Adhesive Properties of the β_3 Integrins: Comparison of GP IIb-IIIa and the Vitronectin Receptor Individually Expressed in Human Melanoma Cells

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Abstract. Glycoprotein IIb-IIIa ($\alpha^{IIb}\beta_3$) and the vitronectin receptor ($\alpha^v\beta_3$), two integrins that share the common β_3 subunit, have been reported to function as promiscuous receptors for the RGD-containing adhesive proteins fibrinogen, vitronectin, fibronectin, von Willebrand factor, and thrombospondin. The present study was designed to establish a cell system for the expression of either GP IIb-IIIa or the vitronectin receptor in an otherwise identical cellular environment and to compare the adhesive properties of these two integrins with those of native GP IIb-IIIa and the vitronectin receptor constitutively expressed in HEL cells or platelets. M21 human melanoma cells lack GP IIb-IIIa and use the vitronectin receptor to attach to vitronectin, fibrinogen, fibronectin, and von Willebrand factor. To study the functional properties of GP IIb-IIIa in these cells, we transfected GP IIb into M21-L cells, a variant of M21 cells (Cheresh, D. A., and R. C. Spiro. 1987. *J. Biol. Chem.* 262:17703–17711), which lack the expression of functional α^v and are therefore unable to attach to vitronectin, fibrinogen, and von Willebrand factor. Transfectants expressing GP IIb were isolated by immunomagnetic beads and surface expression of the GP IIb-IIIa complex was documented by FACS analysis and immunoprecipitation experiments performed with ^{125}I-labeled M21-L/GP IIb cells. Comparative functional studies demonstrated that GP IIb-IIIa expressed in M21-L/GPIIb cells as well as native GP IIb-IIIa constitutively expressed in HEL-5J20 cells (an HEL variant lacking $\alpha^v\beta_3$) mediated cell attachment to immobilized fibrinogen, but not to vitronectin or von Willebrand factor, whereas the vitronectin receptor expressed in M21 cells and HEL-AD1 cells (an HEL variant expressing $\alpha^v\beta_3$) mediated cell attachment to fibrinogen, vitronectin, and von Willebrand factor. Similarly, PGI_2-treated resting platelets attached to immobilized fibrinogen but not to vitronectin or von Willebrand factor, and this attachment could be inhibited by mAb A2A9 (directed against a functional site on the GP IIb-IIIa complex). However, in contrast to platelets, which adhered to vitronectin and von Willebrand factor after stimulation by thrombin or PMA, activation of the protein kinase C pathway in M21-L/GP IIb or HEL cells did not induce cell adhesion to vitronectin or von Willebrand factor. Our results therefore demonstrate (*a*) that while GP IIb-IIIa in its inactive, resting form is capable of mediating adhesion of platelets to immobilized fibrinogen, it does not to other RGD-containing adhesive proteins such as von Willebrand factor and vitronectin, and (*b*) that GP IIb-IIIa expressed in nucleated cells has similar adhesive properties as does GP IIb-IIIa in resting platelets but is not activated by platelet stimuli.

INTEGRINS are a widely distributed family of cell surface proteins that evolved from a common ancestor to perform a variety of cellular adhesion functions (Hynes, 1987). During hemostasis and thrombosis, integrins of the platelet membrane play an essential role in mediating adhesion of platelets to extracellular matrix proteins exposed at the site of injury of the vessel wall, as well as fibrinogen-dependent aggregation of platelets with each other (reviewed in Phillips et al., 1988). Two β_3 integrins of the platelet membrane, GPIIb-IIIa ($\alpha^{IIb}\beta_3$) and the vitronectin receptor ($\alpha^v\beta_3$), share the same β subunit and have α subunits that are 36% homologous (Fitzgerald et al., 1987). Both GP IIb-IIIa and the vitronectin receptor (VnR) have been shown to function as promiscuous receptors for the adhesive proteins fibrinogen, vitronectin, von Willebrand factor, fibronectin, and thrombospondin involved in platelet–subendothelium and platelet–platelet interactions. The multiple ligand-binding capability of these receptors is due to their ability to bind to the Arg-Gly-Asp (RGD) recognition sequence (Ruoslahti and Pierschbacher, 1987). Fibrinogen contains two RGD sequences in its Aα chain, at residues 95–97 and 572–574 (Doolittle et al., 1979). A second site on fibrinogen that

