Biosynthesis and Processing of Platelet GPIIb–IIIa in Human Megakaryocytes

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Abstract. Platelet membrane glycoprotein IIb–IIIa forms a calcium-dependent heterodimer and constitutes the fibrinogen receptor on stimulated platelets. GPIIb is a two-chain protein containing disulfide-linked α and β subunits. GPIIIa is a single chain protein. These proteins are synthesized in the bone marrow by megakaryocytes, but the study of their synthesis has been hampered by the difficulty in obtaining enriched population of megakaryocytes in large numbers. To examine the biosynthesis and processing of GPIIb–IIIa, purified human megakaryocytes were isolated from liquid cultures of cryopreserved leukocytes stem cell concentrates from patients with chronic myelogenous leukemia. Immunoprecipitation of [35S]methionine pulse-chase-labeled cell extracts by antibodies specific for the α or β subunits of GPIIb indicated that GPIIb was derived from a precursor of $M_r$ 130,000 that contains the α and β subunits. This precursor was converted to GPIIb with a half-life of 4–5 h. No precursor form of GPIIIa was detected. The glycosylation of GPIIb–IIIa was examined in megakaryocytes by metabolic labeling in the presence of tunicamycin, monensin, or treatment with endoglycosidase H. The polypeptide backbones of the GPIIb and the GPIIIa have molecular masses of 120 and 90 kD, respectively. High-mannose oligosaccharides are added to these polypeptide backbones co-translationally. The GPIIb precursor is then processed with conversion of high-mannose to complex type carbohydrates yielding the mature subunits GPIIbα ($M_r$ 116,000) and GPIIbβ ($M_r$ 25,000). No posttranslational processing of GPIIIa was detected.

Platelet GPIIb–IIIa is a calcium-dependent heterodimer making up 18% of the platelet membrane-associated glycoproteins (12). GPIIb is composed of two disulfide-linked polypeptide chains: IIbα and IIbβ of apparent molecular masses, on SDS gel electrophoresis, of 116 and 25 kD, respectively (12, 18). GPIIIa is a single chain polypeptide which has an apparent molecular mass of 100 kD when reduced (12). GPIIb–IIIa is implicated in the platelet adhesive reactions. It serves as a receptor for fibronectin (16), fibronectin (19), and von Willebrand factor (25), these interactions being essential for platelet attachment, spreading, and aggregation. Recently, two observations have suggested that GPIIb–IIIa is a member of a broadly distributed family of membrane adhesion receptors which mediate cell adhesion and cell–cell interactions, and for which the designation “cytoadhesins” has been proposed. Firstly, the presence of antigens related to GPIIb–IIIa on the surface of cells other than platelets has been demonstrated. Certain monoclonal and polyclonal antibodies which interact with the platelet GPIIb–IIIa immunoprecipitate similar species from surface-labeled endothelial cells (8) and leukocytes (6). Secondly, GPIIb–IIIa recognizes Arg-Gly-Asp-containing sequences (22), and is thus a member of the widely distributed family of Arg-Gly-Asp-binding receptors (20, 21). Differences in receptor specificity and in the molecular mass of the constituent subunits of these receptors have been observed according to their cellular origin, suggesting that the differences amongst these proteins may result from the transcription of distinct genes. Alternatively, the subunits of these receptors may derive from a single gene via cell-specific posttranscriptional processing mechanisms. The recent observation that a 20-kb genomic fragment (7) appears to contain sequences encoding proteins immunologically related to GPIIb–IIIa as well as to OKM-1 and LFA-1, two other membrane adhesion receptors expressed in leukocytes, is in support of the latter hypothesis. The analysis of the posttranscriptional events which occur during the biosynthesis and processing of these adhesion receptors should provide a valuable insight into the molecular mechanisms which control the expression of these receptors at the surface of these different cell types.

