Role of the Transmembrane and Cytoplasmic Domains in the Assembly and Surface Exposure of the Platelet Integrin GPIIb/IIIa†

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ABSTRACT: Integrins are $\alpha\beta$ heterodimers that play a major role in cell–cell contacts and in interactions between cells and extracellular matrices. Identification of structural domains that are critical for the expression of such receptors at the cell surface in a functional conformation is one of the major issues that has not yet been resolved. In the present study, the role of the cytoplasmic and transmembrane domains of each of the subunits has been examined using platelet GPIIb/IIIa as a prototypic integrin. GPIIb/IIIa (α_{IIb}/β_3) is a member of the integrin family and functions as a receptor for fibrinogen, fibronectin, von Willebrand factor, and vitronectin at the surface of activated platelets. Human megakaryocyte GPIIb and GPIIIa cDNAs were used to create a GPIIb mutant coding for the extracellular GPIIb heavy chain alone (GPIIbΔl) and a GPIIIa mutant lacking the transmembrane and cytoplasmic domains (GPIIIaΔm). Full length and mutant cDNAs were subcloned into the expression vector pECE and used to transfect COS cells. The formation of heterodimers and their cellular localization was analyzed by immunoprecipitation and immunofluorescence labeling using anti-platelet GPIIb/IIIa antibodies. We show here that the extracellular domains of α and β subunits are able to form a heterodimer, although with a lower efficiency, in the absence of the transmembrane and cytoplasmic domains. The presence of the cytoplasmic and transmembrane domains in the α subunit is, however, necessary for expression at the surface of the cell whereas the corresponding domains of the β subunit are not required.

Cell adhesion is an important function in a large number of biological processes including embryogenesis, host defense, differentiation, and metastasis development. Adhesion is mediated by a variety of membrane-associated glycoproteins that function as receptors for circulating or tissue adhesive proteins. Among these receptors, integrins play a major role in cell–cell contact and in interactions between cells and extracellular matrices (Hynes, 1987). Integrins are heterodimers of the $\alpha\beta$ type, which interact with adhesive ligands via the recognition of short amino acid sequences, such as the ubiquitous RGD tripeptide (Pierschbacher et al., 1984), acting in synergy with other motifs (Obara et al., 1988; Andrieux et al., 1989). Since the identification of the integrin family, an increasing number of β and α subunits have been characterized by structural and serological studies, demonstrating the large diversity of this family. At least 7 homologous integrin β subunits and 13 different α subunits have already been described (Hemler et al., 1990). Integrins are currently divided into subfamilies. Each subfamily is distinguished by a common β subunit capable of associating with specific α subunits. Adding further complexity to the organization of integrins, recent work has shown that certain α subunits can combine with more than one β subunit [for a review, see Albelda and Buck (1990)]. All integrins share common structural features. α and β subunits are composed of a large extracellular domain, a transmembrane domain, and a short intracellular domain, but the precise function of these domains is unclear. How these integrins are produced and expressed in a functional conformation at the surface of the cell and what are the bases for the code that controls their specificity are two major issues that have not been resolved yet. With the recent cloning of cDNAs for different β and α subunits, these questions can now be approached. Of particular interest is the identification of structural domains that are critical for the expression of the receptor at the surface of a cell. Recent studies (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990) on the β1 subunit using expression of mutant and recombinant forms have shown that the cytoplasmic domain is required for cytoskeletal organization, though recombinant complexes $\alpha\beta$1 lacking this domain were still exported to the cell surface and expressed the ligand binding activity. Point mutations on the β2 gene, resulting in amino acid substitutions on the extracellular domain of the β2 subunit, have also indicated that regions within these domains are critical for receptor assembly and cell surface expression (Arnaout et al., 1990).

GPIIb/IIIa (α_{IIb}/β_3) is a prototypic integrin which functions as a platelet receptor for fibrinogen (Marguerie et al., 1979; Bennett & Vilaire, 1979), fibronectin (Plow & Ginsberg, 1981; Gardner & Hynes, 1985), von Willebrand factor (Ruggeri et al., 1982), and vitronectin (Pytela et al., 1986; Thiagarajan & Kelly, 1988). In the present study, we have used full length cDNA coding for the platelet integrin GPIIb/IIIa (Frachet et al., 1990) to create mutants devoid of cytoplasmic and transmembrane domains of either subunit in order to verify their importance in the assembly and the intracellular processing of the complex. We have previously shown in human megakaryocytes that assembly of the pro-forms of both subunits is an early posttranslational event. This interaction between the two subunits is required for a correct processing and surface expression (Duperray et al., 1989). We demonstrate here that this assembly involves interactions between regions that are located in the extracellular domains of the two subunits and that transmembrane interactions are not essential. We also demonstrate that while the presence of the transmembrane domain in the α subunit is necessary for the expression at the surface of the cell, the corresponding domain

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of the β subunit is not required.

Experimental Procedures

Materials. The monoclonal antibodies anti-GPIIIa B2A, anti-GPIIb D12A, and anti-GPIIb/IIIa CS9 have already been described (Duperray et al., 1987, 1989; Troesch et al., 1990). Full length cDNA clones for GPIIb and GPIIIa form human megakaryocyte cDNA libraries have already been described (Frachet et al., 1990). Oligonucleotides were synthesized on an Applied Biosystems synthesizer. DNA amplification was performed on a Techne programmable Dry Block PHC-1 (Techne, Inc.) using a PCR amplification kit from Perkin-Elmer Cetus. The expression plasmid pECE (Ellis et al., 1986) containing the earlier promoter and the terminated region of SV40 was used for COS-7 cells transfection (Gluzman, 1981). Carrier-free [^{125}I]- (17 mCi/μg) and [^{35}S]methionine (1000 mCi/mmol) was from Amersham International (Amersham, Buck., U.K.). Endoglycosidase H (endo H), trypsin, and culture medium were from Boehringer (Meylan, France).

