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# Biogenesis of the platelet receptor for fibrinogen: Evidence for separate precursors for glycoproteins IIb and IIIa

**(platelet membrane/fibrinogen receptor/cell-free synthesis/human erythroleukemia cell RNA)**

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ABSTRACT **Congenital absence of platelet glycoproteins IIb and IIIa (GPIIb and GPIIIa) results in a severe bleeding disorder characterized by defective platelet aggregation and failure of fibrinogen to bind to platelets. GPIIb is a two-chain protein containing disulfide-linked $\alpha$ and $\beta$ subunits. GPIIb and GPIIIa are present as a heterodimeric, noncovalent complex in the platelet plasma membrane and function as the fibrinogen receptor. To characterize synthesis of these two proteins, RNA isolated from a human leukemia cell line that contains GPIIb and GPIIIa was translated in a wheat germ cell-free system. Polyclonal antibodies specific for each protein immunoprecipitated distinct [$^{35}$S]methionine-labeled precursors, indicating that GPIIb and GPIIIa are translated from separate mRNAs. Moreover, using specific antibodies against either intact unreduced GPIIb or the $\beta$ subunit, we obtained evidence for synthesis of a common polypeptide precursor for GPIIb$_\alpha$ and GPIIb$_\beta$. Based on experiments using microsomal membranes, it appears that GPIIb is integrated into the platelet membrane with little or no cytoplasmic component. These results suggest that precursors of GPIIb and GPIIIa may be encoded by separate genes and that each precursor is processed before delivery to the plasma membrane.**

Platelet aggregation at the site of vessel injury is a critical step in the maintenance of normal hemostasis. Aggregation requires the binding of fibrinogen to the surface of activated platelets (1, 2). A large body of evidence now suggests that two nonidentical platelet membrane glycoproteins, glycoprotein IIb (GPIIb) and glycoprotein IIIa (GPIIIa), mediate aggregation by acting as receptors for fibrinogen (3–9). More recent evidence suggests that they may also be receptors for von Willebrand factor, fibronectin, and thrombospondin (10–12). As determined by NaDodSO$_4$/polyacrylamide gel electrophoresis (PAGE), unreduced GPIIb has an apparent $M_r$ of 142,000 (13). Electrophoresis of the reduced protein reveals that there are two disulfide-linked subunits: GPIIb$_\alpha$ ($M_r$ = 123,000) and GPIIb$_\beta$ ($M_r$ = 22,000) (13). GPIIIa is a single-chain glycoprotein. When electrophoresed in NaDodSO$_4$/polyacrylamide gels, reduction induces a shift in apparent $M_r$ from 88,000 to 105,000, presumably due to unfolding of the molecule after cleavage of internal disulfide bonds (13). GPIIb and GPIIIa are associated as a noncovalent, calcium-dependent heterodimer (GPIIb-IIIa) in Triton X-100 solutions (14–16) and in the platelet membrane (6–9, 17).

The initial evidence for the role of the GPIIb-IIIa heterodimer in platelet aggregation came from the studies of patients with Glanzmann thrombasthenia, a hereditary bleeding disorder in which platelets fail to aggregate or bind fibrinogen in response to physiologic stimuli (18). The platelets of these patients are deficient in GPIIb and GPIIIa, presumably accounting for the functional defects (18–21). The molecular basis for this unusual genetic abnormality, in which two separate proteins are decreased in parallel, has not yet been defined.

Because platelets are anucleate and make little or no protein (22), study of the biosynthesis of GPIIb and GPIIIa, and hence the molecular defect in Glanzmann thrombasthenia, has been limited. Human megakaryocytes, the bone marrow cells from which platelets are derived, have been difficult to purify in sufficient numbers to study protein synthesis (23). Recently, a human erythroleukemia (HEL) cell line has been described that contains several proteins found in platelets and megakaryocytes, including GPIIb and GPIIIa (24–26). We have used RNA from HEL cells to study the synthesis and membrane insertion of GPIIb and GPIIIa in a cell-free system. Our results suggest that GPIIb and GPIIIa are translated from separate mRNAs. In addition, the $\alpha$ and $\beta$ subunits of GPIIb appear to be derived from a common single-chain precursor. A preliminary report of this work has been published in abstract form (27).