GPIIb–IIIa synthesis is detectable from the early stages of megakaryocytopoiesis (31). As the megakaryocyte represents less than 0.5% of all bone marrow cells and is difficult to purify in large quantities, information on the cellular transit of GPIIb–IIIa in human megakaryocytes has not as yet been
available. Recently we have described the isolation of human megakaryocytes from liquid culture of cryopreserved leukocytes stem cells concentrates from patients with chronic myelogenous leukemia (CML) (3). High numbers of developing megakaryocytes from the megakaryoblast to the polyploid mature megakaryocyte can be obtained in these liquid cultures (4). These cells have been shown to express not only platelet GPIIb-IIIa but also fibrinogen, von Willebrand factor, and GPIb, which are either not expressed or only weakly expressed in established erythroid/blastic cell lines. Thus, these megakaryocytes constitute an excellent tool for studying the specific nuclear and cytoplasmic mechanisms controlling the biosynthesis and processing of platelet factors. In this study, these cells have been used to examine the biosynthesis and processing of GPIIb-IIIa.

Materials and Methods

Materials

Carrier-free $^{35}$S (17 μCi/mg) and [35S]methionine (1,000 mCi/mmol) Amplifier and molecular mass standards were from Amersham International (Amersham, England). P2, an anti-GPIIb-IIIa monoclonal antibody (17) was obtained from Immunotech (Marseille, France). BSA, monensin, tunicamycin, Staphylococcus aureus Cowan I strain (Pansorbin) and lactoperoxidase were from Calbiochem-Behring Corp. (La Jolla, CA). Oligo-DT cellulose was obtained from Bethesda Research Laboratories (Bethesda, MD) and endoglycosidase H (endo H) was from New England Nuclear (Boston, MA). The nuclease-treated rabbit reticulocyte lysate system was from Promega Biotech (Madison, WI). The immunoblot assay kit used for Western blotting was from Bio-Rad Laboratories (Richmond, CA).

Purification of Human Megakaryocytes

Megakaryocytes were obtained from liquid cultures of cryopreserved blood cell concentrates from patients in the chronic phase of CML as previously described (3, 4). Briefly, leukocytes-enriched fractions were obtained from CML patients by leukopheresis, and the leukocytes were separated from platelets by low-speed centrifugation at 300 g for 10 min. The leukocyte pellet was washed twice in Hank’s balanced salt solution (HBSS). Low-density cells containing the megakaryocytic progenitor cells were obtained after centrifugation on a BSA density gradient and stored at −196°C using a two-step freezing technique, as previously described (2). Culturing of thawed cells was performed in T 25 flasks (Falcon Labwares, Oxnard, CA) with 5 x 10⁶ cells per 10 ml RPMI 1640 medium supplemented with 20% human plasma. Incubation was performed at 37°C in a 5% CO₂ fully humidified atmosphere.

GPIIb-IIIa-positive cells were purified by sorting on a fluorescence-activated cell sorter (FACS IV, Becton Dickinson, Mountain View, CA) equipped with a 5-W argon ion laser (Spectra-Physics Inc., Mountain View, CA). Cells were labeled by indirect immunofluorescence using P2, a monoclonal antibody which reacts with GPIIb-IIIa (17). The purity of the sorted cell population was evaluated using standard cytological criteria for megakaryocytic cells (32). After sorting, the cells were cultured in human plasma-supplemented RPMI medium for 24 h before use.

Labeling Procedures

Metabolic labeling of proteins synthesized by the cultured human megakaryocytes was routinely performed in 35-mm plastic dishes containing 2 x 10⁶ cells/ml in methionine-free RPMI 1640 with 20% dialyzed human plasma and 250 μCi. [35S]Methionine was then added and the cells were incubated for up to 8 h at 37°C in an atmosphere of 95% air/5% CO₂. For pulse-chase experiments, cells were initially labeled as above for 30 min with 250 μCi/ml [35S]methionine, and the pulse was terminated by removing the [35S]methionine-containing medium. The cells were washed twice with HBSS and incubated with the media containing 20% human plasma for intervals of up to 48 h. When labeling was performed in the presence of tunicamycin or monensin, the cells were preincubated at 37°C with either 5 μg/ml tunicamycin for 2 h or with 2 μg/ml monensin for 16 h. The drug concentrations were maintained throughout the incubation with [35S]methionine.

Washing and Surface Iodination of Platelets

Platelets were isolated from acid citrate dextrose anti–coagulated fresh human blood by differential centrifugation and gel filtration as previously described (16). The platelets were ultimately suspended in Tyrode’s buffer containing 2% BSA and tested for aggregation and fibrinogen binding using previously described assay systems (15). Washed platelets were surface labeled in the presence of carrier-free $^{125}$I using the lactoperoxidase method (18).

