Biosynthesis and intracellular post-translational processing of normal and mutant platelet glycoprotein GPIb-IX

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The multisubunit leucine-rich glycoprotein (GP) Ib-IX-V complex mediates von Willebrand factor-dependent platelet adhesion at sites of blood-vessel injury. Molecular defects of this receptor are reported to cause the Bernard-Soulier haemorrhagic disorder. To gain insight into the mechanisms controlling expression of normal and defective receptors, we performed pulse-chase metabolic studies and detailed analysis of intracellular processing in GPIb-IX-transfected Chinese-hamster ovary cells. In the native complex, after early subunit association, sugars N-linked to the three subunits are trimmed and sialylated in the Golgi compartment and GPIb α undergoes extensive O-glycosylation. Surface biotinylation during chase demonstrated that only fully processed complexes reach the cell surface. Tunicamycin treatment revealed that early N-glycosylation is not required for O-glycosylation of GPIb α and surface expression of the complex. Biosynthetic studies were then performed on a Bernard-Soulier variant based on previous description of abnormal GPIb α size

and decreased surface expression. The mutant complex associated normally, but displayed defective processing of its N-linked sugars and abnormal O-glycosylation of GPIb α . Confocal immunofluorescence microscopy revealed that the mutant complexes could reach the cell surface but also accumulated intracellularly, while use of compartment specific markers showed strong co-localization in the endoplasmic reticulum (ER) and ER-to-Golgi intermediate compartments ('ERGIC') and only slight labelling of the *cis*-Golgi. Blockade before the Golgi was confirmed by brefeldin A treatment, which restored O-glycosylation and processing of N-linked sugars. The present study has shown that transfer from the ER to the Golgi represents an important step for controlling post-translational processing and surface expression of normal GPIb-IX-V complex.

Key words: Bernard-Soulier, CHO cells, glycosylation, leucine-rich, von Willebrand factor.

INTRODUCTION

It appears that for most multisubunit receptors, modification of a single subunit will affect expression of the entire complex [1–5]. This has important implications in pathology, one relevant example being the four-subunit platelet GPIb-IX-V complex (where GP stands for glycoprotein). Mutations have been described in three of the four subunits which lead to abnormal surface expression of the entire receptor and are responsible for the Bernard-Soulier bleeding defect [6,7]. The GPIb-IX-V complex is formed by the association in 2:2:2:1 stoichiometry of four leucine-rich proteins, namely GPIb α , GPIb β , GPIX and GPV (135, 30, 20 and 82 kDa respectively) [8]. GPIb α is disulphide-linked to GPIb β to form GPIb, which is non-covalently associated with GPIX and GPV [9,10]. Studies of Bernard-Soulier patients and of transfected heterologous cells have indicated that GPIb α , GPIb β and GPIX are necessary for efficient expression of the complex, whereas GPV is non-essential [7,11–13]. Nevertheless, a defect or the absence of a single subunit of GPIb-IX usually allows some assembly of partial complexes and surface expression.

Characterization of the mechanisms underlying the biosynthesis and maturation of GPIb-IX is required to fully understand its role in physiology and pathology. However, studies of the processing of the GPIb-IX complex cannot be easily performed in platelets or megakaryocytes and are limited to transfected cell lines. A recent study of its biosynthesis in

transfected cells delineated the main steps leading to assembly and export of the native receptor [14]. It demonstrated rapid formation of the complex in the endoplasmic reticulum (ER), followed by export through the Golgi apparatus and intracellular degradation of GPIb α that has been transfected in partial complexes with GPIb β or GPIX.

The biosynthesis of a multisubunit receptor requires a succession of well-controlled steps which include: (i) co-ordinate transcription and translation of each gene; (ii) correct folding and post-translational processing of each polypeptide; (iii) timely assembly of the subunits in the correct compartment; and (iv) efficient export and insertion in the outer membrane [1–5]. The post-translational addition and maturation of sugar residues represents a critical step [15], which is further complicated when dealing with multiple subunits and can have important repercussions on the folding, assembly and transport of the entire complex.

Numerous studies aimed at elucidating the biosynthetic pathways and control mechanisms leading to the export of multisubunit receptors have been performed using partial complexes lacking one or more subunits [1–5,14]. There is less information concerning the consequences of more subtle deletions or mutations of structural or functional domains. A number of Bernard-Soulier mutations have been reported, mostly in GPIb α , which were accompanied by detectable expression of substantial amounts of non-functional GPIb-IX-V on the platelet surface and were referred to as variant cases [16–18]. The existence of

Abbreviations used: CHO, Chinese-hamster ovary; Endo, endoglycosidase; ER, endoplasmic reticulum; ERGIC, ER-to-Golgi intermediate compartments; GP, glycoprotein; Mab, monoclonal antibody; TCR, T-cell receptor.