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binds to GP IIb-IIIa is a 12-amino acid sequence located at the carboxy-terminus of the γ chain of fibrinogen (Kloczewiak et al., 1984). This dodecapeptide sequence is not found in other adhesive proteins, but competes with RGD-containing peptides for binding to GP IIb-IIIa (Lam et al., 1987). Equilibrium binding studies have shown that GP IIb-IIIa contains a single RGD binding site (Steiner et al., 1989). On GP IIb-IIIa and the VnR, this RGD binding site appears similar as photoaffinity cross-linking studies with ^{125}I-RGD containing peptides have identified the same domain on the β subunit for both receptors (D'Souza et al., 1988; Smith and Cheresh, 1988). Despite the structural similarities and the apparent identity of the RGD-binding sites of GPIIb-IIIa and the VnR, differences in ligand-receptor interactions individualize the two receptors. In nucleated cells, the VnR function is constitutive, allowing it to function essentially as a cell adhesion receptor, whereas the receptor function of GPIIb-IIIa in platelets is manifest only after cell activation. VnR-mediated attachment of endothelial cells to immobilized fibrinogen uses exclusively the RGD binding site, whereas GPIIb-IIIa mediated attachment of activated platelets to fibrinogen occurs not only via the RGD sites but also the dodecapeptide (Cheresh et al., 1989) which has been found to cross-link primarily to GPIIb (D'Souza et al., 1990).

Receptor–ligand interaction studies for GP IIb-IIIa and the VnR have been performed essentially by comparing the adhesive properties of different cell types expressing a given receptor or by using purified receptor assays. However, a major drawback of whole cell assays is the presence of multiple integrin receptors on a given cell (e.g., platelets express at least six integrins: GP IIb-IIIa, the VnR (Lam et al., 1989), $\alpha^2\beta_1$ (Kunicki et al., 1988), $\alpha^5\beta_1$ (Piotrowicz et al., 1988), $\alpha^6\beta_1$ (Hemler et al., 1988). Purified receptor assays also have limitations as they do not take into account the microenvironment of the receptor in the plasma membrane, such as receptor–phospholipid interactions, receptor–cytoskeleton interactions, or conformational changes of the molecule that might be important for receptor activation and function, and indeed, functional differences depending on the microenvironment have been noted for the VnR $\alpha^v\beta_3$ (Conforti et al., 1990) and for $\alpha^2\beta_1$ (Kirchhofer et al., 1989; Elices and Hemler, 1989).

To gain some further insight in the functional properties of GP IIb-IIIa and the VnR, a clear advantage for receptor–ligand binding studies would be the existence of stable cell models that express either GP IIb-IIIa or the VnR in an otherwise identical cellular environment. With the availability of complete cDNA sequences encoding integrin receptors, the design of such experimental cell models has now become possible and transient expression of recombinant platelet GP IIb-IIIa has been achieved in COS cells (O'Toole et al., 1989) and human embryonic kidney cells (Bodary et al., 1989). However, these cell lines constitutively express a VnR that competes with recombinant GP IIb-IIIa in adhesion assays designed to study functional properties of GP IIb-IIIa. Also, extensive structural studies of the recombinant receptor are limited by the small number of cells expressing the transfected receptor as well as their short life span. Here we describe the establishment of stable cell systems expressing either GP IIb-IIIa or the VnR in the same cellular environment. By studying the functional properties of recombinant GP IIb-IIIa expressed in M21-L cells and native GP IIb-IIIa constitutively expressed in HEL cells and platelets, we demonstrate a new functional role of the inactive, resting form of GP IIb-IIIa in mediating cell attachment to immobilized fibrinogen, but not to other RGD-containing adhesive proteins such as vitronectin or von Willebrand factor.

