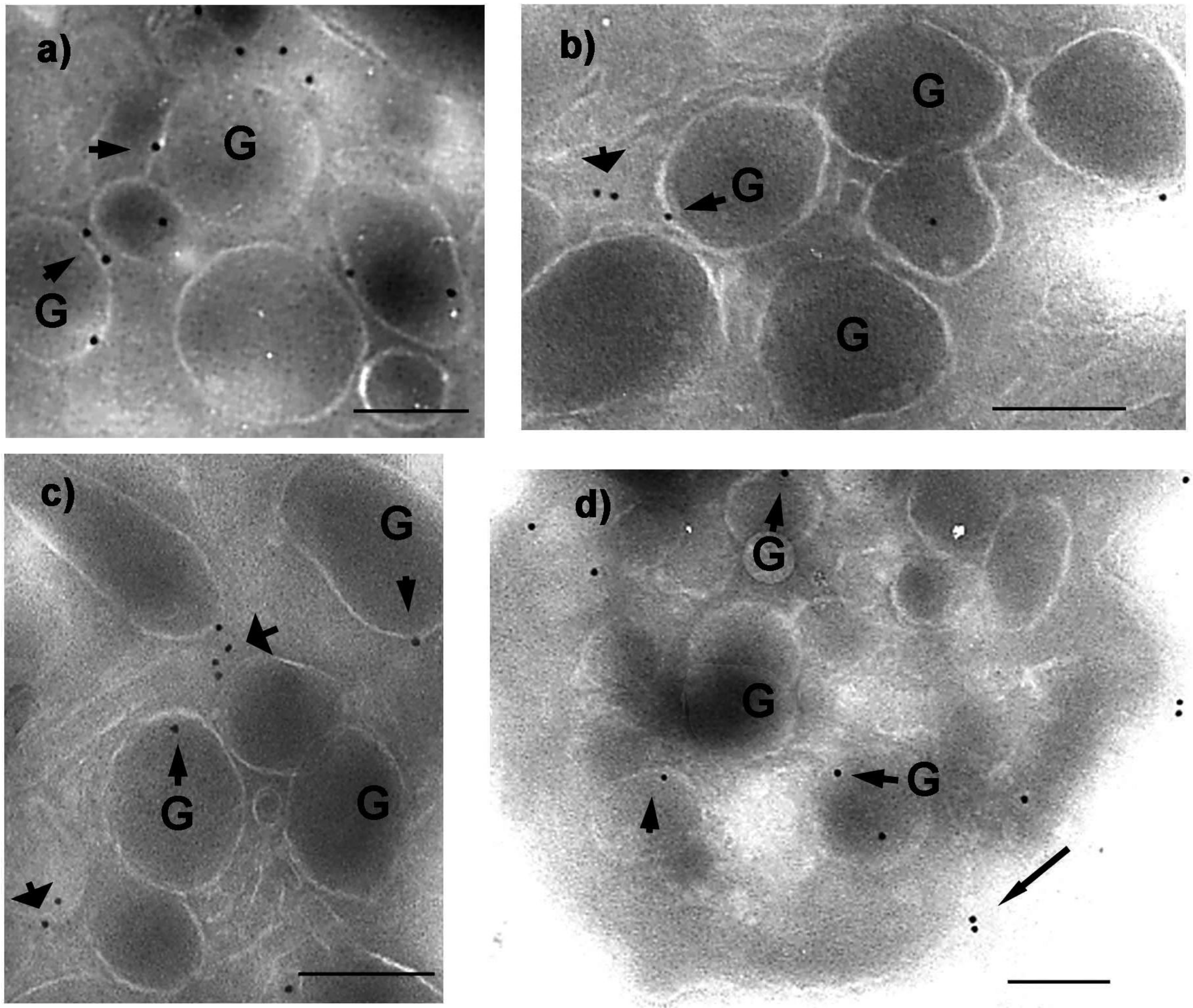

Fig. 1. Localization of TLT-1 in normal platelets by I-EM

Frozen ultrathin sections of glutaraldehyde-fixed normal platelets (a-d) were incubated with rabbit antibody reactive with human TLT-1 followed by a goat antibody monospecific for rabbit IgG adsorbed onto 10 nm gold particles. Note the localization of gold beads in close vicinity to the α-granule (G) membranes (small arrowheads) while occasional beads were also seen on the platelet surface (long arrows). Membranous structures in close proximity to α-granules were also labelled (wide arrowheads). Background staining with the second antibody alone was < 1 gold bead per section. Bar = 0.2 μ