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natural mutations of GPIb α , GPIb β or GPIX in Bernard–Soulier patients offers an excellent model to study the effects of such modifications on the assembly, stability and transport of the complex and the involvement of specific intracellular compartments.

In the present study, we examined the biosynthesis and post-translational modifications of the native GPIb–IX complex in further detail. Understanding the maturation process is particularly important for the GPIb α subunit, which is highly enriched in saccharides. These represent about 40% of its apparent molecular mass [8,14], are mainly in the form of O-linked sugars located at the C-terminus and constitute what is known as the ‘macroglycopeptide region’ [19]. Another relevant, but still unclear, mechanism is the sugar processing of the GPIb β and GPIX subunits. The biosynthesis of normal GPIb–IX complex transfected into Chinese-hamster ovary (CHO) cells was analysed using metabolic labelling and confocal microscopy. GPV was omitted on the basis of previous reports that it is not required for efficient expression of the complex [12,13]. The biosynthetic study was extended to a Bernard–Soulier mutant receptor characterized by a major defect in oligosaccharide processing and transport to the cell surface [20], despite normal synthesis and assembly of its three subunits. Results indicate that tight control is exerted at the exit to the endoplasmic reticulum (ER) and throughout passage through the Golgi structures, thus limiting the expression of abnormal complexes harbouring even subtle mutations on a single subunit.

EXPERIMENTAL

Materials

Monoclonal antibodies (Mabs) ALMA.12 against GPIb α , antibody ALMA.16 against GPIX were produced in our laboratory. Mab WM23 against GPIb α was obtained from Dr Michael Berndt (Baker Medical Research Institute, Prahan, Victoria, Australia). Mab GS28 against GS28 (a specific *cis*-Golgi marker) was purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada), Mab CTR 433 (against an uncharacterized marker of the medial Golgi) and Mab Gi93 [against ER-to-Golgi intermediate compartments (ERGIC) marker] were obtained from Dr Michel Bornens (Institut Curie, Paris, France), and a polyclonal antibody against calnexin (an ER marker) was purchased from Chemicon, Temecula, CA, U.S.A. CHO cell lines expressing wild-type GPIb α associated with GPIb β and GPIX (CHO $\alpha\beta$ IX) or expressing Leu¹⁷⁹-deleted GPIb α associated with GPIb β and GPIX (CHO $\alpha\Delta$ Leu β IX) have been described previously [20].

Metabolic labelling of CHO cells

Cells (5×10^8) were incubated twice in 50 ml of cysteine-and-methionine-free RPMI medium (ICN, Costa Mesa, CA, U.S.A.) without fetal-calf serum for 30 min at 37 °C. The cells were then pulse-labelled for 15 min at 37 °C with 3 mCi (111 MBq) of a [³⁵S]methionine and [³⁵S]cysteine mixture (Amersham Pharmacia Biotech, Uppsala, Sweden). The cells were then diluted in 10 vol. of ice-cold PBS, washed once and chased for different times at 37 °C in RPMI containing 10% (v/v) dialysed fetal-calf serum. At each chase time, cell samples were washed twice in ice-cold PBS and lysed in buffer I [20 mM Tris (pH 7.5)/150 mM NaCl/5 mM EDTA/0.2% (w/v) BSA/1% (v/v) Triton X-100/1 $\times$ Complete Protease Inhibitor Cocktail (Roche Diagnostics G.m.b.H., Mannheim, Germany)].

Biotinylation of surface proteins

Metabolically radiolabelled cells were incubated in ice-cold PBS buffer in the presence of 1 mg/ml EZ-Link biotin Sulfo-NHS or EZ-Link biotin Sulfo-NHS-SS biotin respectively (Pierce, Rockford, IL, U.S.A.), for 20 min at 4 °C. The cells were then washed, quenched twice in 50 mM NH₄Cl in phosphate buffer at 4 °C and lysed in buffer I.