Materials and Methods

Cells and Cell Culture

Platelets were isolated from healthy adult donors by differential centrifugation of whole blood anticoagulated with 1/6 vol of acid/citrate/dextrose (71 mM citric acid, 85 mM sodium citrate, 110 mM glucose) and 1 μM PGI_2 as previously described (Cheresh et al., 1989). The human melanoma cells M21 (called here M21-W for wild type) and M21-L are those described by Cheresh and Spiro (1987). Two subclones of the initial HEL cell line (Martin and Papayannopoulou, 1982) were used: the clone HEL-5J20 has a high surface expression of GP IIb-IIIa and only background expression of the VnR, whereas the clone HEL-AD1 expresses equal amounts of GP IIb-IIIa and the VnR. The cells were grown in RPMI medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Gibco Laboratories), 100 U/ml of penicillin, and 100 μg/ml of streptomycin in 5% CO_2 in a fully humidified incubator at 37°C. COS-7 cells (SV40 transformed African green monkey kidney) (Gluzman, 1981) were grown in DME medium (Gibco Laboratories) supplemented with 10% FCS and antibiotics as described above.

mAbs

The following mAbs were used in this study. S1 was produced in our laboratory and reacts with GP IIbα in Western blot; SZ21 (Amac Corp., Westbrook, ME) is directed against GP IIIa; A2A9 is a complex-specific GP IIb-IIIa antibody (Bennett et al., 1983), LM609 a complex-specific VnR antibody (Cheresh and Spiro, 1987), and BIIG2 a complex-dependent fibronectin receptor antibody (Werb et al., 1989). All mouse mAbs were isolated from ascites fluid and purified on a protein-A Sepharose immunoabsorbent (Pharmacia Fine Chemicals, Piscataway, NJ). The rat BIIG2 mAbs were purified from ascites fluid by ammonium sulfate precipitation followed by chromatography on a DEAE column.

Plasmid Construct

For plasmid construction, we used the expression vector pBJ1, which was kindly provided by Dr. Mark Davis (Stanford University School of Medicine). The pBJ1 vector is a modified form of the pcD-SRα expression cloning vector described by Takebe et al. (1988) that contains the simian virus 40 (SV40) early promoter and the R segment and part of the RU sequence (R-U5') of the long terminal repeat of the human T cell leukemia virus type 1 (HTLV-1). A human cDNA for GP IIb was isolated from a commercially prepared HEL cell cDNA library (Stratagene Corp., La Jolla, CA) using an oligonucleotide probe, which represented the first 42 nucleotides of the published sequence (Poncz et al., 1987). An EcoRI–HindIII fragment was subcloned into M13 mp18 (Boehringer Mannheim Biochemicals, Indianapolis, IN), and the 5'-end of the clone was completed using site-directed mutagenesis (Kunkel, 1985). This involved adding 2 nucleotides, such that the GP IIb cDNA contained nucleotides 1–3203 of the published sequence. The full-length GP IIb coding sequence was inserted into the EcoRI site of the pBJ1 vector. The resulting plasmid, designated pBJ1-GP IIb, was characterized by restriction mapping and transient expression in COS cells.

Transfection of M21-L Cells

M21-L cells were transfected using the lipofection method (Felgner et al., 1987). 48 h before transfection, 10^6 cells were plated on fibronectin-coated 100 mm dishes. At day 0, the cells were washed twice with serum-free RPMI medium and then incubated for 24 h with 20 μg of pBJ1-GP IIb plasmid DNA and 4 μg of pSVneo plasmid DNA, mixed in a 1:1 volume with lipofectin (Bethesda Research Laboratories, Gaithersburg, MD) according to the manufacturer's procedure. The next day, complete medium was added and the cells allowed to grow for 4 more days. The cells were then detached with EDTA buffer, pH 7.4 (1 mM EDTA, 150 mM NaCl, 50 mM Hepes)

and washed twice with RPMI medium. Transfected cells were selected for surface expression of GP IIb-IIIa using mAb S1 and immunomagnetic beads coated with polyclonal rabbit anti-mouse IgG (Dynal Inc., Great Neck, NY). Briefly, the cells (10^6 cells/100 μl) were incubated for 30 min at 4°C with 10 μg of S1-IgG, washed with RPMI and incubated for a further 30 min under gentle agitation at 4°C with 3×10^6 immunomagnetic beads in a final volume of 2 ml. Magnetic bead cell rosettes were separated from negative cells using a magnetic particle concentrator (Dynal Inc.). The selected cells were then plated on fibrinogen-coated dishes. After 24 h, nonadherent cells were washed off. Adherent cells were grown to a higher cell density in the presence of 1 mg/ml of G418-sulfate (Gibco Laboratories) and reselected twice for GPIIb-IIIa expression by fluorescence-activated cell sorting.

