Efficient Surface Expression of Platelet GPIIb-IIIa Requires Both Subunits

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Platelet membrane GPIIb-IIIa is a member of the integrin family of heterodimeric adhesion receptors. Processing and export of certain leukocyte and melanoma integrins is disrupted in cells lacking one subunit. We found that surface expression of GPIIb-IIIa, measured by fluorescent activated cell sorting or by surface labeling, required cotransfection of both subunits. In contrast, surface expression was not detected when the subunits were transfected individually. Immunoprecipitation of metabolically labeled transfected cells confirmed the presence of comparable levels of intracellular protein in all cases. When both subunits were transfected, post-translational cleavage of Pro-GPIIb to yield GPIIb heavy chain was also seen.

The adherence of cells to each other and to the extracellular matrix is important in a number of biologic events including organogenesis, cellular migration, immune recognition, and platelet aggregation. The adhesive reactions underlying these functions are mediated in part by a group of related cell surface receptors termed the integrins.1 Platelet glycoprotein (GP)IIb-IIIa is a platelet integrin that serves as a receptor for the adhesive proteins fibrinogen, fibronectin, and von Willebrand factor.2,3 Typical of members of the integrin family, GPIIb-IIIa is composed of an α subunit (GPIIb) and a β subunit (GPIIIa) that exist as a noncovalent heterodimer on the cell surface.4 Both subunits have small cytoplasmic domains, a transmembrane region, and large extracellular domains. GPIIIa is a single-chain polypeptide (Mr ~ 95,000) that is extensively disulfide bonded.5 GPIIb is synthesized as a single chain precursor but undergoes post-translational cleavage to a disulfide-linked heavy chain (Mr ~ 123,000) and light chain (Mr ~ 23,000).6,7

GPIIb-IIIa plays a central role in hemostasis as evidenced by the bleeding disorder, Glanzmann’s thrombasthenia (GT), associated with its deficiency. In general, this disease is associated with the lack of both GPIIb and IIIa from the cell surface8 and is an autosomal recessive disorder.9 The close proximity of the GPIIb and IIIa genes on chromosome 1712-14 suggests that in some cases a large deletion may account for the combined deficiency. Nevertheless, severe

Glanzmann phenotype may exist without evidence of such a deletion.15 Evidence with variant cell lines has suggested that deficiency in expression or processing of the β subunit of integrins of leukocytes16 and α subunit of melanoma cells17 may lead to a failure to process and surface-express its partner. This suggests the hypothesis that mutations which affect expression of either GPIIb and GPIIIa alone may reduce expression of both subunits. In the present work, transfection of GPIIb and GPIIIa cDNAs into heterologous cells was used to test this hypothesis. Our results directly demonstrate that efficient surface expression and processing of this integrin requires the presence of both subunits.

MATERIALS AND METHODS

Cloning and constructs. Partial GPIIb cDNA clones were isolated from a λgt11 library constructed with mRNA from the human erythroleukemia (HEL) cell line.18 Oligonucleotide probes, based on 5' sequences of the largest clones, were used to screen an oligo dT vector-primed HEL cDNA library constructed by the method of Okayama and Berg.19 A 2.9 kb clone (PGHLL13), which contained the entire coding sequence of mature GPIIb with the exception of 327 nucleotides from the 5' end, was isolated. A 720-bp overlapping clone (pXT1) containing the missing 5' fragment was isolated from a primer extension library with an oligonucleotide probe based on the sequence20 corresponding to the amino terminus of the mature protein. Clones PGHLL13 and pXT1 were spliced together at a unique restriction site in the overlap region to give a single 3,362-bp clone designated BS2b.

The clone encoding a GPIIIa-related protein, designated BS3a, was isolated from a λgt11 library constructed from mRNA from primary or first-passage human umbilical vein endothelial cells21 by hybridization with oligonucleotide probes corresponding to the 5' end of the coding sequence.22 BS3a is 4.2 kb in length and contains the entire coding sequence, the putative signal peptides, and portions of the 5' and 3' untranslated sequences.