Antibodies

A454 is a goat polyclonal antibody raised against a purified preparation of platelet GPIIIa-IIIa. Two rabbit antibodies were raised against purified GPIIIa and GPIIb subunits from human platelets. To purify the two subunits, platelet membrane extracts were applied to a Sepharose affinity column to which CS9, a monoclonal antibody specific for GPIIb-IIIa produced in our laboratory, had been coupled. A fraction containing GPIIb-IIIa was eluted with EDTA at pH 8.9 at 37°C and subjected to electrophoresis on an exponential 5–20% gradient polyacrylamide gel, under reducing conditions. Electrophoretic bands at Iβ and 25 kD corresponding to GPIIb and GPIIIa were excised, and specific antibodies were prepared by subcutaneous injection of rabbits with 20–100 μg of purified subunit emulsified in complete Freund’s adjuvant. The rabbits were given a booster injection 2 wk later with the protein in incomplete adjuvant and then bled 2 wk after the last injection. Monoclonal antibodies, 22C4 and PMI-1, which specifically recognize GPIIIa and GPIIbα, respectively, have been previously described (9, 26).

The specificities of A454, PMI-1, and 22C4 were tested by immunoprecipitation of lactoperoxidase surface-labeled platelet extract and gel electrophoresis. The specificities of the polyclonal antibodies anti-GPIIIα and anti-GPIIbβ were analyzed by Western blot. Briefly, 10 μg of proteins from a 1% Triton X-100 platelet extract were subjected to electrophoresis on a 7-12% linear gradient acrylamide gel with or without reduction with 5% 2-mercaptoethanol. Proteins were transferred onto a nitrocellulose filter, the strips incubated with the antisera at a 1:100 dilution for 1 h, and the antibodies detected using an immunoperoxidase method.

Preparation of Cell Lysates and Immunoprecipitation

After labeling, the cells were washed twice with ice-cold PBS and lysed with lysis buffer (1% Triton X-100 and 2 mM phenylmethylsulfonyl fluoride [PMSF] in PBS, pH 7.4) for 20 min on ice. The lysis was clarified by centrifugation at 11,000 g for 10 min. 1 ml of cell extract was treated with 100 μl of a 10% Staphylococcus aureus Cowan I strain suspension for 30 min at 4°C. The Pansorbin was removed by centrifugation and 100 μl of the cell lysate diluted with 4 vol of washing buffer (1% Triton X-100, 0.5% SDS, 0.5% sodium deoxycholate, 0.5% BSA in PBS, pH 7.4) and incubated for 16 h at 4°C with 5–20 μl of specific antibodies against GPIIIa and GPIIbα. The immunoprecipitate was washed twice with 1 ml washing buffer and then eight times with washing buffer without BSA. After the washing steps, the adsorbed immune complexes were dissociated by boiling for 5 min at 100°C in 50 mM Tris-HCl, pH 6.8, containing 5% mercaptoethanol, 2% SDS, 10% glycerol, and 0.01% bromophenol blue. The samples were clarified by centrifugation at 11,000 g for 5 min and analyzed by SDS-PAGE on either 7.5% gels or linear gradient gels of 7-12%, according to Laemmli (14). After electrophoresis, the gels were fixed for 30 min with 7% acetic acid and treated with Amplify for 30 min, dried under vacuum at 80°C, and exposed to Kodak X-O Mat film at −70°C, using Dupont Cronex Lighting Plus intensifying screens.

Treatment of Proteins with Endo H

[35S]-labeled proteins were isolated by immunoprecipitation with antiserum A454, and fractionated by SDS-PAGE as described above. The [35S]methionine-labeled bands, detected by autoradiography, were excised from the gels using the autoradiograph film as a template. Sample buffer containing SDS and 5% 2-mercaptoethanol was added to each band, and the gel was then crushed. Digestions with endo H were performed in the presence of 0.1 M sodium citrate buffer (pH 5.5) containing 2 mM PMSF and 7 μg/ml of the enzyme. After 18 h of incubation, the reaction was stopped by heating for 2 min at 100°C.