COS Cell Transfection

COS-7 cells were transfected using the lipofection method. Briefly, 5×10^5 cells were plated onto 90-mm culture dishes. When the cells had reached 70% confluency, transfection was performed with 10 μg of pBJ1-GP IIb plasmid DNA as described above. The cells were then metabolically labeled for 24–48 h after transfection and processed for immunoprecipitation experiments as described in the following section.

Immunofluorescence Analysis of Receptor Expression

GP IIb-IIIa and VnR expression was analyzed on M21-L-transfected cells in suspension using indirect immunofluorescence and a fluorescence-activated cell sorter (FACS 440; Becton-Dickinson Co., Mountain View, CA). Adherent cells were detached by incubating the cells for 5 min at 37°C with EDTA buffer. The cells were then immediately washed and resuspended in RPMI medium. These experimental conditions have previously been shown to induce only minimal irreversible GP IIb-IIIa complex dissociation in human platelets (Fitzgerald and Phillips, 1985). The cells were further incubated at 4°C for 30 min at 10^6 cells/100 μl in RPMI medium containing 10 μg of monoclonal mouse IgG. The cells were then washed with RPMI and incubated with FITC-conjugated goat anti-mouse IgG (Cappel Laboratories, West Chester, PA) for 30 min at 4°C. Positive fluorescence was determined on a four-decade log scale and expressed as channel number of mean intensity of fluorescence (MIF).[1] Background fluorescence was determined for each cell population using FITC-conjugated goat anti-mouse IgG alone. The cytometric data were obtained with the assistance of the UCSF Laboratory for Cell Analysis.

Cell Radiolabeling and Immunoprecipitation

For ^{125}I surface labeling, the cells were detached with EDTA buffer, washed twice with PBS and resuspended at 10^7 cells/ml in PBS. Labeling was performed by the lactoperoxidase catalyzed iodination procedure as previously described (Kieffer et al., 1986). For steady state metabolic labeling, adherent cells were washed twice with RPMI medium and then cultured for 30 min at 37°C in methionine and cysteine free RPMI medium supplemented with heat inactivated, dialyzed FCS. After 30 min, 250 μCi [^{35}S]methionine and 250 μCi [^{35}S]cysteine (specific activity >800 Ci/mM; Amersham Corp., Arlington Heights, IL) were added to the medium and the cells cultured for 24 h at 37°C. The cells were then washed twice with RPMI, detached with EDTA buffer, and resuspended at 5×10^7 cells/ml in PBS. For immunoprecipitation, radiolabeled cells were lyzed in lysis buffer, pH 7.4 (10 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM PMSF). Triton X-100 insoluble material was removed by ultracentrifugation, and lysate samples, corresponding to 5×10^6 cells were incubated with 5 μg of mAb IgG. After 3 h at 4°C, 25 μl of protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) were added and the incubation continued for 30 min at 4°C. Protein A-Sepharose beads were washed six times with lysis buffer, and the final pellet resuspended in 50 μl of 10 mM Tris-HCl, pH 7.4, containing 2% (wt/vol) SDS and 5% 2-mercaptoethanol. Bound proteins were eluted by boiling the suspension for 5 min and analyzed by SDS-PAGE according to the method of Laemmli (1970). After electrophoresis, the gels were fixed and when required processed for fluorography with Amplify (Amersham Corp.). The gels were then dried and exposed at −80°C to FUJI RX films.

1. *Abbreviations used in this paper*: GP, glycoprotein; MIF, mean fluorescence intensity; VnR, vitronectin receptor.

