Biosynthesis and Assembly of Platelet GPIIb-IIIa in Human Megakaryocytes: Evidence That Assembly Between Pro-GPIIb and GPIIIa Is a Prerequisite for Expression of the Complex on the Cell Surface

By Alain Duperray, Alain Troesch, Rolande Berthier, Elisabeth Chagnon, Philippe Frachet, Georges Uzan, and Gérard Marguerie

The platelet membrane glycoproteins GPIIb and GPIIIa form a calcium-dependent heterodimer that functions as a receptor for adhesive proteins on stimulated platelets. In this study, we have investigated the kinetics of the assembly reaction that result in GPIIb-IIIa dimerization. Pulse-chase experiments analysis performed on human megakaryocytes obtained from liquid cultures of chronic myelogenous leukemic patients with antibodies specific for GPIIb or GPIIIa demonstrated the existence of a pro-GPIIb-GPIIIa complex and of a large pool (60%) of unassociated GPIIa; nearly all the GPIIb and the pro-GPIIb molecules were found associated with GPIIIa. This free GPIIIa was not exposed on the cell surface. Pulse-chase experiments on a subclone of the human megakaryocytic cell line LAMA-84 revealed that the cells from this subclone produced only the pro-GPIIb, which was neither processed into mature GPIIb nor expressed on the cell surface. The expression of GPIIa in PMA treated cells resulted in the production of the mature GPIIb form and the expression of the GPIIIIa complex on the cell surface. These results indicate that assembly between the early forms of pro-GPIIb and GPIIIa is an obligatory step for the maturation of the heterodimer and its expression on the cell surface.

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Preparation of RNA, DNA dot blot, and RNA gel hybridization. RNA was extracted from cells in guanidium isothiocyanate.\textsuperscript{24} For RNA dot blot hybridization, 2 μg poly(A)\textsuperscript{+} RNA purified once on oligo(dT)-cellulose,\textsuperscript{35} from control and sham, England. Oligo(dT)-cellulose was obtained from BRL (Beltsvnda, MD). Nitrocellulose BA85 (0.45 μm) was from Schleicher & Schuell. BSA, Staphylococcus aureus Cowan I Strain (Pansorbin) and lactoperoxidase were obtained from Calbiochem-Behring Corp (La Jolla, CA). Random primed labeling kit and culture medium were from Boehringer (Meylan, France). RNA ladder was from BRL. Fluorescein-conjugated rabbit anti-mouse IgG were obtained from Jackson Immunolaboratories (Aventdnde, PA).

Preparation of RNA, DNA dot blot, and RNA gel hybridization. RNA was extracted from cells in guanidium isothiocyanate.\textsuperscript{24} For RNA dot blot hybridization, 2 μg poly(A)\textsuperscript{+} RNA purified once on oligo(dT)-cellulose,\textsuperscript{35} from control and PMA treated LAMA-84 cultures, were denaturated in 2.2 mol/L formaldehyde at 65°C for five minutes, serially diluted in TE (10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA) and applied to Hybond-N membranes equilibrated in TE. For RNA gel hybridization, 2 μg of poly(A)\textsuperscript{+} RNA were denaturated in 2.2 mol/L formaldehyde at 65°C for ten minutes, and size-fractionated by electrophoresis in 1% agarose gels containing 2.2 mol/L formaldehyde, transferred and UV cross-linked to Hybond-N membranes, following the supplier's recommendations. Blots were prehybridized for at least one hour at 65°C in 3 x SSC (1 x SSC = 0.015 mol/L Na citrate, 0.15 mol/L NaCl, pH 7.2), 5 x Denhart’s solution, 10% dextran sulfate, 0.5% sodium dodecyl sulfate (SDS) and 100 μg/mL salmon sperm DNA. Hybridization was carried out overnight at 65°C in the same solution by adding [\textsuperscript{32}P]-labeled random primed cDNA probes (2 x 10\textsuperscript{6} cpm/mL, 10\textsuperscript{7} cpm/μg). After hybridization, the blots were washed for 30 minutes at 65°C in 2 x SSC, 0.5% SDS, and stringently washed for two hours, with three to four changes of 0.1 x SSC, 0.1% SDS, and washed in TE. For RNA gel hybridization, the blots were exposed at 65°C in 2 x SSC, 0.5% SDS, and stringently washed for two hours, with three to four changes of 0.1 x SSC, 0.1% SDS, and washed in TE.