Production and Analysis of the Recombinant GPIIb and GPIIIa. Each subunit of the platelet heterodimer GPIIb/IIIa is comprised of an extracellular domain (EC), a hydrophobic transmembrane domain (TM) and a short cytoplasmic domain (CY) (Figure 1). GPIIIa is a single-chain protein, whereas the mature GPIIb (α_{IIb}) is formed with a heavy and a light chain linked by a disulfide bond which originate from a precursor form, the pro-GPIIb (Duperray et al., 1987), containing both the heavy and the light chains.

Construction of the GPIIb and the GPIIIa Expression Vectors. Full length GPIIb and GPIIIa cDNAs were introduced into the pECE expression vector using unique restriction sites. The resulting plasmids were named pECEIIb and pECEIIIa. The pECEIIB plasmid contains a GPIIb cDNA fragment from nucleotide 1 to 3198 ligated into the *Sma*I pECE restriction site. The pECEIIIa plasmid contains a 2603 bp cDNA fragment (from nucleotide −18 to 2585) introduced between the *Eco*RI and *Xba*I pECE restriction sites. The expression vector were then isolated from HB101 transformed cells by the alkaline lysis method and further purified by a CsCl gradient centrifugation (Sambrook et al., 1989). The structure of the pECEIIb and the pECEIIIa plasmids was confirmed by extensive restriction mapping and double-strand DNA sequencing reaction of ligation sites.

Construction of a GPIIb Mutant cDNA Coding Only for the GPIIb Heavy Subunit (Figure 1A). A modified cDNA clone coding only for the GPIIb heavy chain was generated by PCR experiment (Mullis et al., 1987) on the wild-type GPIIb cDNA after digestion at the *Xmn*I restriction site on the plasmid. To prime the first strand, the oligonucleotide 5′-GTGCCGGTCCGGGCAGAGGCC-3′ corresponding to the wild-type GPIIb cDNA containing a unique restriction site (*Rsr*II) at position 2317 was used; a mutant oligonucleotide 5′-TTAGCGATCCCGCTTGTGATGGG-3′ coding for an in-frame stop codon introduced at position 2667 was used to prime the second-strand synthesis reaction. Nucleotide 2667 corresponds to the carboxy terminus of the GPIIb heavy chain when the major proteolytic cleavage site between Arg 858 and Arg 859 (Loftus et al., 1988) is used. After purification of the PCR product, fill-in reaction with Klenow fragment of DNA polymerase I, and digestion with *Rsr*II, the DNA fragment (nucleotides 2317–2667 plus a stop codon) was introduced into the pECEIIb plasmid deleted of the GPIIb region from nucleotide 2317 to 3127 (including the light chain sequence from the Arg 859 to Glu 1008, plus a short nontranslated region from nucleotide 2168 to 2198). The resulting

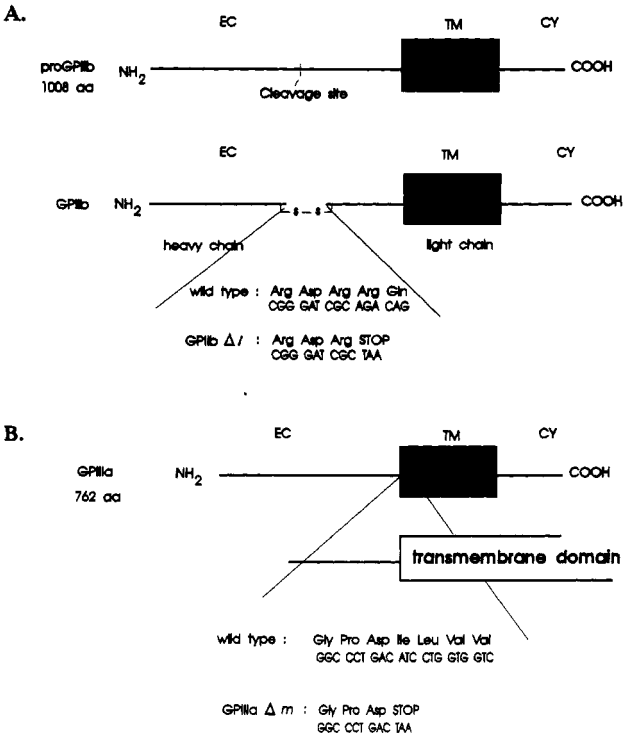

FIGURE 1: Generation of mutant forms of GPIIb/IIIa subunits. The structure of the native GPIIb and GPIIIa is illustrated; the extracellular domain (EC), the transmembrane domain (TM), and the cytoplasmic tail (CY) are indicated. (A) Construction of a GPIIb mutant cDNA coding for a GPIIb subunit truncated from Arg 859 to Glu 1008. The GPIIb heavy and light chains are linked together by a disulfide bond, and their precursor form is named pro-GPIIb (Duperray et al., 1987). As described at the bottom, the pro-GPIIb contains a major proteolytic cleavage site which is used during the maturation of the protein (Loftus et al., 1988). A modified cDNA clone, interrupted at this cleavage site and coding for the GPIIb heavy chain only, was generated by PCR experiment on a wild-type GPIIb cDNA containing plasmid as described under Experimental Procedures. (B) Construction of a GPIIIa mutant. This cDNA construct codes for a GPIIIa subunit truncated from Ile 693 to Thr 762 and thus deleted of the transmembrane and intracellular domains. The resulting expression plasmid was designated pECEIIIaΔm.

mutant GPIIb expression plasmid contains the cDNA region corresponding to the GPIIb heavy chain (1–2667) and was designated pECEIIbΔl. The structure of the resulting plasmid was confirmed by extensive restriction mapping and double-strand DNA sequencing reaction of the PCR fragment and all ligation sites.