Cell Adhesion Assay

M21-L/GP IIb cells and M21-L cells were metabolically labeled overnight with [^{35}S]methionine as described above and were then washed twice with RPMI medium. The cells were detached with EDTA buffer, washed and resuspended in RPMI medium at 10^6 cells/ml. Before the adhesion assay, the cells were incubated with 10 μg of the appropriate mAb or inhibitor for 30 min at 4°C. The cells (5×10^4) were then added to individual wells of microtiter plates previously coated with the adhesive protein (10 μg/ml in RPMI medium). Cell attachment was allowed to occur at 37°C for 3 h, after which microtiter wells were washed with RPMI medium to remove unattached cells. The remaining cells were lyzed with 2% SDS and radioactivity counted in a β scintillation counter (LS1701; Beckman Instruments, Palo Alto, CA). For platelet adhesion studies, platelets were isolated from blood collected in acid-citrate-dextrose anticoagulant containing 1 μM PGI_2 (Sigma Chemical Co., St. Louis, MO). After centrifugation of the blood at 160 *g* for 15 min, the platelet rich plasma was removed and subjected to gel filtration over a Sepharose 2B column. Platelets were then pelleted and resuspended at 2×10^8 cells per ml in 0.002 M Hepes, pH 7.35, containing 0.15 M NaCl, 0.005 M dextrose and 1 mg/ml BSA. Platelets were activated with either 0.5 NIH units/ml of thrombin or 50 μM PMA for 10 min at 22°C or treated with 1 μM PGI_2, and the adhesion assay performed as described by Cheresh et al. (1989). The adhesive proteins fibrinogen, vitronectin, and fibronectin were purified from human plasma according to published procedures (Kazal et al., 1963; Engvall et al., 1978; Yatohga et al., 1988), and were essentially devoid of contaminating proteins as determined by SDS-PAGE and Coomassie blue staining of the gel. BSA (fraction V) was purchased from Sigma Chemical Co.

Results

Transfection of GPIIb cDNA into M21-L Cells

As previously reported by Cheresh and Spiro (1987), M21-L cells are a variant cell clone of the human melanoma cell line M21 and lack the VnR α chain or its mRNA but do produce normal levels of the β_3 chain that accumulates within the cell. A full-length cDNA clone containing the entire coding sequence of GP IIb was constructed as described in the Materials and Methods section, subcloned into the expression vector pBJ1, and introduced into the M21-L adherent cells using lipofectin. To select for transfected M21-L cells expressing a fully complexed GP IIb-IIIa receptor, positive transfectants were immunoselected using GP IIb mAb S1 and immunomagnetic beads coated with rabbit anti-mouse IgG. A typical result is illustrated in Fig. 1, which shows a few rosette-forming cells and a nonnegligible number of negative cells, most likely entrapped in the pellet of free magnetic beads (Fig. 1 *A*). Selected transfectants were thereafter plated on fibrinogen-coated microtiter wells. The rosette forming cells were found to attach and spread, whereas the negative cells did not and thus could be partially washed off (Fig. 1 *B*). The selected cells were then grown to a higher cell density, reselected twice by cell sorting using mAb S1 and finally subcloned by limiting dilution.

Surface Expression of the GP IIb-IIIa Complex in M21-L Cells Transfected with GP IIb

To study the surface expression of the GP IIb-IIIa complex in transfected M21-L/GP IIb cells, indirect immunofluorescence was performed on cells in suspension using monoclonal antibodies specific for either the GP IIb subunit (S1) or the GP IIb-IIIa complex (A2A9) and analyzed on a fluorescence-activated cell sorter. The results shown in Table I are expressed as numeric data of MIF. Nonspecific fluorescence was determined for each cell type by incubating the cells with FITC-conjugated goat anti-mouse IgG alone (MIF