Immunoprecipitation and SDS/PAGE

Cell lysates were clarified by incubating twice with 100 μ l of Protein G–agarose (Sigma) for 1 h at 4 °C. The lysates were then incubated overnight at 4 °C with 10 μ g of the relevant antibodies and 100 μ l of fresh Protein G–agarose. After centrifugation the bead pellets were washed once in buffer I, twice in 0.5% Triton X-100/20 mM Tris (pH 7.5)/150 mM NaCl/5 mM EDTA/0.2% BSA/0.1% (w/v) SDS, three times in 0.5% Triton X-100/20 mM Tris (pH 7.5)/500 mM NaCl/5 mM EDTA/0.2% BSA and twice in 50 mM Tris, pH 7.5. The beads were resuspended in 40 mM Tris (pH 6.8)/2% SDS/2.5% (v/v) glycerol/2 mM *N*-ethylmaleimide/10 mM dithiothreitol and boiled for 5 min. To precipitate surface biotinylated proteins, the immunoprecipitates were incubated for 5 min at 95 °C in 10% SDS and then incubated for 2 h with 80 μ l of streptavidin–agarose beads (Pierce). Proteins were resolved by SDS/7.5–15%-(w/v)-PAGE, the gels were fixed and processed for autoradiography.

Carbohydrate analyses

Immunoprecipitates were dissociated from the beads by incubation in 20 μ l of 50 mM Tris (pH 7.5)/1% SDS/5 mM dithiothreitol for 5 min at 95 °C. Sodium citrate, pH 5.5, was added to a final concentration of 0.1 M before the addition of 1.7 μ g/ml calpain inhibitor and 1 $\times$ protease inhibitor cocktail. Endoglycosidase (Endo) H (5 m-units/sample) or Endo D (2 m-units/sample) (Sigma) was incubated for 6 h at 37 °C. In neuraminidase treatment, the beads were directly resuspended in 20 μ l of 100 mM sodium acetate (pH 5)/1 mM CaCl₂/150 mM NaCl before addition of 10 μ l of neuraminidase (100 m-units) (Roche) and incubation for 6 h at 37 °C.

Tunicamycin and brefeldin A treatment

Tunicamycin (20 μ g/ml) was added to the cells 3 h before labelling and at every step during pulse–chase. In brefeldin A treatment, the cells were pulse-labelled and then chased in medium containing 7 μ g/ml brefeldin A (Sigma).

Confocal microscopy

CHO cells seeded on glass or polylysine-coated coverslips were allowed to adhere in four-well plates for 2 h at 37 °C or for 15 min at room temperature (RT, namely 22 °C) respectively. After washing, the cells were fixed with 3% (w/v) paraformaldehyde and permeabilized with 0.05% (w/v) saponin in blocking solution (PBS containing 0.2% BSA and 1% goat serum). Antibodies were incubated in blocking solution for 45 min at RT using the following concentrations: WM23 and GS28 at 4 μ g/ml, polyclonal anti-calnexin antibody at 1 μ g/ml, CTR433 at 1:10 dilution and Gi93 at 1:1000 dilution. When not directly labelled with a fluorophore, mouse Mabs were revealed

with GAM-Cy3 (Jackson ImmunoResearch, West Grove, PA, U.S.A.) or GAM-Alexa 488 (Molecular Probes, Eugene, OR, U.S.A.) at 1:400 dilution. Anti-calnexin antibody was revealed with a donkey anti-rabbit antibody coupled to Texas Red (Jackson ImmunoResearch). The coverslips were washed with PBS, rinsed with water and mounted in Mowiol 4-88 (Calbiochem–Novabiochem). Labelled cells were examined under a Zeiss laser scanning microscope (LSM 410 invert) equipped with a Planapo oil-immersion lens (63 $\times$ magnification; numerical aperture 1.4). Alexa 488 emission was excited using the 488 nm argon laser line, and Cy3 emission with the 543 nm He/Ne laser line. The signals were filtered with a Zeiss 515–565 nm filter (Alexa 488 emission) or a long-pass 595 nm filter (Cy3 emission). Non-specific fluorescence was assessed by incubating cells with the secondary fluorescent labelled antibodies alone and measuring the average intensity for each fluorochrome. This value was then subtracted from all specific images.

RESULTS

Association and sugar processing of the subunits of native GPIb-IX

CHO cells expressing GPIb α , GPIb β and GPIX (CHO α /IX) were pulse-labelled with [³⁵S]methionine and [³⁵S]cysteine and chased in unlabelled medium. Already after a 15 min pulse (0 chase time) all three labelled subunits were co-precipitated with a monoclonal antibody (Mab) specific for GPIb α (ALMA.12; Figure 1A), indicating early association of the complex following translation. At this time, GPIb α , GPIb β and GPIX had lower apparent molecular masses (85, 21 and 17 kDa respectively) than the mature forms found at the cell surface (125, 24 and 20 kDa respectively; Figure 3 below). The 85 kDa GPIb α band was gradually replaced by a more mature 125 kDa form, and this molecular-mass shift was completed within about 30–45 min of chase. It was mainly due to addition of O-linked sugars, since treatment of the 125 kDa immunoprecipitate with O-glycanase reduced it to 90 kDa (results not shown). Low-intensity intermediate bands were apparent during the first 15 min of chase. GPIb β and GPIX underwent a more modest molecular-mass increase of 1–2 kDa (Figure 1B) and progressively reached their mature sizes after 1 h of chase.