Construction of a GPIIIa Mutant Lacking the Transmembrane and the Intracellular Regions (Figure 1B). The same PCR technique was employed to design this GPIIIa mutant lacking the nucleotide sequence 2155–2364 coding for the sequence between Ile 693 and Thr 762. To prime the first strand, an oligonucleotide 5′-ACTGCAACTGTACCACGCGTACTG-3′ corresponding to the wild-type GPIIIa cDNA and containing the unique restriction site *Mlu*I at position 1763 was used. To introduce a stop codon at position 2155, the oligonucleotide 5′-TTAGTCAGGGCCCTTGGGACACTCTGG-3′was used to prime the other strand DNA during the amplification reaction by PCR. After the fill-in reaction, this PCR product was digested by *Mlu*I. The fragment (1763–2154 plus a stop codon) was then introduced into a pECEIIIa plasmid deleted of nucleotides 1763–2585. Nucleotides 1763–2364 correspond to the Ile 693–Ile 721 transmembrane domain and the His 722–Thr 762 cytoplasmic domain; region 2365–2585 is a 3′ nontranslated tail. The resulting expression plasmid was designated pECEIIIaΔm and

contains the coding region for the extracellular domain (1–2154). The structure of the resulting plasmid was confirmed by extensive restriction mapping and double-strand DNA sequencing reaction.

Transient Expression of Integrin GPIIb/IIIa in COS Cells. The pECE expression vectors containing GPIIb or GPIIIa wild-type cDNA clones (pECEIIIa and pECEIIb) or the mutant complementary DNA were transfected into COS-7 cells. For control experiments, a wild-type pECE vector was used. Confluent cells were passaged the day before transfection, and subconfluent cells were transfected with CsCl-purified plasmid DNA using the DEAE-dextran method (Lopata et al., 1984). Generally, 0.5 μg of each plasmid was used for a 35-mm culture dish. Efficiency of the transfection was determined for each condition by immunologic labeling of permeabilized cells with anti-GPIIb (D12A), anti-GPIIIa, (B2A), and anti-GPIIb/IIIa (CS9) monoclonal antibodies using an immunoalkaline phosphatase method. Under our conditions, 27 ± 2% of the cells were transfected with a single plasmid, and 12 ± 3% were transfected with both plasmids and expressed a GPIIb/IIIa complex.

Immunoprecipitation of Recombinants GPIIb/IIIa. The different GPIIb/IIIa complexes expressed 48 h after COS cell transfection were immunoprecipitated with B2A or CS9 antibodies. Metabolic labeling was performed in methionine-free RPMI medium containing dialyzed fetal calf serum, in the presence of 250 μCi of [^{35}S]methionine/mL. Cells were labeled for 8 h at 37 °C and lysed in PBS containing calcium and magnesium, 1% Triton X100, and 2 mmol/L PMSF (lysis buffer) for 1 h at 0 °C. After clarification by centrifugation at 11000*g* for 10 min, immunoprecipitation was performed by incubating the lysate with the selected antibody. The following steps and the analysis of the recombinant proteins on 7.5% SDS–PAGE under reducing conditions and autoradiography were as previously described (Troesch et al., 1990). Control immunoprecipitation on the culture medium was performed 48 h after the transfection experiment by incubating the selected antibody with 1 mL of clarified culture medium. For pulse–chase experiments, COS cells were pulsed 48 h after transfection with [^{35}S]methionine for 1 h and chased in cold medium containing 2 mmol/L methionine for the time indicated. The intensity of the immunoprecipitated bands after autoradiography was quantified on an Ultroscan XL laser densitometer (LKB). When the anti-GPIIb/IIIa monoclonal antibody was used, the 1:1 stoichiometry was verified for each heterodimer taking into account the methionine content for each mutant subunit.

Surface Labeling of Transfected Cells. Transfected cells were detached with 0.25% trypsin from the culture dishes 48 h after transfection and surface labeled in the presence of carrier-free ^{125}I using the lactoperoxidase method (Phillips & Agin, 1977). After washing and lysis, immunoprecipitation was performed as described above.

Oligosaccharide Digestion. ^{35}S-labeled proteins were isolated by immunoprecipitation with the appropriate antibody, digested with endo H (200 units/mL) 16 h at 37 °C in the presence of 1 mmol/L PMSF, and then analyzed by SDS–PAGE on reducing 7.5% gels (Laemmli, 1970).

Immunofluorescence Labeling. After 2 days of growth in normal DMEM medium, cells were tested for normal or mutant GPIIb/IIIa production by indirect immunofluorescence labeling directly on the chamber slide for tissue culture (Labtek). The cells were washed twice with phosphate-buffered saline (PBS) and fixed by a 10-min incubation with 3% freshly prepared paraformaldehyde in PBS. Immune reactions were performed by incubation for 1 h at 4° C with the primary monoclonal antibody CS9 in PBS, followed by incubation 1 h at 4 °C with the anti-IgG fluorescein-conjugated second antibody (Dako). At each stage, the cells were washed twice with PBS.