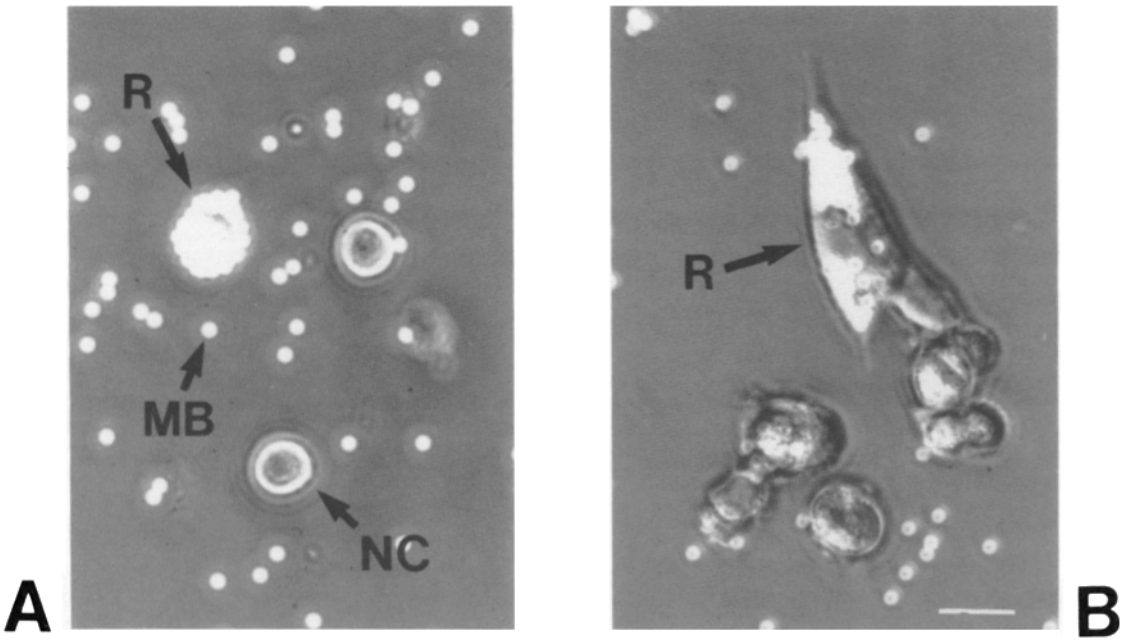


Figure 1. Phase-contrast micrographs of M21-L/GP IIb transfected cells after immunoselection using the GP IIb mAb S1 and goat anti-mouse-coated magnetic beads. (*A*) Phase view of selected cells. The arrow points to a rosette forming cell. (*B*) Selected cells after 24 h spreading on a fibrinogen-coated microtiter plate. The arrow points to an attached rosette. Bar, 22.5 μm. *MB*, magnetic bead; *NC*, negative cell; *R*, rosette-forming cell.

< 40). The transfected M21-L/GP IIb cells were positive with both mAb S1 (MIF 70) and mAb A2A9 (MIF 81), whereas they were negative with the VnR mAb LM609 (MIF 35). Nontransfected M21-L control cells were negative for all three mAbs (MIF 28), whereas M21-wild-type cells exhibited positive fluorescence with mAb LM609 (MIF 77) and were negative with mAb S1 (MIF 32).

Biosynthesis and Posttranslational Processing of GP IIb in M21-L/GP IIb Cells as Compared with COS/GP IIb Cells

As a prelude to functional analysis of the transfected protein, experiments were performed to determine whether posttranslational processing of GP IIb had occurred. A simple procedure to demonstrate posttranslational processing of a glycoprotein is based on the change of its molecular weight, visualized by a change in the electrophoretic mobility of the glycoprotein when submitted to SDS-PAGE analysis. To determine whether posttranslational processing of GP IIb-IIIa occurred in M21-L/GP IIb cells, we compared the apparent molecular weight of GP IIb or GP IIIa immunoprecipitated from transfected M21-L/GP IIb cells and COS/GP IIb cells as well as cells that constitutively express GP IIb-IIIa, such as platelets or HEL cells. In an initial experiment shown in Fig. 2, M21-L/GP IIb cells and platelets were ^{125}I-surface labeled, lysed and immunoprecipitated with the GP IIb-IIIa mAb A2A9. Under nonreducing conditions, the precipitated GP IIb-IIIa from M21-L/GP IIb cells had a molecular mass of 140,000 and 95,000 respectively and comigrated with platelet GP IIb and GP IIIa (lanes *A–C*). After disulfide cleavage, the electrophoretic mobility of both subunits underwent the characteristic shift identical to that observed for platelet GP IIb and GP IIIa, with the appearance of the GP IIbβ subunit (lanes *D–F*). A similar result was obtained when GP IIb-IIIa from M21-L/GP IIb was compared with HEL cell GP IIb-IIIa (data not shown). Thus, GPIIb in M21-L/GP IIb cells had undergone posttranslational cleavage. In contrast, when experiments were performed using metabolically labeled COS cells that had been transfected with the same pBJ1/GP IIb vector, only unprocessed pro-GP IIb was immunoprecipitated with mAb S1, whereas the same mAb precipitated pro-GP IIb as well as the processed GP IIb-IIIa complex from metabolically labeled HEL cells (Fig. 3). These results thus provide evidence that normal posttranslational proteolytic processing of GP IIb into its α and β

Table I. Immunofluorescence Flow Cytometric Analysis of M21 Cell Clones for Surface Expression of GP IIb-IIIa and the VnR