1. Abbreviations used in this paper: CML, chronic myelogenous leukemia; endo H, endoglycosidase H.
Preparation of mRNA and Cell-Free Translation

Total RNA was isolated from cells using the guanidin–hydrochloride method (13) in the presence of laurylsarkosine. Poly (A)-rich RNA was isolated by oligo-dT cellulose chromatography of total RNA (1). 4 μg of Poly (A) + mRNA were incubated with [35S]methionine (2 mCi/ml) and reticulocyte lysate for 90 min at 30°C in a total volume of 100 μl. After incubation, PMSF was added at a final concentration of 2 mM and incorporation of radioactivity in the translation assay was determined by removing 1-μl aliquots and measuring the radioactivity precipitated by 10% TCA.

Results

Specificity of the Antibodies

The specificity of these antibodies is demonstrated in Fig. 1. On immunoprecipitation of lactoperoxidase surface-labeled platelet extract and gel electrophoresis under reduced conditions, A454 precipitated bands at 116 and 100 kD (Fig. 1 A, lane 1) and monoclonal antibodies 22C4 and PMI-1 bands at 100 and 116 kD, respectively (Fig. 1 A, lanes 2 and 3). By Western blotting, the antibody anti–GPIIbα recognized electrophoretic bands at 130 (nonreduced) and 116 (reduced) kD (Fig. 1 B, lanes 1 and 2), while the antibody anti–GPIIbβ blotted electrophoretic bands at 130 (nonreduced) and 25 (reduced) kD (Fig. 1 B, lanes 3 and 4).

Characterization of Purified Megakaryocytes

Large numbers of megakaryocytes were obtained in liquid cultures of cryopreserved blood cell concentrates from patients in the chronic phase of CML (3, 4). The thawed blood
Table 1. Cytological Composition of a Typical Human Megakaryocytes Preparation

| Suspension               | Megakaryocytes | Myeloid cells | Macrophages |
|--------------------------|----------------|---------------|-------------|
| Day-10 liquid cultures   | 45             | 47            | 8           |
| (unseparated)            |                |               |             |
| After sorting            | 92             | 6.5           | 1.5         |

Cell concentrates were cultured for 10–12 d in RPMI medium supplemented with 20% human plasma. When assessed by standard cytological criteria (32), these cultures contained 40–60% megakaryocytic cells. A representative microscopic view of a cell preparation is shown in Fig. 2 A. Further purification of the megakaryocytes was obtained by sorting the cells on a FACS-IV after indirect immunofluorescence labeling with a monoclonal antibody (P2) against GPIIb–IIIa. Sorting resulted in cell suspensions containing >90% megakaryocytes. A representative microscopic view is shown in Fig. 2 B. After purification, the megakaryocytes were cultured for an additional 24 h in human plasma–supplemented RPMI at a cell density of 1 × 10⁶ per ml. The cytological composition of a megakaryocyte preparation before and after sorting is illustrated in Table I. The sorted cell suspension contained <10% myeloid and macrophagic cells, the major contaminating cells in these CML leukocyte cultures.

Analysis of GPIIb and GPIIIa Produced by Human Megakaryocytes

A series of experiments were performed to identify and characterize the GPIIb- and GPIIIa-related proteins produced by the purified megakaryocytes. Initially, the cells were metabolically labeled with [35S]methionine for 8 h and the immunoreactive species precipitated from cell lysates with the various antisera and analyzed by SDS-PAGE under reducing conditions. The results are summarized in Fig. 3 A. Three major electrophoretic bands with estimated molecular masses of 130, 116, and 100 kD (reduced) were detected in the immunoprecipitates of the polyclonal anti–GPIIb–IIIa A454 (Fig. 3 A, lane 1). Monoclonal antibody 22C4, specific for GPIIIa, immunoprecipitated a protein at 100 kD (Fig. 3 A, lane 2), while the monoclonal PMI-1, specific for GPIIbα, immunoprecipitated bands at 130 and 116 kD (Fig. 3 A, lane 3). Controls of nonimmune goat IgG and nonimmune ascite were performed replacing the A454 or the monoclonal antibodies, respectively. No bands were detected in these controls. Fig. 3 A, lane 4, shows the nonimmune goat IgG control. Furthermore, immunocompetitive experiments with the polyclonal antibody in the presence of a large excess of cold platelet membrane extracts resulted in