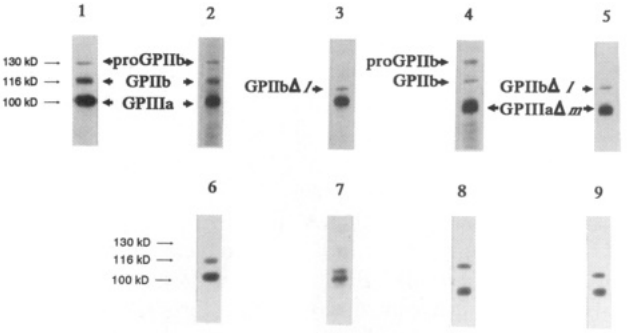

FIGURE 2: Immunoprecipitation of the mutant GPIIb/IIIa complexes produced in transfected COS cells. The pECE expression vectors containing GPIIb or GPIIIa full length cDNA clones (pECEIIIa and pECEIIb) or the mutant complementary DNA (pECEIIIaΔm and pECEIIbΔl, described in Figure 1) were transfected into COS-7 cells. To specify the nature of the different GPIIb/IIIa complexes expressed after COS cells transfection, recombinant proteins were immunoprecipitated with the anti-GPIIIa monoclonal antibody B2A (lanes 1–5) or with the anti-GPIIb/IIIa antibody CS9 (lanes 6–9). Lane 1 shows the immunoprecipitation pattern obtained under the same conditions with metabolically labeled human megakaryocytes; as already described (Troesch et al., 1990), an anti-GPIIIa monoclonal antibody immunoprecipitates GPIIIa and coimmunoprecipitates GPIIb and pro-GPIIb. (Lane 2) The same pattern is obtained from COS cells producing normal recombinant GPIIb/IIIa. (Lane 3) When pECEIIbΔl and pECEIIIa were cotransfected, COS cells produced a GPIIbΔl/IIIa complex as indicated by the coimmunoprecipitation of GPIIbΔl together with GPIIIa. (Lane 4) transfection of normal GPIIb cDNA (pECEIIb) plus the GPIIIa cDNA coding for a mutant subunit without transmembrane and cytoplasmic domains (pECEIIIaΔm) allows the formation of a pro-GPIIb/GPIIIaΔm complex and its processing into mature GPIIb/GPIIIaΔm. (Lane 5) The immunoprecipitation experiment when the two mutant cDNAs were cotransfected and the formation of a GPIIbΔl/IIIaΔm complex. (Lanes 6–9) Reproduction of the experiments of lanes 2–5 with the anti-GPIIb/IIIa monoclonal antibody CS9.

FACS Analysis. Cells were harvested with trypsin (0.25%) and incubated with antibodies as described above. Immunofluorescence staining was analyzed on a FACStar-plus flow cytometer (Becton Dickinson) using a FACScan research software program (Lysis II). Subtraction of histograms corresponding to control transfection experiment (wild-type pECE plasmid transfection) and to GPIIb/IIIa transfected cells was done to determine the number of cells which expressed the recombinant complex. Mean fluorescence intensity was expressed in FACS arbitrary fluorescence value.

Results

Expression of the Recombinant GPIIb/IIIa Complex. Expression of GPIIb/IIIa was achieved by cotransfecting full length cDNA encoding both subunits into COS cells (Gluzman et al., 1981). Immunoprecipitation of GPIIb/IIIa molecular variants with anti-GPIIIa and anti-GPIIb/IIIa antibodies from metabolically labeled cells indicated that both subunits are properly produced and processed (Figure 2, lanes 2 and 6). As shown in Figure 2, the electrophoretic mobility of the pro-GPIIb was consistent with a molecular mass of 130 kDa. This synthesized pro-GPIIb associated with a 100-kDa recombinant GPIIIa and was correctly processed to form the mature GPIIb/IIIa heterodimer. Immunoprecipitation of surface ^{125}I-labeled cells (Figure 3, lane 1) and immunofluorescence labeling of nonpermeabilized cells (Figure 4A) indicated that the recombinant GPIIb/IIIa was expressed at

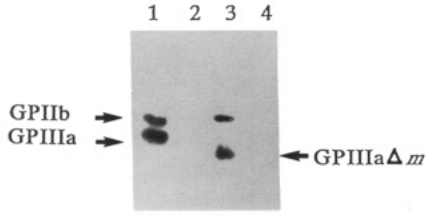

FIGURE 3: Immunoprecipitation of surface-expressed ^{125}I-labeled recombinants GPIIb/IIIa. After surface labeling with ^{125}I, cells were lysed and immunoprecipitation was performed with the CS9 antibody, specific for the GPIIb/IIIa complex. (Lane 1) COS cells were transfected with wild-type GPIIb and GPIIIa cDNAs. (Lane 2) pECEIIbΔl and pECEIIIa were cotransfected. (Lane 3) pECEIIb and pECEIIIaΔm were coexpressed. (Lane 4) Cotransfection of pECEIIbΔl and pECEIIIaΔm.

the surface of the cell. These results were consistent with previously published observations using metabolically labeled human megakaryocytes (Duperray et al., 1989) and indicated that biosynthesis of GPII/IIIa in COS cells and in megakaryocytes is similar. The electrophoretic mobility of GPIIb and GPIIIa produced in both cells were identical, suggesting that processing of the two subunits, including glycosylation, was correct in COS cells (Figure 2, lanes 1, 2, and 6). This processing was further confirmed by analysis of the endo H sensitivity of these proteins. Endo H specifically removes unprocessed high-mannose oligosaccharide chains but does not remove complex-type carbohydrates (Tarentino & Maley, 1974). Consistent with our previous observations (Troesch et al., 1990), GPIIIa and the pro-form of GPIIb were sensitive to endo H, since their molecular masses were decreased, respectively, by 10 and 5 kDa (Figure 5, lanes 1 and 2). As observed in megakaryocyte, recombinant GPIIb heavy chain was fully processed since treatment with endo H did not affect its molecular mass (Figure 5, lanes 1 and 2). These results are consistent with those reported by O'Toole et al. (1989) and indicate that the expression of GPIIb/IIIa on transfected COS cells is normal.

Quantitation of transfected COS cells expressing the recombinant GPIIb/IIIa at the surface level was determined using a FACScan research software program (Lysis II, Beckton Dickinson). Subtraction of histograms corresponding to control transfection experiment (wild-type pECE plasmid transfection) and to GPIIb/IIIa transfected cells was done to determine the number of cells which expressed the recombinant complex. The results indicated that, 48 h after transfection, 12 ± 3% of intact cells (mean of five experiments) expressed the GPIIb/IIIa complex on their surface. A typical histogram is reproduced on Figure 4A. The mean fluorescence intensity of this subpopulation is increased by 7.0 times when compared to control cells (293.0 compared to 41.4 in arbitrary fluorescence value).