Full-length cDNAs were excised from BS2b and BS3a by digestion with Xba I or Hinc II, respectively. Overhangs were made blunt with T4 DNA polymerase and ligated to BstXI linkers (Invitrogen, CA). Linkered cDNAs were ligated to the BstXI-cut eukaryotic expression vector CDMA23 and used to transform competent MC1061/p3. Recombinants were grown and analyzed by restriction enzyme digestion and DNA sequencing. Those constructs possessing the proper orientation (CD3a and CD2b) were grown and purified in CsiG gradients.

COS cell transfection. COS cells were transfected by a modified DEAE-dextran procedure.24 Briefly, two days prior to transfection, COS-7 cells were seeded into 60 mm dishes at 3 x 10⁵ cells/mL. Cells were washed and put into transfection medium consisting of

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serum free RPMI 1640 plus 250 μg/mL DEAE-dextran. Five micrograms of the appropriate plasmid DNAs were added with gentle swirling and transfections continued for 12 hours. Cells were subjected to a glycerol shock (15% glycerol for two minutes) and put into normal growth media (RPMI 1640, 5% fetal bovine serum). Cells were harvested after 48 hours with one change of media. This procedure has proved optimal with respect to the number of cells, amount of DNA, length of transfection, type and length of post-transfection shock, and time following transfection at which measurements were made.

Fluorescence-activated cell sorting. Transfected cells were harvested after 48 hours with 3.5 mmol/L EDTA in PBS, washed, and counted. An aliquot containing 5 x 10⁶ cells was pelleted, resuspended in RPMI 1640, and incubated with the first antibody for 20 minutes on ice. Cells were pelleted, washed, and resuspended in RPMI 1640 containing the second antibody, FITC-conjugated goat anti-mouse IgG (Tago, Burlingame, CA). Following a 20-minute incubation on ice, the cells were pelleted, resuspended in 0.5 mL of RPMI 1640, and subjected to flow cytometric analysis on a FACS IV analyzer (Becton Dickinson, San Jose, CA).

Surface labeling and immunoprecipitation. Human platelets were isolated by differential centrifugation and gel filtration. Isolated platelets and COS cell transfectants were surface labeled with ¹²⁵I (Amersham, Arlington Heights, IL) by the lactoperoxidase-glucose oxidase method. Labeled cells were lysed and precleared with normal rabbit serum. The samples were immunoprecipitated and the precipitates analyzed by SDS gel electrophoresis.

Metabolic labeling and immunoprecipitation. At 36 hours after transfection, COS cells were harvested with trypsin and washed with serum-free RPMI 1640. Cells were starved in 0.5 mL of methionine-free RPMI 1640 for 15 minutes, pelleted, and resuspended in 0.5 mL RPMI 1640 containing 1 mCi/mL of ³⁵S-methionine (ICN, Costa Mesa, CA). The pulse was continued for ten minutes, at which time the cells were washed, resuspended, and recultured in normal media. At chase times, the cells were harvested with EDTA and lysed. Cell extracts were immunoprecipitated with an antibody reactive with both subunits, subjected to SDS-PAGE, and autoradiographed. Densitometric analysis was performed with an Ultrosan XL Densitometer (LKB, Piscataway, NJ).

Antibodies. GPIIb-IIIa complex specific monoclonals, 4F10, AP2, and 10E5 were obtained from Virgil Woods (University of California, San Diego), Thomas Kunicki (The Blood Center of Southeastern Wisconsin), and Barry Coller (State University of New York, Stony Brook), respectively. Subunit specific monoclonals Tab and AP3 were supplied by Rodger McEver (University of Texas, San Antonio) and Peter Newman (Blood Center of Southeast Wisconsin, Milwaukee). MAb LM609, specific for the vitronectin receptor, was kindly supplied by Dr David Cheresh (Research Institute of Scripps Clinic, La Jolla, CA). Other polyclonal or monoclonal antibodies were obtained as previously described.