## MATERIALS AND METHODS

**Wheat Germ.** Wheat germ extract was prepared according to the method of Erickson and Blobel (28).

**Membranes.** Microsomal membranes were prepared from freshly excised dog pancreas similar to the method of Walter *et al.* (29) with the variation described previously (30).

**Purification of GPIIb and GPIIIa and Preparation of Antibodies.** The GPIIb-IIIa heterodimer was isolated from Lubrol-PX-solubilized human platelet membranes by monoclonal antibody affinity chromatography (6). The individual GPIIb and GPIIIa subunits were then separated by preparative NaDodSO$_4$/PAGE under nonreducing conditions as described (31, 32). Subunits were recovered electrophoretically (33). The same procedure was used for the isolation of reduced subunits, except that GPIIb-IIIa was first reduced and alkylated (34) and then applied to a discontinuous preparative NaDodSO$_4$/polyacrylamide gel. The purity of the isolated subunits was confirmed by analytical NaDodSO$_4$/PAGE. Polyclonal antibodies were prepared by injecting rabbits subcutaneously with 20–100 $\mu$g of purified subunit emulsified in complete Freund's adjuvant. The rabbits were given booster injections subcutaneously twice at 2-week intervals with protein in incomplete adjuvant and then bled 1 week after the second boost. Antibody titers as assessed by immunoblotting ranged from 1:1000 to 1:5000.

**Radiolabeling of Platelets.** Platelets were surface labeled with Na$^{125}$I by the lactoperoxidase method (35).

**Cell Culture.** HEL cells were grown as described (24).

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Abbreviations: GPIIb, glycoprotein IIb; GPIIIa, glycoprotein IIIa; HEL, human erythroleukemia.

**Immunoblotting.** Platelets and HEL cell membranes were prepared as described (26). Proteins solubilized in 2% $NaDodSO_4$ were electrophoresed on a 7–15% polyacrylamide gel (36) and then transferred to nitrocellulose (37, 38). Bound antibody was then detected by an ELISA system ("Vectastain," Vector Laboratories, Burlingame, CA).

**Cell-Free Translation.** Total RNA was extracted from the HEL cells by the guanidinium thioisocyanate method (39) with a final phenol/chloroform extraction. RNA, at a concentration of 0.2 $\mu g/\mu l$ of cell-free translation, was then incubated with [$^{35}$S]methionine in a wheat-germ translation system for 90 min at 27°C as described (40). In some experiments translation mixtures were supplemented with dog pancreatic microsomal membranes at a concentration of 3.75 $A_{280}$ units/ml. After incubation of translation mixtures for 90 min, phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM (except in proteolysis experiments).

**Posttranslational Proteolysis.** After translation, aliquots were adjusted to 10 mM $CaCl_2$, and then trypsin was added to a final concentration of 0.1 mg/ml. Digestion was carried out for 1 hr at 22°C. In some cases, detergent (final concentration of 1% Triton X-100) was added before the digestion. Proteolysis was terminated by the addition of phenylmethylsulfonyl fluoride and Trasylol (final concentration, 1 mM and 0.1%, respectively).

**Carbonate Extraction.** Protein processing into microsomal membranes was analyzed by treatment with $Na_2CO_3$ according to the method of Fujiki *et al.* (41). The translation products were treated with 100 mM $Na_2CO_3$ (pH 11.5) for 30 min on ice. The sample was then centrifuged at 200,000 × *g* for 2 hr. The resulting supernatant or pellet was immunoprecipitated.

**Immunoprecipitation.** Aliquots of translation products were diluted with Triton buffer (1% Triton X-100/100 mM Tris·HCl, pH 8.0/100 mM NaCl/10 mM EDTA). Two microliters of nonimmune or immune antiserum was added to equal volumes of translated material, and samples were incubated at 4°C for 12 hr. Protein A-Sepharose was added, and samples were agitated at 4°C for 1 hr, then pelleted, washed, and eluted (30). Samples were electrophoresed in either $NaDodSO_4$/7–15% gradient polyacrylamide or $NaDodSO_4$/7% polyacrylamide gels, and then fluorography performed (42).