Expression of a Mutant Lacking the GPIIb Light Region. A GPIIb mutant (GPIIbΔl) was made by deletion of the sequence from Arg 859 to Glu 1008 (Figure 1A), corresponding to the entire light chain of GPIIb. Consequently, GPIIbΔl was deleted of the cytoplasmic, the transmembrane, and a short extracellular domain. The production of mutant complexes containing GPIIbΔl was analyzed by immunoprecipitation of transfected COS cells lysate and showed two distinct bands (Figure 2, lanes 3 and 7). Association of GPIIbΔl and GPIIIa was demonstrated by the coimmunoprecipitation of the two subunits using the anti-GPIIIa monoclonal antibody B2A and by the anticomplex antibody CS9 (Figure 2, lanes 3 and 7). An apparent molecular mass of 110 kDa was found for the GPIIbΔl. This molecular mass is lower than that obtained with the normal GPIIb (Figure 2, lanes 2 and 6) and corresponds to the molecular mass of a GPIIb heavy chain with unprocessed oligosaccharide side chains (Troesch et al., 1990). This result was confirmed by analysis of the endo H sensitivity of this mutant. After endo H treatment, the molecular mass of the GPIIbΔl subunit decreased by 5 kDa (Figure 5, lanes 3 and 4). This result indicated that the oligosaccharide chains of GPIIbΔl were not fully processed. Immunoprecipitation of surface-labeled cells (Figure 3, lane 2), immunofluorescence labeling, and FACS analysis (Figure 4B) demonstrated that this heterodimer was not detected at the cell surface. Thus, the region corresponding to the GPIIb light chain is necessary for the maturation of GPIIb and for surface exposure of the complex.

Expression of a Mutant Lacking the GPIIIa Transmembrane and Cytoplasmic Domains. GPIIIaΔm was a mutant that did not contain the GPIIIa sequence from the amino acid residue Ile 693 to the carboxy-terminal amino acid Thr 762 (Figure 1B). This deleted sequence corresponds to the cytoplasmic and the transmembrane domains of the GPIIIa subunit. Immunoprecipitation with the anti-GPIIIa showed that GPIIIaΔm was expressed and able to associate with pro-GPIIb and GPIIb (Figure 2, lane 4). The presence of the mature GPIIb heavy chain indicated that the processing of the pro-GPIIb/IIIaΔm complex to the GPIIb/IIIaΔm form was achieved. These two events, formation of an heterodimer and its maturation, were confirmed by using the anti-GPIIb/IIIa antibody CS9 (Figure 2, lane 8).

As shown in Figure 2, lanes 4 and 8, the GPIIIaΔm variant was produced with a molecular mass of 90 kDa, compatible with the removal of 70 amino acid residues from the structure of GPIIIa. This variant associated with wild-type GPIIb to form the pro-GPIIb/IIIaΔm complex. In this complex the GPIIIaΔm was sensitive to endo H treatment whereas the associated GPIIb subunit was not. This indicated that the mutant heterodimer, GPIIb/IIIaΔm, was normally processed. Furthermore, this GPIIb/IIIaΔm heterodimer was expressed at the surface of the cell as demonstrated by surface immunoprecipitation experiment (Figure 3, lane 3), immunofluorescence labeling, and FACS analysis (Figure 4C). Quantitation of the subpopulation expressing GPIIb/IIIaΔm indicated that 12 ± 4% of the cells (mean of five experiments) expressed the mutant complex at the surface level with a mean fluorescence intensity increased by 3.5 times compared to the control (143.4 compared to 41.4 in arbitrary fluorescence value).

GPIIbΔl and GPIIIaΔm Are Able To Form an Intracellular Heterodimer. When the two mutants were cotransfected, the GPIIbΔl and the GPIIIaΔm variants were still able to associate together, as demonstrated by the immunoprecipitation of a complex with the anti-GPIIIa antibody (Figure 2, lane 5) and with the anti-GPIIb/IIIa (Figure 2, lane 9). The oligosaccharide chains of the GPIIbΔl were still not fully processed (Figure 5, lanes 7 and 8) as indicated by their endo H sensitivity, suggesting that this heterodimer did not undergo a normal cellular traffic and probably remained inside the cell, without maturation in the Golgi system. This was confirmed by surface immunoprecipitation experiment (Figure 3, lane 4), which showed that this complex was not found at the cell surface. Control experiments also indicated that this heterodimer was not detected by immunoprecipitation in the culture medium under our conditions (data not shown).

The Intensity of the Immunoprecipitated Bands May Reflect a Differential Association Efficiency and/or a Lower Stability of the Mutant Complexes. The low intensity of