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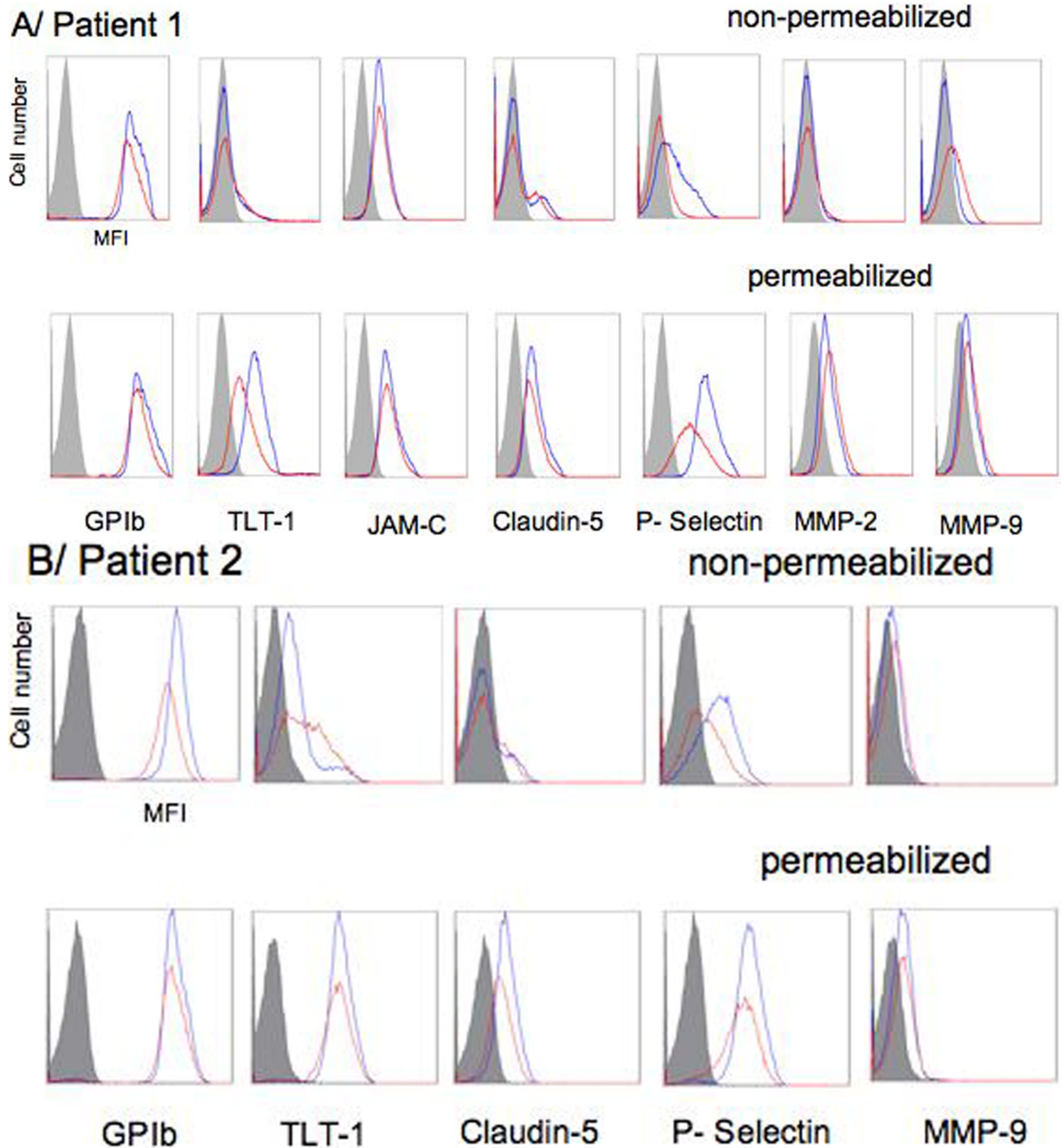