## RESULTS

**Characterization of Antisera.** Immunoblotting was used to characterize the specificities of the antibodies employed in this study (Fig. 1). In Fig. 1*A*, antiserum raised against unreduced GPIIb reacted with a single protein of $M_r$ = 142,000 in unreduced $NaDodSO_4$/polyacrylamide gels of HEL cell membrane and platelet proteins. When HEL cell membrane and platelet proteins were reduced, the antiserum reacted with two proteins in each preparation of $M_r$ = 123,000 and 22,000, corresponding to the disulfide-bonded $\alpha$ and $\beta$ subunits of GPIIb (13). In Fig. 1*B*, antiserum against the $\beta$ chain of GPIIb was shown to react only with intact GPIIb in nonreduced samples and the $\beta$ subunit in reduced samples of HEL cell membrane and platelet proteins. The anti-GPIIb$_\beta$ antibody showed no detectable crossreactivity with GPIIb$_\alpha$. Two different antisera to GPIIIa were prepared (Fig. 1*C*). Antiserum raised against nonreduced GPIIIa reacted strongly with a HEL cell membrane protein (lane 1) and with platelet GPIIIa ($M_r$ = 90,000, lane 2). Antiserum raised against reduced and alkylated GPIIIa (lanes 3 and 4) reacted with a single protein of $M_r$ = 105,000 in reduced gels of HEL cell membrane and platelet proteins, corresponding to reduced GPIIIa. Of note, antiserum to nonreduced GPIIIa reacted poorly with the reduced protein, whereas antiserum

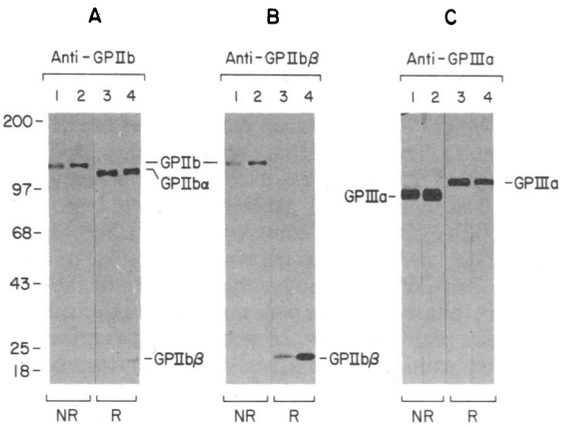


FIG. 1. Specificity of antibodies against HEL cell and platelet GPIIb, GPIIb$_\beta$, and GPIIIa. After solubilization of HEL cell membranes or platelets in 2% $NaDodSO_4$ with or without reduction with 5% 2-mercaptoethanol, proteins were electrophoresed in a $NaDodSO_4$/exponential 7–15% polyacrylamide gel and then electrophoretically transferred to nitrocellulose membranes. Strips were then incubated with specific antisera (1:1000 dilution) for 1 hr. Bound antibodies were detected by a commercial immunoperoxidase technique (Vectastain). Lanes 1 and 3, 25 $\mu g$ of HEL cell membrane proteins; lanes 2 and 4, 10 $\mu g$ of total platelet proteins. (*A*) Anti-GPIIb antisera. (*B*) Anti-GPIIb$_\beta$ antisera. (*C*) Antisera to nonreduced GPIIIa (lanes 1 and 2) or reduced and alkylated GPIIIa (lanes 3 and 4). NR, nonreduced; R, reduced. Molecular weights are shown as $M_r \times 10^{-3}$.

to reduced/alkylated GPIIIa reacted poorly with the nonreduced protein (not shown).