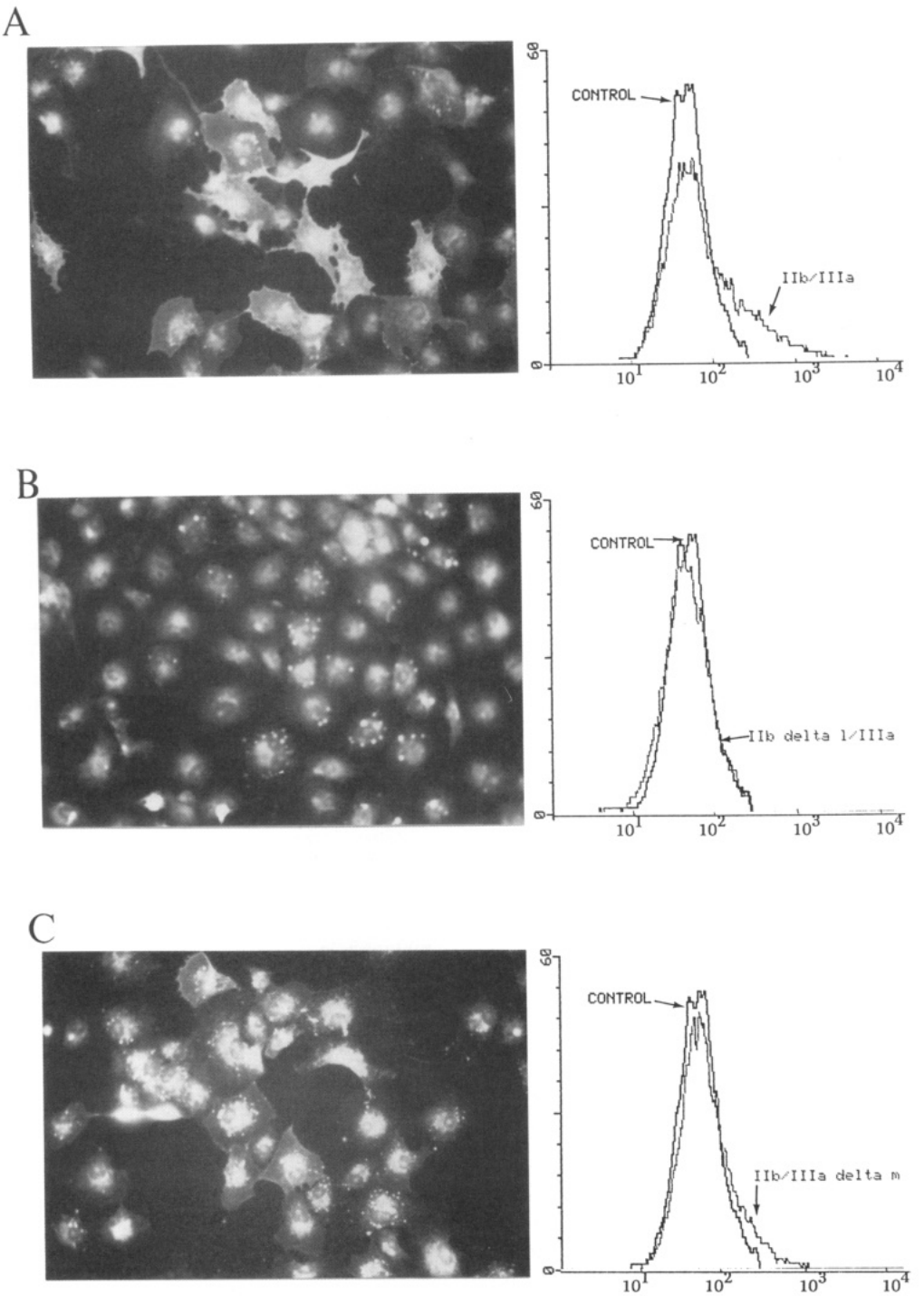

FIGURE 4: Detection of mutant GPIIb/IIIa complexes by immunofluorescence labeling and FACS analysis. The anti-GPIIb/IIIa monoclonal antibody CS9 was used for indirect immunofluorescence labeling and FACS analysis under all conditions. On FACS histograms, the *x* axis is fluorescent intensity and the *y* axis is counts full scale. The control histogram corresponding to COS cells transfected with a wild-type pECE plasmid (without insert) is reported in panels A, B, and C. Each histogram correspond to 10 000 analyzed cells. (A) Full length cDNAs for GPIIb and GPIIIa were cotransfected, and GPIIb/IIIa was detected at the surface of the cell. (B) pECEIIbΔl was cotransfected with pECEIIIa; the cells were not stained. (C) pECEIIIaΔm corresponding to the mutant deleted of the transmembrane and the cytoplasmic domains was cotransfected with pECEIIb; the cells were surface labeled.

electrophoretic bands corresponding to the different GPIIb forms when compared to GPIIIa molecules was due to the fact that the anti-GPIIIa antibody immunoprecipitated the complexed form as well as the free GPIIIa expressed in COS cells expressing only GPIIIa. For this reason a careful quantitative analysis was performed with the anti-GPIIb/IIIa complex antibody CS9.

Immunoprecipitation experiments with anti-GPIIIa or anti-GPIIb/IIIa monoclonal antibodies show a lower quantity of heterodimers composed of mutant subunits (Figure 2, lanes 2–9 and Table I). A fine analysis of the GPIIb and GPIIIa immunoprecipitated bands indicated a 50–60% decrease of their intensity when GPIIIaΔm or GPIIbΔl was included in the mutant complexes (Figure 2, lanes 7–9 and Table I) compared to the wild-type GPIIb/IIIa (Figure 2, lane 6). These decreased intensities could not be explained only by the loss of methionine residues of deleted subunits compared to the wild-types (GPIIbΔl contains nine methionines, as the GPIIb heavy chain and GPIIIaΔm have 13 residues compared to 14 for GPIIIa) and rather suggest a lower stability or a

Table I: Summary of the Expression of GPIIb/IIIa Normal and Mutant Subunits in COS Cells

plasmid transfected	complexes expressed[a]	surface expression[a]	FACS intensity (arbitrary value)	quantification of immunoprecipitated bands (%)[a]
pECE	None		41.4	
pECEIIb + pECEIIIa	IIb/IIIa	yes	293.0	100
pECEIIbΔl + pECEIIIa	IIbΔI/IIIa	no		45 ± 5
pECEIIb + pECEIIIaΔm	IIb/IIIaΔm	yes	143.4	45 ± 5
pECEIIbΔl + pECEIIIaΔm	IIBΔI/IIIaΔm	no		45 ± 5

[a] Transfection experiment, FACS analysis, and immunoprecipitation are described under Experimental Procedures. Different complexes were detected by immunoprecipitation with anti-GPIIIa and anti-GPIIb/IIIa antibodies. Surface expression was analyzed by immunofluorescence labeling. Quantification of immunoprecipitation was determined by scanning GPIIb and GPIIIa bands of the autoradiograms; the maximum (100%) was arbitrarily fixed for wild-type GPIIb/IIIa expressed on COS cells.

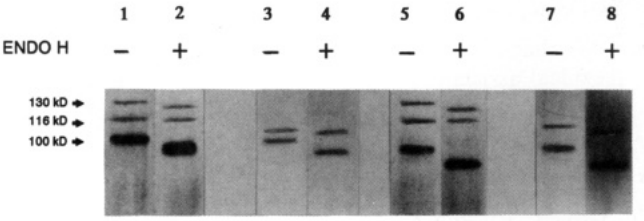

FIGURE 5: Endo H sensitivity analysis. COS-7 cells were transfected with the expression vectors; 48 h after transfection, immunoprecipitation was performed on the cell lysate with the B2A antibody, and the recombinant proteins were digested with endo H (+) or not (–) and then separated by SDS–PAGE on a 7.5% reducing gel. (Lanes 1 and 2) GPIIb/IIIa. (Lanes 3 and 4) GPIIbΔl/IIIa. (Lanes 5 and 6) GPIIb/IIIaΔm. (Lanes 7 and 8) GPIIbΔl/GPIIIaΔm.