Fig. 2. Flow cytometric analysis of selected target antigens in normal platelets and those from two patients with GPS

Platelets were fixed with PFA and analyzed without and after permeabilization with 0.1 % Triton X-100 to access internal antigen pools. Histograms for control platelets (blue tracings) and those for each patient (red tracings) are superimposed and compared to the background obtained in the absence of primary antibody (full gray histograms). Results for patient 1 are

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shown in panel A. While GPIbα is essentially a surface marker, much of JAM-C is also accessible on intact platelets. In contrast, P-selectin, TLT-1, claudin 5, MMP-2 and MMP-9 require permeabilization for antibody accessibility. Note the decreased presence of P-selectin and TLT-1 in platelets from the patient whereas JAM-C, Claudin-5, MMP-2 and MMP-9 are normally present. Results for patient 2 are shown in panel B. Note the now normal presence of P-selectin and TLT-1 in platelets from the patient.

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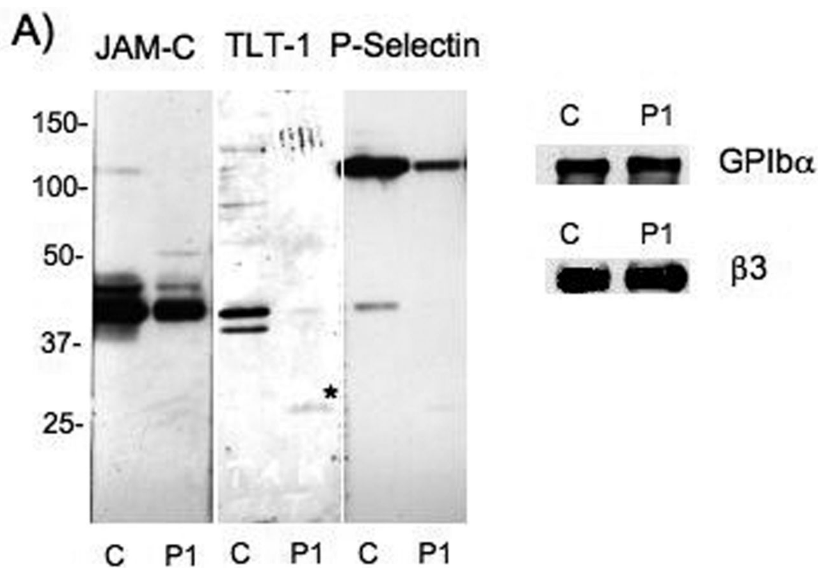