RESULTS

Characterization of antibodies. A polyclonal antiserum was isolated from a rabbit polyclonal antiserum directed against human platelet membrane glycoproteins by the method of Olimsted,\textsuperscript{37} with minor modifications.\textsuperscript{28} Briefly, a total platelet protein preparation was applied on a 7.5% slab gel under nonreducing conditions. After electrophoretic transfer (100 V, 90 minutes), a vertical sheet strip was removed for staining. GPIIb was revealed on this strip after incubation for one hour with the monospecific anti-GPIIb-IIIa B2A, which was then detected using an immunoperoxidase method. The region corresponding to GPIIIa was excised from the blot, and used as immunoadsorbant to purify the specific GPIIIa antibodies.

Washing and labeling of human platelets. Platelets were isolated from acid dextran-anti-coagulated fresh human blood by differential centrifugation and gel filtration as previously described.\textsuperscript{29} Washed platelets were surface labeled in the presence of carrier-free [\textsuperscript{125}I] using the lactoperoxidase method.\textsuperscript{29}
Table 1 summarizes the specificity of all these antibodies. No protein was detected with the monoclonal CS9 GPlIIa (lane 6), whereas the polyclonal anti-GPIIb Lyl 1 (lane 10), demonstrating that the epitope recognized by this antibody was present only on the native GPIIb-IIIa complex. Under nondissociating conditions, no protein was detected (Table 2). In contrast, only 30% of the GPIIIa reacted (Table 2). For the formation of the native GPIIb-IIIa complex was first analyzed. Human megakaryocytes were metabolically labeled with [35S]-methionine for ten minutes, and chased for up to 48 hours. The GPIIb and GPIIIa forms produced were immunoprecipitated from the cell lysates under nondissociating conditions and analyzed on reduced polyacrylamide gels. At time 0, the anti-GPIIIa Bipl immunoprecipitated GPIIIa together with the precursor form of GPIIb (pro-GPIIb) (Fig 2A). Consistent with previously published observations, a gradual increase in the intensity of the electrophoretic band corresponding to the mature GPIIb heavy subunit and a concomitant decrease of the pro-GPIIb was observed over a 24-hour period. During this chase period the intensity of the labeled GPIIIa remained constant. When the same experiment was performed with the anti-GPIIb Lyl 1, a similar pattern was observed except that the intensity of the labeled GPIIIa increased during the first two hours of the chase (Fig 2B). These results indicated the existence of a pro–GPIIb-IIIa complex and suggested the presence of an intracellular pool of unlabeled GPIIIa.

To further analyze this association between GPIIIa and pro-GPIIb, the different electrophoretic bands corresponding to pro-GPIIb, GPIIb, and GPIIIa were excised from the gels and quantified by liquid scintillation counting. At the beginning of the chase, the anti-GPIIIa Bipl immunoprecipitated 80% of the pro-GPIIb obtained with the anti-GPIIb Lyl 1 and no significant amount of mature GPIIb was detected (Table 2). In contrast, only 30% of the GPIIIa reacting with the anti-GPIIIa Bipl were immunoprecipitated by the anti-GPIIIa Lyl 1. Thus, immediately after a ten-minute pulse, a large quantity of the pro-GPIIb is

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Bip1</th>
<th>Ly11</th>
<th>CS9</th>
<th>D33C</th>
<th>B2A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>GPIIIa</td>
<td>GPIIb</td>
<td>GPIIb-IIIa complex</td>
<td>GPIIb</td>
<td>GPIIIa</td>
</tr>
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</table>