When samples were electrophoresed under nonreducing conditions in 7% polyacrylamide gels, HEL cell GPIIIa migrated slightly slower than platelet GPIIIa (not shown). The molecular basis for this difference remains to be determined. With this exception, our results confirm the molecular and immunologic similarities of platelet and HEL cell GPIIb and GPIIIa reported previously (26).

**Immunoprecipitation of HEL Cell Proteins Synthesized *in Vitro*.** Fig. 2 demonstrates synthesis of $^{35}$S-labeled precursors

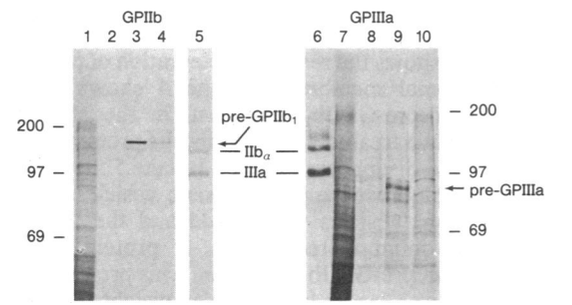


FIG. 2. Cell-free synthesis of GPIIb and GPIIIa using HEL cell RNA. HEL cell RNA was translated in a wheat-germ system using [$^{35}$S]methionine. $^{35}$S-labeled proteins were immunoprecipitated, reduced, and electrophoresed in $NaDodSO_4$/polyacrylamide gels, and fluorography was performed. Lanes 1–4 and 7–10 represent separate 100 $\mu l$ translation mixtures and immunoprecipitations with anti-GPIIb and anti-GPIIIa, respectively. Lanes 1 and 7, samples of total translation products. Lanes 5 and 6, $^{125}$I-surface-labeled platelets. Lanes 2 and 8, nonimmune serum. Lane 3, anti-GPIIb antisera. Lane 4, same as lane 3 except that 10 $\mu g$ of purified platelet GPIIb$_\alpha$ was added prior to the antiserum. Lane 9, antiserum against reduced and alkylated GPIIIa. Lane 10, same as lane 9 except that 10 $\mu g$ of purified reduced and alkylated platelet GPIIIa was added prior to the antiserum. In lanes 2, 3, 8, and 9, equal amounts of the buffer in which the purified GPIIb and GPIIIa were suspended (lanes 4 and 10) were added to the incubation mixture prior to immunoprecipitation. Molecular weights are shown as $M_r \times 10^{-3}$.

of GPIIb and GPIIIa in a cell-free system using HEL cell RNA. After translation, immunoprecipitates were reduced and electrophoresed in $NaDodSO_4$/polyacrylamide gels. Antiserum to GPIIb (lane 3) precipitated a protein larger in molecular weight by ≈5000 than mature platelet $^{125}$I-labeled $GPIIb_\alpha$ (lane 5). To confirm that this translation product was actually related to GPIIb, we used several controls. First, nonimmune serum did not precipitate labeled proteins of similar mobility (lane 2). Second, when purified unlabeled platelet $GPIIb_\alpha$ was added to the translation mixture prior to adding the anti-GPIIb antibody, the intensity of the labeled immunoprecipitated protein was markedly diminished (lane 4). Thus, pure platelet $GPIIb_\alpha$ competed for the antibody with the cell-free translation product, hereafter termed "pre-$GPIIb_1$." As an additional control (data not shown), when the erythrocyte protein band 4.1 was added to the translation mixture before adding the antibody, immunoprecipitation of pre-$GPIIb_1$ was not inhibited.

In our initial experiments we were unable to precipitate a $^{35}$S-labeled translation product with antibody to nonreduced GPIIIa. We therefore prepared a polyclonal antibody to the reduced and alkylated molecule. This antibody precipitated a protein (lane 9) smaller in molecular weight by ≈10,000 than mature platelet $^{125}$I-labeled GPIIIa (lane 6). This band was not seen with nonimmune serum (lane 8). In addition, this band was not present when purified reduced and alkylated platelet GPIIIa was added prior to adding the anti-GPIIIa antisera (lane 10). Therefore, this translation product represents a protein specifically related to GPIIIa, termed "pre-GPIIIa." Bands other than pre-GPIIIa are seen in lane 9. Those that are present in all three lanes (8–10), and hence do not undergo competition by purified GPIIIa, represent nonspecific interaction of proteins with our polyclonal antibody. One minor band of lower molecular weight than pre-GPIIb (Fig. 2, lane 3) and pre-GPIIIa (Fig. 2, lane 9) was seen. Since each of these bands underwent competition by unlabeled purified protein, they may represent incompletely translated peptides.