Figure 3. SDS-PAGE of platelet GPIIb and GPIIIa produced by human megakaryocytes. After metabolic labeling for 8 h with [35S]methionine, the cells were solubilized in PBS containing 1% Triton X-100 and 2 mM PMSF. The newly synthesized GPIIb- and GPIIIa-related proteins were immunoprecipitated and analyzed on 7.5% SDS–polyacrylamide gels. (A) The samples were immunoprecipitated immediately after labeling and reduced before electrophoresis. Lane J, Precipitation with A454; lane 2, precipitation with 22C4; lane 3, precipitation with PMI-1; lane 4, precipitation with A454 in the presence of 10 μg proteins from platelet membrane extract; lane 5, precipitation with goat nonimmune serum. (B) Reduced and nonreduced samples were compared. (Lanes 1 and 2) Immunoprecipitation was performed with A454 immediately after labeling and the samples were either reduced (lane J) or nonreduced (lane 2). (Lanes 3 and 4) The cells were cultured in medium containing cold methionine for an additional 72 h after labeling, and the immunoprecipitates were reduced (lane J) or nonreduced (lane 4). (C) Subunit composition of the 130- and 90-kD nonreduced species. The two bands at 130 and 90 kD from B, lane 4, were excised from the gel, reduced, and analyzed separately on a 7–12% linear gradient polyacrylamide gel. Lane J shows the analysis of the 130-kD band and lane 2 shows the analysis of the 90-kD band. The fluorograms were obtained as described in Material and Methods. Molecular masses are shown as \( M_r \times 10^3 \).
Figure 4. Time course of biosynthesis of GPIIb and GPIIIa in human megakaryocytes. (A) Purified human megakaryocytes were pulsed for 30 min with $[^{35}S]$methionine and chased as described in Material and Methods. The proteins were immunoprecipitated from cells extracts at selected time points with A454. The samples were analyzed on a linear gradient from 7-12% SDS-polyacrylamide gels after reduction with 5% 2-mercaptoethanol. (B) The fluorogram was scanned densitometrically with a Vernon spectrophotometer and the relative intensities of the 130-, 116-, and 25-kD proteins were plotted as a function of the chase time. (C) Megakaryocytes were metabolically labeled for 8 h in the presence of $[^{35}S]$methionine. Immunoprecipitations were performed in the presence of anti-GPIIb (lane 2) or anti-GPIIb (lane 1) either after reduction and alkylation of the cell lysates and analyzed with a 7-12% linear gradient polyacrylamide gel (lane 1 and 2) or before reduction and analysis with a 7.5% polyacrylamide-SDS gel. Due to the low intensity of the 25-kD band, the lower part of the gel was autoradiographed for 7 d. The upper portion of the fluorogram was obtained after 1 d of exposure. Molecular masses are shown as $M_r \times 10^{-3}$.

Duperray et al. Platelet GPIIb–IIIa in Human Megakaryocytes 1669
Figure 5. (A) Endo H treatment of the 130-, 116-, and 100-kD forms of GPIIb and GPIIIa. Megakaryocytes were labeled with [35S]methionine for 8 h and immunoprecipitation was performed with A454. The samples were reduced with 5% 2-mercaptoethanol and subjected to electrophoresis on 7% SDS-polyacrylamide gels (lane 1). The electrophoretic bands at 130-, 116-, and 100-kD were excised, treated separately with 7 μg/ml endo H for 16 h, and reelectrophoresed. The lane 2 represents the 130-kD band, lane 3 the 116-kD band, and lane 4 the 100-kD band after treatment with endo H. (B) Cell-free translation of a megakaryocyte mRNA preparation. The mRNA were translated as described and the newly synthesized proteins were immunopurified with nonimmune IgG (lane 1), A454 (lane 2), PMI-I (lane 3), and electrophoresed on 7.5% SDS-polyacrylamide gels in the presence of 2-mercaptoethanol. Molecular masses are shown as Mr × 10⁻³.

The Journal of Cell Biology, Volume 104, 1987 1670
to 120 kD. The 120-kD protein was immunoprecipitated from a cell-free translation system programmed with megakaryocytic mRNA using both A454 and the monoclonal anti-GPIIb, PMI-1 (Fig. 5 B, lanes 2 and 3). A minor band of low molecular mass can be seen, possibly representing incompletely translated peptides. Thus the 120-kD protein, derived from the 130-kD species by endo H treatment, probably represents fully deglycosylated pro-GPIib. The GPIIIa species, produced by the megakaryocytes, was also sensitive to endo H. Treatment by this enzyme reduced its apparent molecular mass from 100 to 90 kD under reducing conditions.