The three subunits detected after the 15 min pulse (0 chase time) were sensitive to digestion with Endo H (Figure 2A). As resistance to Endo H is acquired in the medial-Golgi compartment, this means that the immature N-glycosylated GPIb-IX complexes reside in the ER or *cis*-Golgi and maturation takes place mainly in late-Golgi compartments. The 85 kDa GPIb α band was precipitated by jacalin, a lectin specific for O-linked sugars [21] (results not shown). At later chase times, all three subunits became resistant to Endo H, indicating trimming of their N-linked sugars in the medial-Golgi network. In addition, GPIb α , GPIb β and GPIX acquired sensitivity to neuraminidase (Figure 2B). Hence the three glycoproteins had reached the *trans*-Golgi, where addition of sialic acid residues occurs. Since immature N-glycosylated complexes are retained ahead of the medial Golgi, this compartment could represent a regulatory checkpoint in the biosynthesis of GPIb-IX.

The low-molecular-mass immature bands detected at 0 chase time were N-glycosylated, as they further decreased to 71, 18 and 14 kDa respectively after tunicamycin treatment (Figure 2C). Since tunicamycin treatment did not prevent co-precipitation of GPIb α , GPIb β and GPIX, assembly of the complex was independent of the presence of N-linked sugars. When tunicamycin was used, GPIb α nevertheless matured to a molecular mass of

A

MoAb : ALMA.12

B

MoAb : ALMA.16

Figure 1 Maturation of the GPIb-IX complex

CHO α /IX cells were pulse-labelled for 15 min with a [³⁵S]methionine + [³⁵S]cysteine mixture as described in the Experimental section and then chased for the indicated times in unlabelled medium. Cell lysates prepared at each time point were immunoprecipitated with Mabs ('MoAb') ALMA.12 (**A**) or ALMA.16 (**B**). (**A**) GPIb α progressively shifted from an early immature 85 kDa form to a mature 125 kDa form. The shift was detected at 5 min and completed in approx. 30–45 min. The band at $\approx$ 60 kDa is unrelated to GPIb α and is also present when immunoprecipitations are performed with control non-immune IgG1 (results not shown). (**B**) Analysis of the lower portion of a SDS/7.5–20%-(w/v)-PAGE gel revealed a slight increase in the molecular masses of GPIb β and GPIX. These results indicate that the three subunits of the complex undergo post-translational modifications following their early assembly and N-glycosylation. In this and subsequent Figures the molecular masses in kDa are shown to the right of the gels.

120 kDa, which corresponds to the addition of O-linked sugars. The complex must therefore be able to move through the different intracellular compartments without prior N-glycosylation. Cleavage by neuraminidase confirmed that such complexes in fact reach the *trans*-Golgi where the O-linked residues of GPIb α are sialylated. After tunicamycin treatment, GPIb β and GPIX displayed no increase in molecular mass over the 180 min chase period, indicating that maturation of these two glycoproteins is limited to processing of their N-linked sugars.

Time to reach the cell surface

To determine the time required for newly synthesized GPIb-IX complexes to reach the cell surface, metabolically labelled cells were biotinylated at different chase times, and proteins which had

Figure 2 Sugar processing during maturation of GPIb-IX

Cells, treated (**C**) or not (**A** and **B**) with tunicamycin, were pulse-labelled, chased and immunoprecipitated as described in the legend to Figure 1, and the immunoprecipitates prepared at each time point were analysed for Endo H (**A** and **C**) and neuraminidase (**B** and **C**) sensitivity. '+' denotes enzyme or tunicamycin treatment and '-' the untreated control. (**A**) At early chase times, Endo H treatment decreased the molecular masses of immature N-glycosylated GPIb α , GPIb β and GPIX. Maturation of the three subunits to their higher-molecular-mass forms led to Endo H resistance, indicating trimming and maturation of their N-linked sugars. (**B**) The immature forms of GPIb α , GPIb β and GPIX immunoprecipitated after the pulse period were resistant to neuraminidase treatment. At later chase times, neuraminidase decreased the molecular masses of all three subunits, indicating addition of terminal sialic acid residues. (**C**) Tunicamycin treatment decreased the molecular masses of immature GPIb α , GPIb β and GPIX