**Processing of GPIIb.** Because the *in vitro* translated proteins were of different molecular weights than mature glycosylated platelet or HEL cell proteins, we next examined the processing of pre-$GPIIb_1$ in rough endoplasmic reticulum using dog pancreatic microsomal membranes. Translations were carried out in the presence or absence of membrane vesicles. Fig. 3 shows the results of integration of pre-$GPIIb_1$ within microsomal membranes. Lane 4 shows immunoprecipitation of pre-$GPIIb_1$ translated in the absence of membranes. When treated with trypsin, the protein is digested, and the band disappears (lane 2). In lane 5 the results of translation in the presence of membrane vesicles are seen. After translation, vesicles were lysed and the translation products were immunoprecipitated. A protein migrating more slowly than pre-$GPIIb_1$ was seen. This protein, termed "pre-$GPIIb_2$," was larger in molecular weight by ≈22,000 than mature, reduced platelet $GPIIb_\alpha$ ($M_r$ = 123,000) shown in lane 1. Since core glycosylation occurs in the rough endoplasmic reticulum, the shift in molecular weight was presumably due to insertion of the protein into the microsomal membrane and addition of carbohydrate (43). This interpretation was confirmed by treating immunoprecipitates of the pre-$GPIIb_2$ precursor with endoglycosidase H and observing that it migrated with pre-$GPIIb_1$ (unpublished data).

To further characterize membrane processing of translated pre-$GPIIb_1$, trypsin was added to the incubation mixture after translation (lane 6). Proteins that were not integrated within or translocated across the membrane vesicle would have been accessible to digestion by trypsin. Since the pre-$GPIIb_2$ band was intact, it represented proteins either integrated within or translocated across the membrane and, hence,

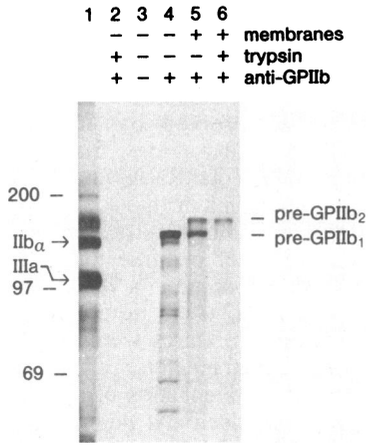


FIG. 3. Fluorograph demonstrating integration and processing of GPIIb into microsomal membranes. HEL cell RNA was translated in a wheat-germ system using [$^{35}$S]methionine either in the presence or absence of dog pancreatic microsomal membranes. Aliquots were then treated with trypsin or buffer, immunoprecipitated, and electrophoresed, and fluorography was performed. Lane 1, $^{125}$I-surface-labeled platelets. Lanes 2–6, immunoprecipitates of $^{35}$S-labeled translation products electrophoresed in a 7% polyacrylamide gel. Translations were carried out in the presence (lanes 5 and 6) or absence (lanes 2–4) of dog pancreatic microsomal membranes. Lanes 2 and 4–6, anti-GPIIb antisera. Lane 3, nonimmune serum. Lanes 2 and 6 represent the results of posttranslational proteolysis. Trypsin was incubated with translation products at a concentration of 0.1 mg/ml, for 1 hr at 22°C, prior to immunoprecipitation. Molecular weights are shown as $M_r \times 10^{-3}$.

protected from digestion by trypsin. Pre-$GPIIb_1$, which represented proteins not inserted into membranes and therefore not protected from digestion by trypsin, was not seen. When detergent (1% Triton X-100) was added to the incubation mixture with trypsin, no bands were immunoprecipitated (data not shown), indicating that an intact membrane vesicle is necessary to prevent digestion of pre-$GPIIb_2$.