**Fig 1.** Characterization of antibodies. Surface iodinated platelets were washed and lysed in PBS containing 1% Triton X100 and 2 mmol/L PMSF. An aliquot (1 x 10⁶ cpm) of this lystate was incubated with the selected antibody for 16 hours. Immune complexes were collected with fixed staphylococci and were examined on reduced 7.5% polyacrylamide slab gels and autoradiography of the dried gels. Immunoprecipitation was performed with the anti-GPIIIa Bip1 (lanes 1 and 3), the monoclonal anti-GPIIb D33C (lanes 2 and 4), the anti-GPIIIa B2A (lanes 5 and 6), the anti-GPIIb Ly11 (lanes 7 and 8), and the anti–GPIIb-IIIa complex CS9 (lanes 9 and 10). On lanes 3, 4, 6, 8, and 10, dissociation of the GPIIb-IIIa complex was performed by treatment with 10 mmol/L EGTA, pH 8.7, at 37°C for 30 minutes before immunoprecipitation. On lane 2, the supernatant obtained after immunoprecipitation with the anti-GPIIIa Bip1 was subjected to a second immunoprecipitation with the monoclonal anti-GPIIb D33C. Molecular masses are shown as molecular weight x 10⁴.
associated with GPIIIa, while 70% of the labeled GPIIIa remains unassociated with the labeled pro-GPIIb. After a four-hour chase period the same amount of labeled pro-GPIIb and GPIIb heavy chain was immunoprecipitated by the anti-GPIIIa Bip1 and the anti-GPIIb Ly11. In contrast, only 40% of the total immunoprecipitable GPIIIa reacted with the anti-GPIIb. These results indicated that after a four-hour chase period both the GPIIb and its precursor form are associated with GPIIIa and that 60% of labeled GPIIIa is still in the free form.

The nonassociated GPIIIa is not expressed on the cell surface. In order to determine whether these unassociated GPIIIa molecules were expressed on the cell surface or remained inside the cells, the megakaryocytes were labeled for six hours with [35S]-methionine and then incubated with the anti-GPIIIa Bip1 (Fig 2, lane 1), the anti-GPIIb Bip1 (lane 2), or the anti–GPIIb–IIIa complex CS9 (lane 3) for two hours. After washing away excess serum, the cells were lysed into lysis buffer containing an excess of unlabeled lysate. The bound antibodies were fixed with Pansorbin. After electrophoresis and autoradiography, the bands corresponding to GPIIb and GPIIIa were quantified. For the three antibodies, similar quantities of GPIIb and GPIIIa were obtained and the ratio between GPIIb and GPIIIa was almost the same (1.13 for Ly11, 0.95 for Bip1, 1.07 for CS9 after correction for the methionine content). This demonstrated that GPIIb and GPIIIa are present on the cell surface only as heterodimer, in a molecular ratio of 1:1. Lane 4 represents the control experiment: the same cells were lysed before immunoprecipitation with Bip1, and a great excess of GPIIIa, as compared with GPIIb, was still obtained. In addition, this experiment indicated that the GPIIb–IIIa complex was not dissociated by these antibodies.

Selection of a stable LAMA-84 subclone with decreased expression of platelet GPIIb–IIIa. The human chronic myeloid leukemic cell line LAMA-84 has already been described. Previous studies have indicated that 25% of the native cells express the heterodimer while 90% of the cell population is positive for the GPIIb–IIIa complex after treatment with the phorbol ester PMA. To investigate the

![Graph](https://example.com/graph.png)

Table 2. Quantitative Immunoprecipitation of the Different Forms of GPIIb and GPIIIa