Figure 3 Time course of the surface appearance of GPIb-IX

CHO α /IX cells were pulse-labelled and chased as indicated in Figure 1 and cell samples taken at each time point were divided into two aliquots. One was processed directly for immunoprecipitation with ALMA.12 ('Total'). The second was surface-biotinylated, immunoprecipitated with ALMA.12 and the immunoprecipitated proteins were incubated with streptavidin-agarose beads to select surface proteins ('Surface'). Parallel analysis of the total and surface labelling showed that only fully mature GPIb-IX complexes reached the cell surface. These complexes appeared after only 15 min of chase and the surface export of GPIb-IX was completed within 30–45 min.

reached the cell membrane were identified by sequential precipitation with anti-GPIb α (ALMA.12) and streptavidin beads (Figure 3). Only the fully processed high-molecular-mass forms of GPIb α , GPIb β and GPIX were detected as radiolabelled proteins in the streptavidin-precipitated fraction, and only after 15–30 min of chase. Thus the complex appeared at the cell surface, exclusively in its mature form, within approx. 30 min and did not seem to be retained in the late-Golgi compartments. Comparison of the total and surface labelling revealed little or no degradation during up to 2 h of chase, by which time most of the newly synthesized receptor had reached the cell surface.

Biosynthesis of the Δ Leu¹⁷⁹ GPIb-IX complex

The above results suggested that complete sugar processing of the complex occurs after the *cis*-Golgi and might require association or correct folding of the subunits. Therefore, to better characterize the steps necessary for normal maturation, we studied a mutant receptor, the Bernard-Soulier Nancy I variant, previously described with defective glycosylation due to the deletion of Leu¹⁷⁹ of GPIb α [20]. This mutation was reproduced in CHO cells (CHO Δ Leu¹⁷⁹/IX), and the association and maturation of the mutant receptor was followed by ³⁵S metabolic labelling (Figure 4). After a 15 min pulse, immunoprecipitation with a Mab against GPIb α revealed an immature 85 kDa form

(0 chase time). At later times, tunicamycin completely blocked the N-sugar processing of GPIb β and GPIX, but allowed the addition of O-linked sugars to GPIb α , which shifted to 120 kDa and became sensitive to neuraminidase. Comparison with cells not treated with tunicamycin indicated a molecular-mass difference of 12–15 kDa due to the lack of N-linked sugars. Tunicamycin treatment nevertheless did not prevent complex formation throughout the chase period.

Figure 4 Abnormal maturation of ΔLeu GPIb-IX in $\text{CHO}\Delta\text{Leu/IX}$ cells

(A) Cells were pulsed for 15 min with a [^{35}S]methionine + [^{35}S]cysteine mixture, chased for the indicated times and immunoprecipitated with ALMA.16 (upper panel) or ALMA.12 (lower panel), and the immunoprecipitates were treated (+) or not (–) with Endo H before separation by SDS/PAGE and autoradiography. The band corresponding to GPIb α progressively increased from 85 to 95 kDa during the 4 h chase period, while GPIb β and GPIX showed no changes in molecular mass. The three subunits were sensitive to Endo H throughout the 4 h of chase, indicating a lack of N-linked sugar processing. The apparent decrease in molecular mass of GPIb β and GPIX over time is caused by uneven drying of the gel. (B) Cells were pulse-chased and immunoprecipitated with ALMA.12, and the immunoprecipitates were treated (+) or not (–) with neuraminidase. The molecular-mass changes of GPIb α , GPIb β and GPIX were identical with those of (A) in untreated cells, but were not modified by neuraminidase treatment. Hence there was no addition of terminal sialic acid residues.

identical with that of wild-type GPIb α . However, chase for up to 4 h showed delayed and incomplete maturation of ΔLeu GPIb α , resulting in a 95 kDa band instead of the 125 kDa band of normal GPIb α . No further increase in molecular mass was observed during 7 h of chase (results not shown). This agrees with the molecular-mass decrease found in the patient's platelets and demonstrates that it arises from abnormal maturation rather than intracellular degradation.

Despite this defect, GPIb β and GPIX were co-precipitated with ΔLeu GPIb α at early chase times and underwent N-glycosylation, as demonstrated by a decrease in molecular mass after tunicamycin treatment (results not shown). Hence the mutation had no effect on the association of the subunits or their early N-glycosylation. The GPIb β and GPIX subunits co-precipitated with ΔLeu GPIb α lacked processing of their N-linked sugars, since their molecular masses remained unchanged for up to 4 h of chase. The maturation defect was confirmed by persistent Endo H-sensitivity (Figure 4A) and lack of sialylation (Figure 4B), even after extended chase times. These results suggest that the mutant GPIb-IX complex did not reach the *trans*-Golgi network where sialic acid residues are added and might even have been retained before the medial Golgi, where trimming of N-linked sugars occurs. The small molecular-mass