RESULTS

Surface expression of the α or β subunits of GPIIb-IIIa requires the presence of both. The expression of GPIIb-IIIa following transfection was assessed by FACS analysis and by surface radioiodination followed by specific immunoprecipitation. When stained with a complex specific monoclonal antibody (4FlO), the population of cells transfected with both GPIIb and GPIIIa was shifted to higher fluorescence intensity relative to wild type CDM8 transfectants (Fig I). Similar staining was observed with two other complex specific monoclonals, 10E5 and AP2 (not shown). When stained with a nonreactive mouse monoclonal antibody, the histogram patterns of GPIIb-IIIa cotransfectants and CDM8 transfectants were identical (Fig 1). Cells transfected with GPIIb only or GPIIIa only exhibited levels of fluorescence intensity characteristic of wild type CDM8 transfectants.
COS cell transfections and pulse chase experiments were done as described in Materials and Methods. Lysates from the chase time points listed below were immunoprecipitated with a GPllb-llla specific antiserum and analyzed by SDS-PAGE on a reducing gradient gel. The transfectant type is listed above each panel of the autoradiogram.

**Fig 2.** SDS-PAGE analysis of immunoprecipitates from surface labeled transfectants. COS cell transfectants and isolated platelets (pits) were surface iodinated as described in Materials and Methods. Lysates were immunoprecipitated with GPllb-llla specific antibody (lanes 1 through 7) or a preimmune rabbit serum (NRS, lane 8). The products were electrophoresed on nonreducing (lanes 1, 2, 5 through 8) or reducing (lanes 3 and 4) 6.5% polyacrylamide gels in the presence of SDS, and autoradiographed. The cell or transfectant type is listed below each panel.

The expressed protein is similar to authentic GPIIb-IIIa. In order to confirm our FACS results and address the nature of surface-expressed proteins, COS cells transfected with individual subunits, both subunits, or wild type CDM8 were harvested after 48 hours and surface labeled. The cell lysates were subjected to immunoprecipitation with anti GPIIb-IIIa or preimmune serum and the immunoprecipitates were then analyzed by SDS PAGE. Immunoprecipitation of lysates from cells transfected with both subunits yielded two bands that had similar mobilities to those immunoprecipitated from surface-labeled platelets on both nonreduced and reduced gels (Fig 2). In contrast, when cells were transfected with individual subunits only or CDM8, no bands were seen after immunoprecipitation. Long exposure did reveal faint bands of equal intensity with the GPIIb, GPIIIa, and CDM8 transfectants (not shown), suggestive of background COS cell proteins.

**Efficient processing and stability of the α subunit requires the correct β subunit.** The FACS and surface labeling results suggest that cotransfectants express a protein that is similar to platelet GPIIb-IIIa. The lack of surface expression of individually transfected subunits may be due to the absence of intracellular protein or its inefficient or improper processing. To address these questions, biosynthetic labeling experiments were performed with the transfectants. Whether transfected into COS cells individually or together, transiently expressed GPIIb or GPIIIa were detectable following 15 minutes of chase (Fig 3). Furthermore, when analyzed by densitometric scanning, these initial levels of intracellular GPIIIa (Absorbance [Abs] ~ 1.52) or GPIIb (Abs ~ 1.02) in individual subunit transfectants were comparable to intracellular GPIIIa (Abs ~ 1.57) and GPIIb (Abs ~ 1.27) observed in cotransfectants (Fig 3). Surprisingly, the immunoprecipitation of lysates from GPIIb transfectants resulted in two bands (Fig 3). The upper band had a mobility consistent with Pro-GPIIb (Mr ~ 140,000); and, while the identity of the lower band is unknown at this time, its migration is typical of a β subunit (Mr ~ 100,000 reduced) and thus may represent an endogenous COS cell β subunit. By four hours of chase, three immunoprecipitable bands were observed for GPIIb-IIIa cotransfectants (Fig 3).
bands were observed with the cotransfectants (Fig 3). This presumably signifies the post-translational cleavage of GPIIb into heavy and light chains. The presence of a third band was not detected in GPIIb transfectants suggesting that, when transfected alone, the subunit does not undergo this characteristic post-translational cleavage. In addition to being unprocessed, this pro GPIIb also appears to be less stable. By 14 hours of chase, <3% of the original band remains, while 70% of the remaining GPIIb in the cotransfectants is processed into the heavy chain. Thus, the correct processing, stability, and surface expression of GPIIb appears to require GPIIIa.