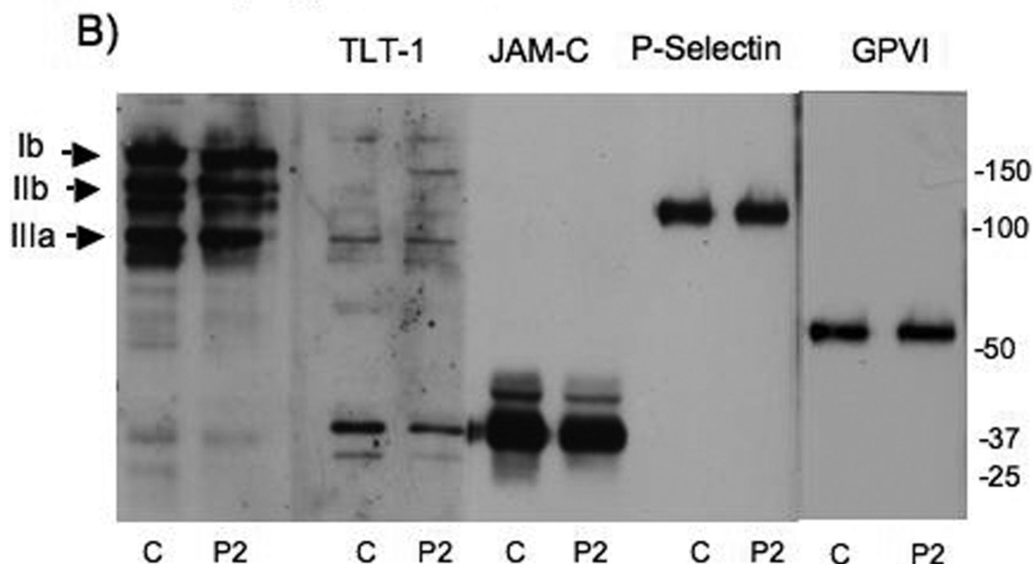

Fig. 3. Western blotting confirming TLT-1 and P-selectin deficiencies in the platelets of patient 1 compared to patient 2

SDS-soluble extracts (10 µg protein) of platelets from patients 1 (P1, part A) and 2 (P2, part B) and control donors (C) were separated by SDS-PAGE without disulfide reduction and transferred to nitrocellulose membrane. For P1, the membranes were probed for JAM-C, TLT-1 and P-Selectin (see Methods). Inserts are also shown for GPIbα and β3. For P2, GPIbα, αIIb and β3 were detected simultaneously while GPVI (MoAb 5.5)) was additionally detected. Bound antibodies were located using a chemiluminescence procedure. Migration of molecular weight markers is shown (kDa).

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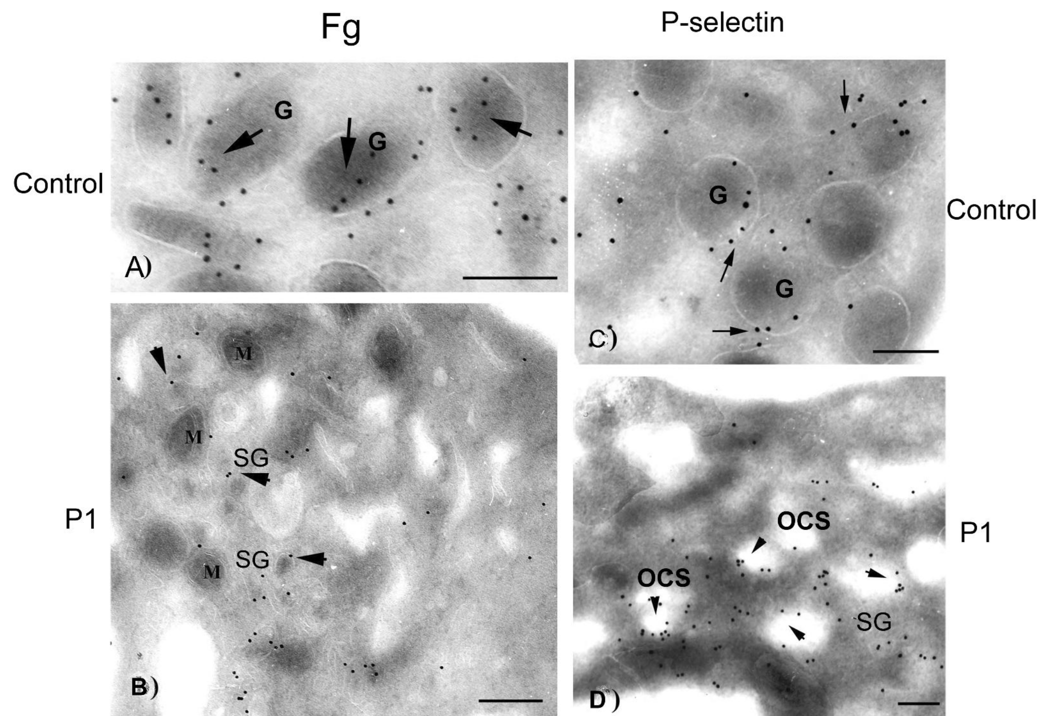