Figure 5 Effect of brefeldin A on GPIb-IX complex maturation in $\text{CHO}\Delta\text{Leu/IX}$ (A) and $\text{CHO}\Delta\text{Leu/IX}$ (B) cells

Cells treated (+) or not (–) with brefeldin A as described in the Experimental section were pulse-chased for the indicated times. At each chase time, cell lysates were immunoprecipitated with ALMA.12 and the immunoprecipitates were treated or not with Endo H or neuraminidase before separation of the proteins by SDS/PAGE. A brefeldin A-untreated control is shown at 6 h of chase. (A) In the presence of brefeldin A, GPIb α matured from 85 to 105 kDa through addition of O-linked sugars, but did not reach the size of mature GPIb α from untreated cells. Maturation of GPIb β and GPIX proceeded without apparent changes in molecular mass, in contrast with the untreated control. The three subunits became progressively resistant to Endo H and sensitive to neuraminidase, indicating trimming of their N-linked sugars and addition of terminal sialic acid residues. (B) Treatment of $\text{CHO}\Delta\text{Leu/IX}$ cells with brefeldin A restored maturation of N-linked sugars and O-glycosylation to the same levels as in brefeldin A-treated $\text{CHO}\Delta\text{Leu/IX}$ cells.

increase observed in ΔLeu GPIb α at later chase times was not due to sialic acid addition, but could have resulted from some O-glycosylation, as was found in Endo H- or tunicamycin-treated samples and was revealed by jacalin precipitation (Figure 4A and results not shown).

A majority of ΔLeu^{179} GPIb-IX complexes do not leave the *cis*-Golgi compartments

The abnormal maturation of the complex could be due to its retention in the *cis*-Golgi or to a defect in sugar processing caused by the mutation. To discriminate between these two possibilities, pulse-chase experiments were performed in the presence of brefeldin A, which disrupts the Golgi apparatus and redistributes *cis*- and medial-Golgi enzymes within the ER [22]. In the presence of brefeldin A, ΔLeu GPIb α displayed a 20 kDa increase in molecular mass at late chase times (Figure 5B). This band was resistant to Endo H and sensitive to neuraminidase, indicating maturation through the action of Golgi enzymes. In parallel, the GPIb β and GPIX subunits co-precipitated by

Figure 6 Intracellular localization of Δ Leu GPIb-IX complexes

CHO $\alpha\beta$ IX and CHO $\alpha\Delta$ Leu β IX cells were detached and immobilized on glass (**A**) or polylysine-coated (**B**) coverslips. After fixation and permeabilization, the cells were double-labelled with Mab WM23 against GPIb α (green) and a marker (red) of the ER (polyclonal antibody against calnexin), ERGIC (Mab Gi93), *cis*-Golgi (Mab GS28), or medial Golgi (Mab CTR 433). The cells were analysed by immunofluorescence confocal microscopy using filters adapted for Alexa 488 (WM23), Cy3 (ERGIC, *cis*- and medial Golgi) or Texas Red (ER). In CHO $\alpha\beta$ IX cells, GPIb α labelling was mainly associated with the plasma membrane, and no co-localization was observed with the ER, ERGIC (**A**) or Golgi markers (**B**). In CHO $\alpha\Delta$ Leu β IX cells, Δ Leu GPIb α was detected at the cell periphery, but also intracellularly with a diffuse vesicular appearance. Δ Leu GPIb α strongly co-localized (yellow) with the ER and ERGIC markers (**A**). This is better illustrated in the images on the right panel, which show the labelling patterns obtained by selecting the pixels double-labelled with Alexa 488 and Texas Red or Cy3 in the two-dimensional scatter histograms of grey values constructed from the different WM23 and compartment-specific labellings recorded simultaneously in the same optical section. Some co-localization was also observed with the *cis*-Golgi, but not the medial-Golgi marker (**B**).

ALMA.12 became resistant to Endo H and sensitive to neuraminidase, indicating processing of their N-linked sugars. Maturation was thus identical with that of the native GPIb-IX complex in the presence of brefeldin A (Figure 5A), which suggested that the glycosylation defects were due to blockade of transfer to the Golgi compartments rather than to defective recognition by the Golgi enzymes. Brefeldin A did not, however, reproduce the normal glycosylation process, since GPIb α did not reach their mature sizes, while GPIb β and GPIX remained resistant to neuraminidase.