**PreGPIIb$_2$ Is Integrated Within the Microsomal Membrane.** Since pre-$GPIIb_2$ did not shift in molecular weight after treatment with trypsin, it could represent either an integral membrane protein with little or no cytoplasmic component or a secretory protein completely translocated across the membrane. Fig. 4 shows the results of carbonate extraction of GPIIb integrated within microsomal membranes. Proteins were translated in the presence of membranes, which were then treated with $Na_2CO_3$ (pH 11.5) and centrifuged, and the resulting supernatant (lane 1) and pellet (lane 2) were immunoprecipitated. Carbonate extraction converts membrane vesicles into sheets, releasing secretory proteins, leaving only those proteins integrated into the bilayer to sediment with the membrane remnants (41). The pre-$GPIIb_2$ band was localized entirely to the pelleted membrane fraction, indicating that it was integrated into the microsomal membrane. The

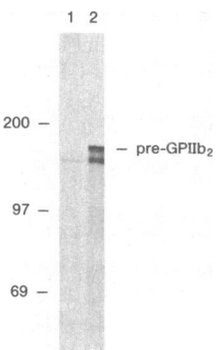


FIG. 4. Carbonate extraction of GPIIb integrated within microsomal membranes. Cell-free synthesis of $^{35}$S-labeled proteins in the presence of microsomal membranes was followed by treatment with 100 mM $Na_2CO_3$ for 30 min at 0–4°C. The sample was centrifuged at 200,000 × *g* for 2 hr. Lane 1, immunoprecipitation of the supernatant with anti-GPIIb antisera. Lane 2, immunoprecipitation of the solubilized pellet. Molecular weights are shown as $M_r \times 10^{-3}$.

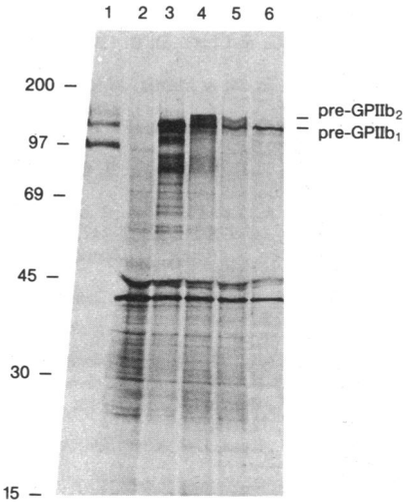


FIG. 5. Immunoprecipitation of HEL cell translation products with anti-GPIIb and anti-GPIIb$_\beta$ antisera. Translations were performed in the presence (lanes 4 and 5) or absence (lanes 3 and 6) of microsomal membranes. A fluorograph of a NaDodSO$_4$/7–15% polyacrylamide gel is shown. Lane 1, $^{125}$I-labeled platelets. Lane 2, nonimmune serum. Lanes 3 and 4, anti-GPIIb antisera. Lanes 5 and 6, anti-GPIIb$_\beta$ antisera. Molecular weights are shown as $M_r \times 10^{-3}$.

pre-GPIIb$_1$ protein was shown to distribute between the supernatant (lane 1) and pellet (lower band, lane 2) fractions in a fashion characteristic of a nonintregrated membrane protein (unpublished observation).

**Synthesis of a Common Precursor of the $\alpha$ and $\beta$ Subunits of GPIIb.** Further processing of the glycosylated precursor of GPIIb must occur since in reduced gels it is larger in molecular weight by ≈20,000 than mature GPIIb$_\alpha$. This difference is approximately equal to the molecular weight of the $\beta$ subunit of GPIIb. The possibility that the $\alpha$ and $\beta$ subunits of GPIIb are derived from a single precursor was explored by immunoprecipitating translation products with a polyclonal antibody that recognizes the $\beta$ but not the $\alpha$