**Effect of Glycosylation on the Processing of GPIIb and GPIIIa**

Tunicamycin, an antibiotic which blocks co-translational addition of N-linked oligosaccharide to the growing polypeptide (30) affected GPIIb and GPIIIa processing. Fig. 6 A shows that tunicamycin inhibited the formation of the 130-, 116-, and 100-kD bands and two major proteins of 120 and 90 kD were immunoprecipitated. In the presence of tunicamycin, some minor bands were observed and represent non-specific immunoprecipitated proteins.

Monensin which blocks the processing of N-linked oligosaccharides from high-mannose to the complex type in the Golgi cisternae (27, 29), also affected GPIIb biosynthesis (Fig. 6 B). Immunoprecipitation experiments with A454 showed that the mature GPIIb was not produced in the presence of monensin (Fig. 6 B, lane 2). PMI-1 immunoprecipitated the pro-GPIIb at 130 kD (Fig. 6 B, lane 3) and 22C4 the GPIIIa at 100 kD (Fig. 6 B, lane 4). These data support the hypothesis that glycosylation and carbohydrate processing is a prerequisite for the conversion of pro-GPIIb to GPIIb.

**Mature GPIIb Form is Expressed on the Cell Surface While the Pro-GPIIb is an Intracellular Form**

To assess whether both the pro-GPIIb and GPIIb were expressed at the surface of megakaryocytes, the cells were subjected to lactoperoxidase-catalyzed surface labeling followed by immunoprecipitation with A454. The results are shown in Fig. 7. This antibody immunoprecipitated reduced electrophoretic bands corresponding to the mature form of GPIIb and GPIIIa from surface-labeled megakaryocytes (Fig. 7, lane 2). The presence of the pro-GPIIb form was simultaneously detected in metabolically labeled megakaryocytes (Fig. 7, lane 1). Control experiments with surface-labeled platelets indicated that the GPIIb and GPIIIa of platelets and megakaryocytes were of similar molecular masses (Fig. 7, lane 3). These results suggest that only the mature GPIIb and GPIIIa forms are accessible at the cell surface.

**Discussion**

The present study indicates that human megakaryocytes produce platelet GPIIb–IIIa. Previous studies using either enriched bone marrow megakaryocytes (23) or megakaryocytes from human committed megakaryocytes progenitor cells (11) have demonstrated the synthesis of GPIIb and GPIIIa in these cells. The source of megakaryocytes used in the present study was cryopreserved blood of CML patients. These samples offer the distinct advantage that large numbers of developing megakaryocytes (20–100 × 10^6) can be obtained for the study of the biosynthesis of platelet factors. Furthermore, these cells can be purified by fluorescence-activated cell (FAC) sorting to >90% homogeneity and exhibit a capacity to differentiate (4). The present study has demonstrated that human megakaryocytes synthesize the glycoproteins Ib and IIIa separately. No precursor form, common to both the GPIIb and the GPIIIa, could be detected even during short time pulse–chase experiments. In contrast the α and β subunits of GPIIb were derived from the same precursor. Bray et al. have reported cell-free translation studies in the erythroblastic cell line, HEL, and obtained a similar biosynthetic system for the GPIIb–IIIa (5). Thus, the GPIIb and GPIIIa are the end products of separate genes while a single gene codes for the α and β subunits of GPIIb.

GPIIIa is synthesized as a single chain glycoprotein having an apparent molecular mass of 100 kD (reduced) or 90 kD (nonreduced). These values are similar to previously published molecular masses of the reduced and nonreduced forms of platelet GPIIIa. The protein did not exhibit detectable changes in its estimated molecular mass during pulse–chase experiments over a 48-h period, suggesting that the oligosaccharide side chains added co-translationally are not further processed. In contrast, mature GPIIb derives from a posttranslational processing of a single polypeptide chain, the pro-GPIIb moiety, with an apparent molecular mass of 130 (reduced) or 120 (nonreduced) kD. This difference in molecular mass suggests that intrachain disulfide bonds influence the conformation of the nonreduced form of pro-GPIIb.