To determine the subcellular distribution of normal and mutant GPIb-IX complexes, CHO $\alpha\beta$ IX and CHO Δ Leu β IX cells were examined by immunocytochemistry and confocal microscopy. Normal GPIb α was restricted to the cell membrane, whereas mutant GPIb α was found at the cell surface, but also accumulated intracellularly with a diffuse vesicular appearance (Figure 6). GPIb β and GPIX displayed important intracellular accumulation in both normal and mutant transfected cells, consistent with their overexpression in the parental CHO β IX cell line [11]. Double-labelling with the Mab WM23 and a series of intracellular markers was used to identify the compartments containing mutant GPIb α . The Mab CTR433, a marker of the medial Golgi compartment, gave a distinctive cisternae-like labelling, but did not co-localize with mutant GPIb α (Figure 6B). On the other hand, co-localization was observed with WM23 and with GS28, a *cis*-Golgi marker. Similarly, the ER protein calnexin and Gi93, which reacts with ERGIC, gave a distinctive labelling of intracellular granules which co-localized with Δ Leu GPIb α (Figure 6A). Lysotracker Red, a marker of acidic endosomal compartments, also labelled a granular pool which was, however, distinct from that containing Δ Leu GPIb α (results not shown). None of the markers tested co-localized with normal GPIb α , consistent with its restriction to membrane expression. Thus the processing of Δ Leu GPIb α appears to be blocked at an early stage following translation, probably during transfer to the Golgi, which explains its slow and incomplete post-translational modifications.

DISCUSSION

The present study examined the biosynthesis of the subunits forming the GPIb-IX complex and identified intracellular compartments involved in controlling the expression of this prototype multisubunit adhesive receptor. Following early assembly of the complex and its N-glycosylation, the N-linked sugars of GPIb α , GPIb β and GPIX undergo maturation in the medial/late-Golgi compartments, while GPIb α becomes highly O-glycosylated. N-glycosylation is not essential for the complex to form and reach the cell surface or for the O-glycosylation of GPIb α . Surface expression of the complex is achieved within 15–30 min, immediately after addition of sialic acid residues in the late Golgi. Studies of a Bernard-Soulier mutant receptor revealed that transfer from the ER to the Golgi and normal O-glycosylation of GPIb α are crucial steps for efficient export of the GPIb-IX complex. Finally, a comparison of the normal and mutant receptors supported the idea that association of GPIb α with the GPIb β and GPIX subunits (GPIb β -GPIX subcomplex) is necessary, but not sufficient, for efficient surface expression and confers resistance to intracellular degradation.

Our pulse-chase experiments in CHO cells transfected with the normal complex support the general mechanism of early post-translational subunit assembly, as observed in transfected L cells [14] and previously reported for other multisubunit receptors [1–5]. Complex formation was followed by a series of critical maturation steps in the Golgi compartments. Endo H treatment

confirmed the early N-glycosylation of GPIb α and demonstrated that GPIb β and GPIX also undergo early addition of N-linked sugars. These last two proteins share a single consensus for N-glycosylation [8], which on the basis of tunicamycin treatment appeared to be functional. A new observation was the processing of the N-linked sugars on GPIb β and GPIX, as revealed by a slight (~ 1 kDa) increase in molecular mass and progressive acquisition of Endo H-resistance and neuraminidase-sensitivity.

A distinctive feature of GPIb-IX as compared with other multisubunit receptors is its very high content of O-linked sugars in the GPIb α subunit, which confers adhesive properties as found in mucins [23] and could influence the stability and export of the receptor. Somewhat surprising was the reactivity of the 85 kDa immature form with jacalin, suggesting a partial O-glycosylation of GPIb α before its arrival in the Golgi. Some reports have indicated that initiation of O-glycosylation can occur in the ER and ERGIC [24,25] and that it may therefore proceed continuously throughout the secretory pathway [26,27]. Pulse-chase experiments showed that the majority of O-glycans were added to immature GPIb α between 15 and 45 min of chase and led to a 40 kDa increase in molecular mass. Experiments performed in the presence of tunicamycin established that N-linked sugars are not required for correct subunit assembly and that normal O-glycosylation and surface expression of the complex are not dependent on N-glycosylation. Overall, this GPIb α maturation pattern agrees with that previously described in transfected L-cells [14], despite some differences. Firstly, immature GPIb α displayed a molecular mass 15 kDa higher than the 70 kDa mass reported in L cells. Secondly, full maturation of GPIb α took place in less than 15 min, which is faster than the 90 min estimated in L cells. Although these kinetic differences are not easily explained, they could be due to the different origins of the cell lines used for transfection and/or to the absence of the GPV subunit.