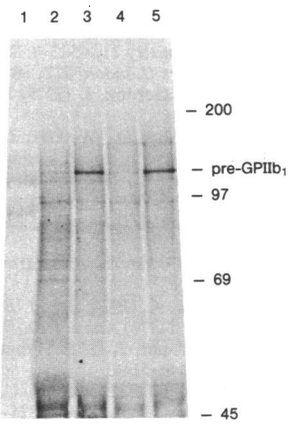


FIG. 6. Effect of purified unlabeled GPIIb$_\alpha$ and GPIIb$_\beta$ on immunoprecipitation of cell-free synthesized proteins with anti-GPIIb$_\beta$. Prior to immunoprecipitation of translated proteins, either purified unlabeled platelet protein or the buffer in which the proteins were suspended was added to the samples. A fluorograph of a NaDodSO$_4$/7–15% polyacrylamide gel is shown. Lane 1, $^{125}$I-labeled platelets. Immunoprecipitates of translation products are represented in lanes 2–5. Lane 2, nonimmune serum. Lanes 3–5, anti-GPIIb$_\beta$ antisera. Platelet GPIIb$_\beta$ (lane 4, 1 $\mu$g), platelet GPIIb$_\alpha$ (lane 5, 5 $\mu$g), and the buffer in which the purified proteins were suspended (lane 3) were added to 50 $\mu$l of the mixture of synthesized proteins prior to immunoprecipitation. Molecular weights are shown as $M_r \times 10^{-3}$.

subunit. In Fig. 5, antisera against GPIIb$_\beta$ (lanes 5 and 6) and intact GPIIb (lanes 3 and 4) immunoprecipitated $^{35}$S-labeled proteins with the same mobilities of pre-GPIIb$_1$ and pre-GPIIb$_2$ in the NaDodSO$_4$/polyacrylamide gel. This fluorogram has more nonspecific bands because it was exposed longer in an attempt to identify mature GPIIb$_\beta$, which would presumably have had fewer [$^{35}$S]methionine molecules incorporated. To confirm that the precursor precipitated by anti-GPIIb$_\beta$ contains the $\beta$ subunit, competition experiments with unlabeled purified platelet GPIIb$_\alpha$ or GPIIb$_\beta$ were performed (Fig. 6). Immunoprecipitation of $^{35}$S-labeled pre-GPIIb$_1$ is seen in lane 3. Purified platelet GPIIb$_\beta$ inhibits immunoprecipitation of pre-GPIIb$_1$ (lane 4), whereas purified platelet GPIIb$_\alpha$ does not (lane 5). These results indicate that GPIIb is synthesized as a single high molecular weight polypeptide containing the $\alpha$ and $\beta$ subunits.

Despite use of antibodies to the unreduced and reduced/alkylated protein, we have been unable to demonstrate incorporation of GPIIIa into membrane vesicles. The experimental conditions required to demonstrate incorporation of GPIIIa into membranes in the *in vitro* system remain to be determined.

## DISCUSSION

We have described the *in vitro* synthesis of separate precursors for GPIIb and GPIIIa. Since immunoprecipitations were performed in the presence of protease inhibitors, it is unlikely that GPIIb and GPIIIa are proteolytic cleavage products of a common polypeptide precursor. Furthermore, we have immunoprecipitated separate $^{35}$S-labeled precursors synthesized by intact cultured HEL cells (unpublished data). Our findings therefore suggest that GPIIb and GPIIIa are translated from separate mRNAs. The simplest interpretation of the data is that GPIIb and GPIIIa are encoded by separate genes that transcribe separate mRNAs. Alternatively, a single gene might transcribe a precursor mRNA that is processed into separate mRNAs for GPIIb and GPIIIa. Alternative mRNA splicing has been described for a number of proteins, including fibronectin (44), and the $\gamma$ chain of fibrinogen (45). In each case, the alternatively translated polypeptides share substantial N-terminal amino acid sequence and immunologic crossreactivity. Since GPIIb and GPIIIa are immunologically distinct and have dissimilar tryptic peptide maps (31, 46), it is unlikely that they are synthesized in this manner. However, direct analysis of RNA with DNA probes will be required to definitively exclude a common mRNA for GPIIb and GPIIIa.