<table>
<thead>
<tr>
<th>Time (H)</th>
<th>Pro-GPIIb</th>
<th>GPIIb</th>
<th>GPIIIa</th>
</tr>
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<tbody>
<tr>
<td>10</td>
<td>1,262/931</td>
<td>0/23</td>
<td>3,561/3,134</td>
</tr>
<tr>
<td>14</td>
<td>1,477/1,333</td>
<td>50/33</td>
<td>1,039/1,038</td>
</tr>
<tr>
<td></td>
<td>277/196</td>
<td>1,598/1,767</td>
<td>3,894/4,388</td>
</tr>
</tbody>
</table>

The labeled proteins corresponding to the times zero (10) and four hours (14) of the pulse–chase experiment shown in Fig 2 were excised from the gels and quantified by liquid scintillation counting. The quantifications obtained from two immunoprecipitations of the same pulse–chase experiment are illustrated. The results are expressed as dpm, after subtraction of the blank and normalization for the methionine content.

![Diagram](https://example.com/diagram.png)

The labeled proteins corresponding to the times zero (10) and four hours (14) of the pulse–chase experiment shown in Fig 2 were excised from the gels and quantified by liquid scintillation counting. The quantities obtained from two immunoprecipitations of the same pulse–chase experiment are illustrated. The results are expressed as dpm, after subtraction of the blank and normalization for the methionine content.
mode of regulation of the GPIIb-IIIa biosynthesis, clones with minimal expression of GPIIb-IIIa were selected by indirect immunofluorescence using the monoclonal antibody P2. Quantitative flow cytometric analysis, performed on the less reactive clones, allowed the selection of a clone with a very low reactivity with P2 when compared with the parental cell line. Recloning of these cells allowed the selection of a stable clone (LAMA-84A) with minimal expression of GPIIb-IIIa.

To study the expression of GPIIb-IIIa in this cell line, intact or permeabilized LAMA-84A cells were analyzed by indirect immunofluorescence before or after induction with PMA. As shown in Fig 4, permeabilized unstimulated cells expressed GPIIb epitopes (A) but failed to react with the GPIIIa antibody B2A (C), whereas nonpermeabilized cells were negative with the monoclonal anti-GPIIb D33C (B), as well as with the anti–GPIIb-IIIa complex CS9 (D). After treatment with 80 nmol/L PMA for three days, the cells were reactive with all antibodies, whether they were permeabilized (Fig 4E and G) or not (Fig 4F and H).

These results indicate that GPIIb is synthesized by native LAMA-84A cells but is not expressed on the cell surface. GPIIIa is not detected in permeabilized cells and the GPIIb-IIIa complex is not exposed on the surface of these cells. In contrast when the cells are treated with PMA, GPIIIa is produced and the GPIIb-IIIa complex is expressed on the cell surface.

GPIIIa mRNA is not transcribed in native LAMA-84A

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**Fig 4.** Indirect immunofluorescence analysis of GPIIb and GPIIIa expression on intact or permeabilized LAMA-84A cells. Native LAMA-84A cells (A to D), or three-days PMA induced cells (E to H) were fixed with paraformaldehyde and in some cases were permeabilized with 1% Triton X100; A and E represent permeabilized cells, labeled with the anti-GPIIb D33C. B and F are non-permeabilized cells incubated with the monoclonal anti-GPIIb D33C. C and G represent permeabilized cells, treated with the anti-GPIIIa B2A. D and H are nonpermeabilized cells labeled with the anti–GPIIb-IIIa complex CS9. Bar, 50 μm.
cells. We further analyzed the expression of GPIIb-IIIa in LAMA-84A cells by Northern and dot blot hybridization studies, using [32P]-labeled human GPIIb and GPIIIa cDNA probes. The results of these experiments are shown in Fig 5. Northern blot experiments were performed with mRNA extracted from unstimulated cells to compare the expression of GPIIb and GPIIIa mRNA in LAMA-84 and in the LAMA-84A subclone. As seen on lane 1, the labeled GPIIIa cDNA probe hybridized in LAMA-84 with a single mRNA species of 6.2 kb, consistent with the GPIIIa mRNA size described by Bray et al in HEL cells. In contrast, no GPIIIa mRNA was detected in LAMA-84A cells (lane 2). When Northern blots were hybridized with the GPIIb cDNA probe, the GPIIb mRNA of 3.4 kb was detected in both LAMA-84 (lane 3) and LAMA-84A (lane 4) cells. Thus, the lack of expression of GPIIIa in LAMA-84A cells is due to the absence of the GPIIIa mRNA.