lower association efficiency of the different mutants. Consequently, the stability of free GPIIbΔl and GPIIIaΔm subunits was examined and compared to normal GPIIb and GPIIIa. COS cells were transfected with one of either plasmid, metabolically labeled with [^{35}S]methionine for 1 h, and chased for up to 24 h. In these experiments, the pro-GPIIb or the GPIIbΔl forms were immunoprecipitated with the anti-GPIIb D12A antibody which immunoprecipitates the nonassociated form of GPIIb. The different GPIIIa molecules were immunoprecipitated with the anti-GPIIIa antibody B2A. For the quantitation of proteins, the intensity of labeled bands was determined with a densitometer. Figure 6 shows that wild-type pro-GPIIb and GPIIbΔl were produced in COS cells with a comparable half-life value (7 h). In contrast, GPIIIaΔm exhibited a decreased half-life of 5 h compared to 14 h for GPIIIa. These differences in stability may explain the lower quantity of heterodimer formed in the case of mutant heterodimers formed with the GPIIIaΔm chain.

Discussion

The present study was designed to verify the importance of the cytoplasmic and the transmembrane domains of the GPIIb and the GPIIIa subunits in the formation of the heterodimer and its exposure at the surface of the cell. Full length cDNAs containing the complete coding sequence of both subunits were inserted into an expression vector and cotransfected in COS cells. As a result of this cotransfection, different populations of COS cells were present in the culture, including nontransfected cells, cells which had been transfected with either one of the subunits, and cotransfected cells expressing both subunits. Using a monoclonal antibody specifically directed against the GPIIb/IIIa complex, the formation of the different mutants and the relative quantity of complex formed in each condition could be evaluated. Cytofluorimetric analysis indicated that 12 ± 3% of the cells in the culture were effectively cotransfected. Consistent with observations from an independent laboratory (O'Toole et al., 1989), we found that, in those cells, GPIIb and GPIIIa were correctly synthesized and processed, with the expected molecular mass.

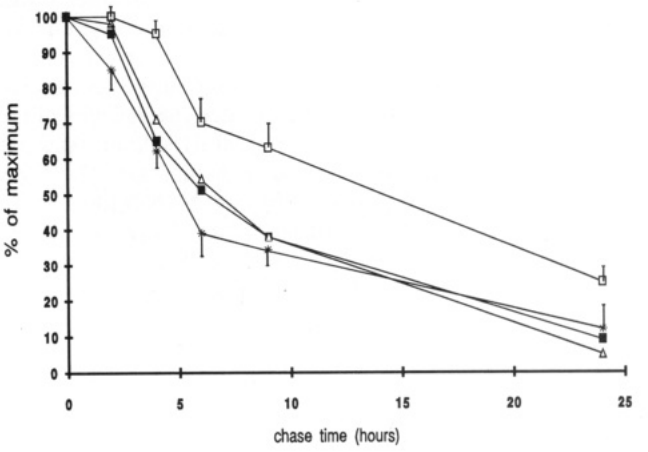

FIGURE 6: Stability of mutant subunits after COS cells transfection. COS-7 cells were transfected with one of the recombinant plasmids pECEIIb, pECEIIIa, pECEIIbΔl, or pECEIIIaΔm. After the pulse–chase experiment, the proteins were immunoprecipitated using anti-GPIIIa (B2A) or anti-GPIIb (D12A) antibodies, and the immunoprecipitated bands were quantified by densitometry. Points represent the mean of four assays, and the standard deviation is represented for GPIIIa and GPIIIaΔm. Symbols: (■) pro-GPIIb, (△) GPIIbΔl, (□) GPIIIa, and (*) GPIIIaΔm.

The first series of information obtained in the present study concerns the role of the GPIIb light chain. This light polypeptidic chain composes the cytoplasmic domain, the transmembrane domain, and 104 amino acid residues of the extracellular domain. A complete deletion of this light chain to produce the GPIIbΔl variant did not prevent the formation of a GPIIbΔl/IIIa complex. This observation is consistent with those of Lam et al. (1987), who demonstrated that purified GPIIb which was deleted of the light chain retains its capacity to associate with GPIIIa and to interact with RGD peptide affinity column. The results obtained in the present study indicate, however, that such a complex does not reach the surface of the cell. Cytofluorimetric analysis as well as measurement of the endo H sensitivity of the complex demonstrated the absence of this mutant at the cell surface and that the carbohydrate chains of GPIIbΔl were not processed. From these observations, we conclude that the light chain of GPIIb is not essential for the formation of the heterodimer but is necessary for the cellular transit and the surface exposure of the subunit associated with GPIIIa. Although the cotransfected cells produced 50% less of GPIIbΔl/IIIa mutant when compared to the wild-type IIb/IIIa (Table I), deletion of the light chain did not significantly affect the half-life of the mutant (Figure 6). Thus the lower amount of mutant complex produced and immunoprecipitated may reflect a decrease in the affinity of the CS9 antibody for the heterodimer or in the assembly efficiency.

The second series of information obtained from the present study concerns the role of the transmembrane and cytoplasmic

domains of the β3 subunit. The GPIIIaΔm mutant was deleted of both domains and was still able to form a complex with pro-GPIIb. This pro-GPIIb/IIIaΔm complex was normally processed into a GPIIb/IIIaΔm mutant and was exposed at the surface of the cell. Quantitatively, the number of cells expressing this mutant was identical to the number of cells expressing the GPIIb/IIIa complex (12%), indicating a similar transfection efficiency with the mutant, but the mutant heterodimer was expressed with a lower efficiency. Treatment with endo H further indicated that the pro-GPIIb associated with this GPIIIa variant was normally processed in the Golgi system.

From these results, we conclude that the transmembrane and the cytoplasmic domains of GPIIIa are not essential for the association with the pro-GPIIb form, a correct cellular transit, and exposure of the complex at the surface of the cell. Recently, Buck et al. (1990) demonstrated that deletion of the transmembrane domain of the integrin β1 subunit does not affect its association with various α subunits of the VLA subfamily. Hayashi et al. (1990) and Marcantonio et al. (1990) have also shown that deletion or mutation of the cytoplasmic domain of the β1 subunit does not inhibit heterodimer formation and surface exposure. The present study extends these observations to the β3 subunit and suggests the existence of a similar implication of β subunits in the formation of integrins.