Fig. 4. Localization of TLT-1 in platelets of patient 2 by I-EM

Frozen ultrathin sections of glutaraldehyde-fixed platelets from patient 2 (a-c) were incubated with rabbit antibody reactive with human TLT-1 followed by a goat antibody monospecific for rabbit IgG adsorbed onto 10 nm gold particles. Note the localization of gold beads in close association with channels of the OCS or vacuoles as well as on the surface of the platelets (arrow heads). Membrane-proximal staining of occasional small granules (SG) is also seen in panel c. Background staining with the second antibody alone was < 1 gold bead per section. Bar = 0.2 μ

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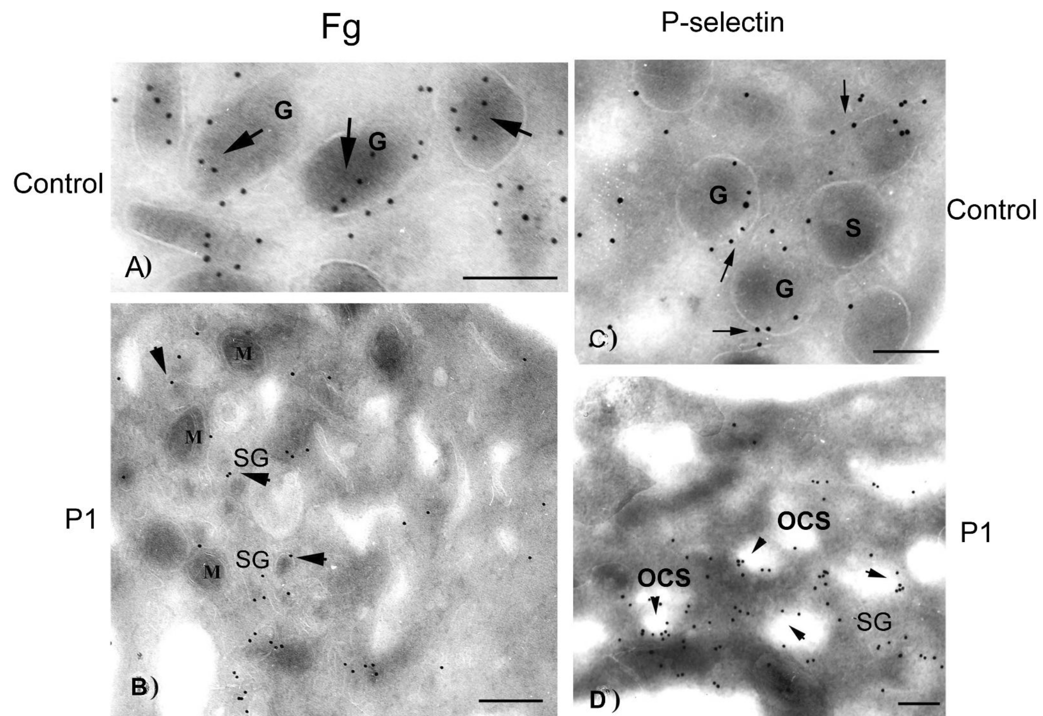

Fig. 5. Further immunogold labelling of platelets from patient 1 and a normal donor

Experiments were now performed using rabbit antibodies to Fg (a, b) and P-selectin (c, d) and typical results are illustrated for control platelets (a, c) and patient 1 (b, d). Whereas many α-granules (G) from control platelets were labelled with antibodies to Fg (arrows) (a), only small granules or vesicles (SG) and membranous structures were labelled for patient 1 (arrow heads) (b). This distribution of gold beads was different for P-selectin where the bulk of the labelling concerned the membranes of α-granules for control platelets (arrows) (c) and for the patient discrete zones were seen with P-selectin largely confined to membranes of empty vacuoles or channels of the OCS (arrow heads), and small vesicular structures (d). Bar = 0.2 μ