**DISCUSSION**

The major findings of this work are (1) efficient surface expression of a prototype integrin, GPIIb-IIIa, requires both subunits, and (2) the processing and stability of the α subunit is dependent upon the β subunit. In addition, as defined by apparent subunit Mr and antibody reactivity, the transfected protein is similar to authentic platelet GPIIb-IIIa. Finally, since the GPIIIa clone was derived from endothelial cells whose only known cytoadhesin is the vitronectin receptor, this study proves that the vitronectin receptor β subunit can form a heterodimer with GPIIb.

As expected, the cotransfection of COS cells with GPIIb and GPIIIa results in synthesis and surface expression of both subunits. The proteins expressed in cotransfected cells are properly processed and exhibit migration patterns similar to those of authentic GPIIb-IIIa from platelets. On the other hand, though intracellular synthesis of each subunit was detected, individual subunit transfectants exhibited only background levels of surface expression. Although our data show individual subunits do not accumulate on the surface, we cannot exclude the possibility that they transiently reach the cell surface but are rapidly degraded.

One unexpected finding in our pulse chase experiments was the presence of two immunoprecipitable bands from COS cells transfected only with GPIIb (Fig 3). Although the identity of the lower band is not known, its migration is similar to known β subunits and thus may represent an endogenous COS cell β subunit that may complex with GPIIb. Interestingly, a polypeptide of slightly larger size was immunoprecipitated with CDM8 transfectants (Fig 3). It is not clear whether these two bands represent two COS cell integrin β subunits or other COS cell proteins. While a complex was detected with GPIIb transfectants, GPIIIa transfectants failed to exhibit such a complex, as only a single protein species was observed upon immunoprecipitation. The absence of a second band indicates that no COS cell α subunit stably complexes with GPIIIa.

In cotransfectants, GPIIb was proteolytically processed; in megakaryocytes, such processing involves a post-translational cleavage at pairs of basic amino acids in the carboxyl terminal end of GPIIb. In contrast, no such cleavage was observed in cells transfected with GPIIb alone. Formation of a complex with GPIIIa seems to precede cleavage of GPIIb and our data indicate that the presence of GPIIIa and presumably complex formation are crucial for light-heavy chain cleavage. The formation of an authentic and appropriately processed complex may also have an effect on protein stability. While the GPIIb-IIIa complex was formed and processed throughout the chase time course, the complex observed with GPIIb transfectants was lost during this period. This disappearance is also in contrast to the GPIIIa transfectants where the single band remains relatively stable throughout the chase period. It is not clear whether the relative instability of GPIIb transfectants is due to the formation of an aberrant complex or the lack of processing, or both.

The concept that in integrins, a common β subunit may be shared among several α subunits arose because of the immunochemical, partial sequence, and peptide map similarities among β subunits in each subfamily. Nevertheless, determined complete amino acid sequences of β subunits have not been reported. Our GPIIa clone was isolated from endothelial cells that do not detectably express GPIIb protein or mRNA and is probably the vitronectin receptor β subunit. Because it was able to form a complex with GPIIb, these data provide the first direct proof that the vitronectin receptor β subunit can act as the GPIIIa moiety of GPIIb-IIIa.

Recent analyses of GT patients indicate a variable content of GPIIb and GPIIIa polypeptides. Nevertheless, most severe GT patients lack virtually all surface GPIIIa. Moreover, an Iraqi Jewish kindred has been found to lack detectable GPIIIa and have no gross abnormality of GPIIb or GPIIIa gene structure. Members of this kindred’s platelets contain small quantities of unprocessed Pro GPIIb. Similarly, a preliminary report has described a variant megakaryocytic cell line that fails to express GPIIIa and in which Pro GPIIb is not processed. Our finding that processing and expression of GPIIb-IIIa requires both subunits provides a ready explanation for these results. Moreover, these findings also provide an alternative explanation for the deficiency of both glycoproteins in patients who may bear mutations affecting only one of them.

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