The CHO expression system is obviously not identical with that of native megakaryocytes. This is attested by differences in post-translational modification such as the 10 kDa lower molecular mass of GPIb α in CHO $\alpha\beta$ IX cells (the present study; [28]). It should be mentioned that this size discrepancy is not affected by the presence or absence of the GPV subunit and is also found in GPIb-V-IX transfected K562 cells [29] and in the Dami megakaryocytic cell line (results not shown). Despite these limitations, the overall maturation of the GPIb-IX complex observed in CHO cells is very similar to that described in megakaryocytes, and Bernard-Soulier mutations introduced in CHO cells reproduce with good fidelity the patient's phenotype [20,30,31].

Surface biotinylation in pulse-chase experiments allowed the accurate temporal detection of labelled GPIb-IX as it arrived at the cell membrane. This demonstrated that surface exposure coincides with the completion of sugar processing, which took approx. 15–30 min in CHO cells. These results indicate that transit through the Golgi and completion of sugar processing are critical for the efficient membrane insertion of a normal complex. Conversely, abnormal entry into or transit through the Golgi complex could result in little or no surface expression of a mutant receptor like those encountered in Bernard-Soulier patients.

The single deletion (Δ Leu) at position 179 of GPIb α represented a good model to examine the consequences of a conformation defect in one of the subunits for intracellular processing of the whole complex. Pulse-chase experiments confirmed that the major defect of Δ Leu GPIb α resides in the abnormal addition of O-linked sugars to the macroglycopeptide region [20], and several lines of evidence indicated that this is caused by retention of the complex in a pre-Golgi compartment. Firstly, treatment

with brefeldin A, which redistributes Golgi enzymes to the ER [22], restored normal O-glycosylation. Secondly, when complexed with Δ Leu GPIb α , the GPIb β and GPIX subunits did not undergo trimming of their N-linked sugars and addition of sialic acid residues. Thirdly, confocal microscopy revealed retention of mutant GPIb-IX in the ER and ERGIC. An obvious conclusion is that the abnormal complex is recognized by the so called 'quality control' system [32], which blocks or slows its entry into the Golgi, despite efficient assembly in the ER.

This control mechanism restricting membrane export to correctly folded proteins is only partially effective and allows the surface exposure of abnormally glycosylated GPIb-IX, as revealed by immunofluorescence microscopy and surface biotinylation of non-metabolically labelled cells [20]. The fact that the GPIb β and GPIX subunits co-precipitated with Δ Leu GPIb α from the cell surface are resistant to Endo H suggests that at least some of the Δ Leu GPIb-IX complexes must follow the usual secretory route. Normal processing is, however, prevented, as shown by the abnormal O-glycosylation of Δ Leu GPIb α . Moreover, transfer to the cell membrane is a slow and inefficient process, as no labelled surface proteins were detected in a 4 h pulse-chase biotinylation experiment (results not shown). It is possible that the sugar residues, added to Δ Leu GPIb α while the complex is retained in a pre-Golgi compartment, prevent normal processing when this subunit finally passes through the Golgi. No degradation of Δ Leu GPIb α was observed, even after long chase periods, which was unexpected in view of the reported instability of GPIb α in partial complexes [14], but suggests that the association of Δ Leu GPIb α with GPIb β and GPIX protects it from intracellular degradation. This would agree with a significant intracellular content of GPIb α observed in the patient's platelets [19].

Several aspects of GPIb-IX biosynthesis still remain to be clarified and, in particular, the mechanisms and domains controlling early assembly of the subunits are still unknown. Ordered addition has been observed for instance in the case of the T-cell receptor (TCR), where CD3 molecules associate with one another before binding to individual chains [33]. In the light of the available results, a probable hypothesis is that GPIb β -IX serves as a subcomplex to which GPIb α is added, and GPIb α in turn controls the level of intact complex expression on the platelet surface. A further analogy with the TCR would suggest that the sites required for subunit assembly lie in their transmembrane domains. This is supported by earlier work showing that the intracellular domains of the subunits are not needed for GPIb-V-IX complex assembly [29,33,34]. The testing of these hypotheses will require carefully targeted mutagenesis and biosynthetic studies in transfected cells.

In conclusion, the present study has provided new information on the biosynthesis and intracellular maturation of normal and mutant GPIb-IX and has revealed that this multisubunit adhesive receptor passes through a series of complex and finely regulated post-translational processing steps. A point mutation on a single subunit, which alone was not sufficient to prevent complex assembly in the ER, was recognized by the 'quality control' system, with subsequent intracellular retention and low surface expression of the mutant complex. This recognition was probably triggered by incorrect folding of the Leu-rich domain of GPIb α , a defect which could be present in other variant cases of Bernard-Soulier syndrome.

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