In order to determine if the PMA induced modifications in the GPIIb-IIIa biosynthesis in LAMA-84A cells were due to changes in levels of GPIIb and/or GPIIIa mRNA, we performed RNA dot blot hybridization analysis (Fig 5B). In native LAMA-84A cells, GPIIb mRNA was transcribed (left panel), but no GPIIIa mRNA was detected (right panel). After stimulation for 12 to 48 hours with PMA, the GPIIIa cDNA probe hybridized with mRNA extracted from LAMA-84A cells, and a time-dependent increase was observed. A slight increase was seen for GPIIb mRNA after two days of PMA treatment. To further investigate the expression of these glycoproteins in this cell line, pulse-chase experiments before and after induction by PMA were performed.

Native LAMA-84A cells synthesize only pro-GPIIb; effect of PMA. After a ten-minute pulse with [35S]-methionine and immunoprecipitation with the polyclonal anti-GPIIb Ly11, only the 130 Kd pro-GPIIb form was detected (Fig 6A). The intensity of this band progressively decreased during the subsequent chase, but the transformation of this precursor into the mature GPIIb did not occur. No protein was immunoprecipitated under the same conditions with the anti-GPIIIa Bipl (data not shown). When the same experiment was performed after a three-day exposure to 80 nmol/L PMA, the anti-GPIIIa Bipl immunoprecipitated the pro-GPIIb (130 Kd) and GPIIIa (100 Kd) (Fig 6B) at the zero time point of the chase (ten minutes of pulse). During the subsequent chase, the relative amount of GPIIIa did not vary, while the pro-GPIIb was rapidly converted into the mature form of GPIIb (116 Kd). Taken together with the results obtained from immunofluorescence studies, these data suggest that assembly between pro-GPIIb and GPIIIa is necessary for the conversion of the precursor form into the mature form of GPIIb.

DISCUSSION

The results presented in this report provide a model for the biosynthesis of GPIIb-IIIa and establish that the maturation of pro-GPIIb and the exposure of the GPIIb-IIIa complex on the cell surface are controlled by an early assembly of the two proteins.

To demonstrate this assembly of GPIIIa with pro-GPIIb, an immunopurified anti-GPIIIa, Bipl, reacting with both the free and the associated forms of GPIIIa, and a polyclonal antiserum specific for GPIIb, Ly11, were used for pulse-chase analysis of human megakaryocytes obtained from chronic myelogenous leukemic patients. The results obtained after quantification of labeled immunoprecipitated proteins indicated that nearly all the pro-GPIIb chains were associated with GPIIIa, while GPIIIa was synthesized in excess and 60% of the newly synthesized GPIIIa remained unassociated. These unassociated GPIIIa molecules were not expressed on the cell surface, as demonstrated by incubating the intact cells with the antibodies before lysis and immunoprecipitation. This experiment also confirmed our previous result that pro-GPIIb was not expressed at the cell surface.

We have further examined the biosynthesis of GPIIb-IIIa in LAMA-84A, a subclone of the megakaryocytic cell line
Fig 6. Pulse-chase kinetic analysis on native and PMA-induced LAMA-84A cells. Cells were pulsed for ten minutes with [35S]-methionine and chased for the indicated length of time. The proteins were immunoprecipitated from cell extracts with the anti-GPIIb Ly11 (A) or with the anti-GPIIIa Bip1 (B) and analyzed on a 7.5% SDS-polyacrylamide gel after reduction with 5% 2-mercaptoethanol. (A) Native cells. (B) Cells were incubated with PMA (80 nmol/L) for three days before the pulse-chase experiment. Molecular masses are shown as molecular weight \times 10^{-3}.