Immunoprecipitation of pre-GPIIb$_1$ with anti-GPIIb$_\beta$ antibody suggests that the $\alpha$ and $\beta$ subunits of GPIIb are synthesized as a common precursor. The observation that purified GPIIb$_\alpha$ does not inhibit precipitation of pre-GPIIb$_1$ by anti-GPIIb$_\beta$ implies that there are no significant antigenic similarities in the $\alpha$ and $\beta$ subunits and that immunoprecipitation of the precursor for GPIIb by anti-GPIIb$_\beta$ is not due to crossreactivity of the $\alpha$ and $\beta$ subunits with the antibody. This is consistent with the lack of detectable crossreactivity of the antibodies on immunoblots and strengthens our conclusion that there is a common precursor for GPIIb$_\alpha$ and GPIIb$_\beta$. *In vivo*, this common precursor, pre-GPIIb$_2$, must undergo proteolytic cleavage, resulting in formation of a two-chain molecule with the $\alpha$ and $\beta$ subunits seen in mature platelet and HEL cell GPIIb. GPIIb is thus analogous to several other molecules consisting of two or more disulfide-linked chains, such as the secretory component of glandular epithelial cells (47) and the insulin receptor (48), which are derived from single-chain precursors.

We have also demonstrated membrane insertion and glycosylation of GPIIb *in vitro*. This system lacks the ability to add complex carbohydrate chains and apparently lacks the enzyme responsible for subunit cleavage. The observation

that pre-GPIIb$_2$ incorporated into microsomal membranes did not change in molecular weight after treatment with trypsin suggests the lack of an available trypsin cleavage site on the cytoplasmic domain of this integral membrane protein. Since GPIIb contains numerous trypsin-sensitive sites (31, 46), this indicates that it has little or no cytoplasmic component. Parise and Phillips recently demonstrated incorporation of purified GPIIb-IIIa into phospholipid vesicles (49). Electron micrographs of the incorporated heterodimer suggested that GPIIb attached indirectly to the membrane by the GPIIIa "tail," although GPIIb remained attached to vesicles after dissociation of the GPIIb-IIIa complex with EDTA. Our findings support the conclusion that the bulk of GPIIb is oriented external to the membrane but also indicate that it is stably incorporated into the membrane.

The explanation for our inability to demonstrate incorporation of GPIIIa into membrane vesicles is unclear. The conditions used for cell-free synthesis may have lacked a critical component necessary for insertion of GPIIIa into the membrane. In preliminary experiments, we have demonstrated synthesis of GPIIIa in intact [$^3$H]mannose-labeled HEL cells (unpublished data). Thus, GPIIIa as well as GPIIb are translocated *in vivo* across the endoplasmic reticulum where initial glycosylation and processing occur. Presumably the two molecules are assembled as heterodimers after entering the endoplasmic reticulum while en route to the plasma membrane.

Study of the synthesis of platelet and megakaryocyte proteins has been difficult because megakaryocytes represent only about 0.05% of the cells in the bone marrow and are therefore difficult to analyze in large numbers or high degrees of purity (23). We have demonstrated the ability to use a continuous cell line with megakaryocytic properties for studies of synthesis of platelet proteins. HEL cells may prove useful in studies of other platelet proteins in the future.

It remains to be determined why GPIIb and GPIIIa are absent or decreased in parallel in platelets from patients with Glanzmann thrombasthenia. If separate genes encode each protein, it is possible that transcription of both is coordinately regulated. If so, a defect in a regulatory element could affect the transcription of both genes. Alternatively, a molecular defect resulting in deficiency of either GPIIb or GPIIIa could result in instability or improper processing of the other protein. For example, transport of both proteins to the cell surface may require a sorting signal from one protein for transport of the other, as is the case with the HLA heavy chain and $\beta_2$-microglobulin (50, 51). Detailed studies of the genetic regulation of synthesis of GPIIb and GPIIIa should provide insight into these issues.

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