This conclusion seems to be in apparent contradiction with a recent report from Newman et al. (1991). These authors have analyzed a large population of Iraqi Jews with Glanzmann's thrombasthenia using the PCR technology. They were able to show that the molecular defect in this population is due to the deletion of 11 bp at position 2049–2059 in the GPIIIa coding sequence, inducing a stop codon at position 2067, which removes the cytoplasmic and the transmembrane domains plus 34 amino acid residues amino-terminal to the site of truncation used in the present study. In the platelets of these patients, the variant form of GPIIb/IIIa is not surface exposed, and GPIIb is only expressed as its precursor form. This suggests that the missing sequence of 34 amino acids is probably involved in the association beteween the two subunits. Alternatively, the lack of transmembrane and cytoplasmic domains within the structure of GPIIIa may considerably affect the assembly efficiency or the metabolism of this subunit. Observation that the half-life of the mutant form GPIIIaΔm was twice lower than the half-life of GPIIIa is in support of the latter hypothesis. Despite similar transfection efficiencies, COS cells which were cotransfected with GPIIb and GPIIIaΔm cDNAs were able to produce only 40–50% of the mutant compared to the wild-type IIb/IIIa complex (Table I).

Altogether these results suggest that the extracellular domains of the α and the β subunits of an integrin contain sufficient structural information for the formation of the heterodimer. However, the transmembrane and cytoplasmic domains may play a role in the formation of an integrin, as suggested by the lower quantity of heterodimer formed with deleted mutants. This is consistent with earlier observations indicating that assembly is an early event (Duperray et al., 1989) and suggests that complex formation is a cotranslational reaction implicating the N-terminal domains of the subunits. In support of this conclusion is the observation that GPIIb and GPIIIa which are both deleted of their cytoplasmic and transmembrane domains can still form a complex. This mutant complex, however, remains intracellularly located and is not transported into the Golgi system to be normally processed.

In summary, the results presented in the present study, taken together with observations from independent laboratories on the β1 and β2 subclasses of integrins (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990; Arnaout et al., 1990), indicate that the cytoplasmic tail of the β subunit which is involved in the interaction with the cytoskeleton and interacts with the protein fibulin (Argraves et al., 1989) is certainly required for the cytoskeletal organization but is not essential for the expression of the heterodimer at the surface of the cell. In contrast, the same domain of the α subunit appears to be critical for the cellular transport of an integrin and its expression at the cell surface.

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Solubilization and Molecular Characterization of Active Galanin Receptors from Rat Brain†

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ABSTRACT: Galanin receptors were solubilized from rat brain using the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS). Binding of ^{125}I-galanin to the soluble fraction was time- and temperature-dependent, saturable, and reversible. Scatchard analysis of binding data indicated that the soluble extract contained a single class of galanin binding sites with a K_d of 0.8 nM and a B_{max} of 26 fmol/mg of protein. Unlabeled galanin and its fragments galanin(2–29) and galanin(1–15) antagonized the binding of ^{125}I-galanin to CHAPS-solubilized extracts with relative potencies similar to those observed with membrane receptors. Galanin(3–29) was found inactive. Binding of ^{125}I-galanin to CHAPS extracts was inhibited by guanine nucleotides with the following rank order of potency: GMP-P(NH)P > GTP > GDP. Molecular analysis of the soluble galanin receptor by covalent cross-linking of ^{125}I-galanin to CHAPS extracts using disuccinimidyl tartarate and further identification on SDS–PAGE indicated that the soluble galanin binding site behaves as a protein of M_r 54 000. After incubation of CHAPS extracts with ^{125}I-galanin, gel filtration on Sephacryl S-300 followed by ultracentrifugation on sucrose density gradient revealed a binding component with the following hydrodynamic parameters: Stokes radius, 5 nm; $s_{20,w}$, 4.5 S; M_r, 98 000; frictional ratio, 1.6. GMP-P(NH)P treatment of CHAPS extracts gave rise to a molecular form with the following characteristics: Stokes radius, 4 nm; $s_{20,w}$, 3.3 S; M_r, 57 000; frictional ratio, 1.4. Assuming one molecule of ^{125}I-galanin (M_r 3000) is bound per molecule of receptor, these data suggest that brain galanin receptor consists of a M_r 54 000 protein associated with the α subunit of a G protein. The availability of this CHAPS-soluble receptor from rat brain represents a major step toward the purification of this newly characterized receptor.

Galanin, a 29 amino acid peptide isolated from the porcine intestine (Tatemoto et al., 1983), is widely distributed in the central and peripheral nervous system of numerous species (Ch'ng et al., 1985). It exerts various biological effects, including inhibition of transmitter release from neurons (Rökaeus, 1987; Fisone et al., 1987), contraction of smooth muscle (Rökaeus, 1987; Ekblad et al., 1985), control of hypothalamic–anterior pituitary functions (Ottlecz et al., 1988), and regulation of gastric and pancreatic endocrine secretions (Kwok et al., 1988; McDonald et al., 1985; Silvestre et al., 1987). According to the potent inhibitory effect of galanin on insulin secretion (McDonald et al., 1985), the first galanin receptors to be discovered originated from a hamster-transplantable pancreatic β-cell tumor (Amiranoff et al., 1987). Using a rat insulin-secreting pancreatic β-cell line in culture, Rin m5F, we demonstrated thereafter that the galanin receptor behaves as a glycoprotein coupled to a pertussis toxin sensitive G_i[1] protein mediating inhibition of adenylyl cyclase in the plasma membrane (Amiranoff et al., 1988, 1989a; Lagny-Pourmir et al., 1989a). Meanwhile, galanin receptors were discovered in rat brain membranes by our group (Servin et

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DST, disuccinimidyl tartarate; CHS, cholesteryl hemisuccinate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; GMP-P(NH)P, guanyl-5′-yl imidodiphosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; G_i, inhibitory regulatory GTP binding protein of adenylyl cyclase.

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