LAMA-84. Indirect immunofluorescence studies on intact or Triton X100 permeabilized cells and pulse-chase experiments revealed that native cells synthesized only pro-GPIIb but no GPIIIa. This pro-GPIIb form was not cleaved into the mature form of GPIIb and progressively disappeared without being expressed on the cell surface. Results obtained from Northern and dot blot hybridization experiments indicated that the absence of GPIIIa in resting LAMA-84A cells was due to an absence of GPIIIa mRNA. These results suggested that in the absence of GPIIIa, the GPIIb chains were neither processed nor transported to the cell surface. After stimulation with PMA, the GPIIIa chains were produced and, as in megakaryocytes, assembly of the pro-GPIIb with GPIIIa was observed with a concomitant processing of pro-GPIIb into the mature GPIIb and expression of the GPIIb-IIIa complex on the cell surface. In these conditions, dot blot experiments revealed a time-dependent increase of GPIIIa mRNA. This suggested that in this cell line the GPIIb and GPIIIa genes are not coordinate regulated.

Taken together, results obtained in human megakaryocytes from chronic myelogenous leukemia patients and in the megakaryocytic cell line LAMA-84A suggested that the assembly between pro-GPIIb and GPIIIa was a necessary event in the subsequent processing and transport of the proteins to the cell surface after their synthesis in the rough endoplasmic reticulum. This conclusion is supported by the failure of unassociated GPIIIa in human megakaryocytes to reach the cell surface, and by the fact that in native LAMA-84A, free pro-GPIIb chains were neither processed nor expressed at the surface of the cell. However, we cannot exclude the possibility that a fraction of the unassociated proteins, too small to be detected, could reach the cell surface. All these studies have been performed with human megakaryocytes from chronic myelogenous leukemia patients and with a megakaryocytic cell line, but preliminary results from our laboratory indicate that in human megakaryocytes cultured from normal bone marrow, GPIIIa is also over-expressed.

The relationship between assembly of multichain complexes, processing reactions, and intracellular transport seems to be a general one. This has been shown for surface expression of the acetylcholine receptor46 surface immunoglobulin,47 class I48 and class II49 major histocompatibility complex molecules, and for the T cell antigen receptor.50\textsuperscript{\textdagger}5\textcircled{3} Within the integrin family, Ho and Springer51 have initially demonstrated that complex formation between the \(\alpha\) and the \(\beta\) precursors of Mac-1, a member of the leukocyte adhesion receptors, precedes the processing reactions. We have previously reported a similar biosynthetic pathway for the vitronectin receptor present on endothelial cells.24 Chereshe and Spiro51 have studied the biosynthesis of the adhesion receptor synthesized by M21 human melanoma cells. In this model, in the absence of synthesis of \(\alpha\) chains, free \(\beta\) chains were neither processed nor exposed on the cell surface. Thus an assembly between the \(\alpha\) and the \(\beta\) subunits in the endoplasmic reticulum seems to be a general mechanism for the expression of the different members of the adhesion receptor family. It remains to be established whether this mode of assembly and biosynthesis has implications for the specificity of the receptor or if the receptor specificity is due only to an intrinsic property of the subunit structure. Chereshe and Harper56 have shown that the precursor of the \(\alpha\) subunit of the adhesion receptor synthesized by M21 human melanoma cells interacts with immobilized RGD containing peptides in the absence of the \(\beta\) subunit. This suggests that in these cells assembly of the precursor forms does not have a functional implication.

The model demonstrated in the present study for the LAMA-84A cell line provides a molecular basis for the congenital deficiency of GPIIb-IIIa in Glanzmann's thrombasthenia.57 One may speculate that the absence of the GPIIb-IIIa complex in some of the thrombasthenic patients could be due to a lack of production of GPIIIa. Patients with Leu-CAM deficiencies have already been described as having a defect in the production of the \(\beta\) subunit,58 which supports such a proposal.

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