The precursor–product relationship between pro-GPIIb and GPIIb was established from the following observations: (a) immunoprecipitation of pro-GPIIb with PMI-1, a monoclonal antibody specific for the GPIIb; (b) pulse–chase experiments showing the gradual disappearance of the precursor and the simultaneous production of the α and β subunits of GPIIb; (c) the acquisition of endo H resistance with time; and (d) the capacity of the precursor to interact with specific antibodies directed against GPIIbα and GPIIbβ. This last observation confirmed that the reduced form of pro-GPIIb

![Figure 7. Immunoprecipitation of surface labeled GPIIb and GPIIIa](image-url)
contains both GPIIb subunits. Thus, the transition between pro-GPIIb and mature GPIIb involves a posttranslational proteolytic event. The pulse–chase experiments indicate that the production of mature GPIIb is a slow process, 48 h being necessary for the total disappearance of the precursor.

GPIIb–IIIa is a glycoprotein and glycosylation influences its biosynthesis and expression at the cell surface. Tunicamycin, which inhibits N-glycosylation but not O-glycosylation, induces the formation of the glycoforms of pro-GPIIb and GPIIIa, but mature GPIIb was not produced when its precursor was not glycosylated. The 120-kD form of pro-GPIIb synthesized in the presence of tunicamycin is most probably the nonglycosylated pro-GPIIb, as a similar nonglycosylated 120-kD pro-GPIIb can be identified in a cell-free translation mixture of megakaryocyte mRNA. Furthermore, the pro-GPIIb is sensitive to endo H and treatment with this enzyme yields a single polypeptidic chain of 120 kD. Similar results were obtained for GPIIIa. Both tunicamycin and endo H treatments generated a single electrophoretic band of 90 kD. This value is in agreement with that previously reported for the nonglycosylated form of GPIIb synthesized by mRNA from the HEL cell line (5) and may well represent the fully deglycosylated form of GPIIIa. We were unable to immunoprecipitate the aglyco-form of GPIIIa in cell-free translation experiment with A454. A454 was raised against nonreduced pro-GPIIb-IIIa and, as pointed out by Bray et al. (5), the translation product of the GPIIIa mRNA is not recognized by this type of antibody. Thus, it can be concluded that pro-GPIIb and GPIIIa contain N-linked oligosaccharides of the high-mannose type. In contrast, mature GPIIb was endo H resistant, suggesting that the processing of the oligosaccharide side chains of this glycoprotein occurs in the Golgi cisternae. This is supported by the results of experiments in the presence of monensin. Monensin interferes with the intracellular transport and processing of glycoproteins. It has no effect on the production of glycosylated pro-GPIIb and GPIIIa, again suggesting that GPIIIa carbohydrates moieties are not processed. In contrast, the production of mature GPIIb was stopped, indicating that either monensin interferes with the proteolytic cleavage which transforms the pro-GPIIb into the two chain GPIIb, or that the processing of the N-linked high-mannose side chains is a prerequisite in the precursor–product transition. The proteolytic cleavage occurred either before or after the passage through the Golgi system. This type of processing is not unique to the GPIIb and has also been described for the other proteins (10, 24). The pulse–chase kinetic analysis suggests that 1–3 h are necessary for processing the high-mannose oligosaccharide side chains of pro-GPIIb.

The present study gives some insight into the details of the biosynthesis of GPIIb and GPIIIa in human megakaryocytes. GPIIb–IIIa resembles the majority of the membrane glycoproteins in their pattern of N-linked oligosaccharide biosynthesis. Differences in the glycosylation of the two subunits have been identified. The glycosylation of GPIIIa occurs posttranslationally and the glycoprotein then undergoes no further processing. In contrast, the production of mature GPIIb is dependent upon the processing of the carbohydrate moiety and this is probably the rate-limiting step in GPIIb–IIIa production. The present study does not however determine whether GPIIIa associates with pro-GPIIb or solely with the mature form of GPIIb. These possibilities are now under investigation. The results obtained using surface-labeled megakaryocytes indicate that only the mature form of GPIIb is expressed at the cell surface. Experiments to demonstrate binding of fibrinogen to megakaryocyte were unsuccessful (our unpublished results). Thus the acquisition of the fibrinogen receptor activity may well occur postsynthetically during platelet formation.

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