	M21 cell clone		
Antibody	M21-W	M21-L	M21-L/GP IIb
Negative control (second Ab alone)	34	23	30
Anti-GP IIb (α^{IIb}) (S1)	32	23	70
Anti-GP IIb-IIIa ($\alpha^{IIb}\beta_3$) (A2A9)	34	27	81
Anti-VnR ($\alpha^{v}\beta_3$) (LM 609)	77	28	35

Flow cytometry analysis of M21-W-, M21-L-, and M21-L/GP IIb-transfected cells for surface expression of the β_3 integrins GP IIb-IIIa and the VnR. Washed, unfixed cells (10^6) were stained by indirect immunofluorescence and analyzed by flow cytometry analysis as described in Materials and Methods. Each profile was generated from an analysis of 10,000 cells. Relative values of MIF were derived from gated computerized histogram analysis and expressed as channel number of the histogram axis. Nonspecific fluorescence measured with the second fluorescein isothiocyanate antibody alone was found <channel 40 and considered as negative.

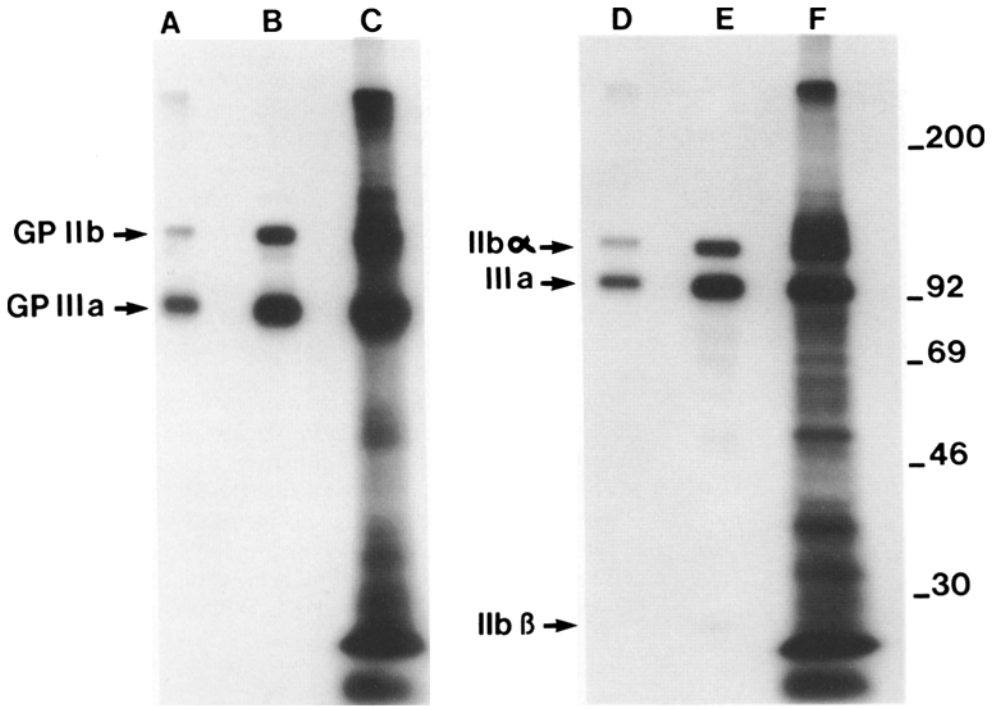


Figure 2. Immunoprecipitation of GP IIb-IIIa from ^{125}I-surface labeled M21-L/GP IIb cells and platelets. Detergent extracts of ^{125}I-surface labeled M21-L/GP IIb cells and platelets were incubated with GP IIb-IIIa mAb A2A9 and the immune complexes precipitated with protein A-Sepharose. Precipitated proteins were analyzed on a 7–12% SDS-PAGE gel under nonreducing conditions (lanes *A–C*) or reducing conditions (lanes *D–F*). Lanes *C* and *F*, total profile of ^{125}I-surface labeled platelets; lanes *B* and *E*, GP IIb-IIIa immunoprecipitated from platelets; lanes *A* and *D*, GP IIb-IIIa immunoprecipitated from transfected M21-L/GP IIb cells.

subunits occurred in M21-L/GP IIb cells, but did not in COS/GP IIb cells. Also, comigration of GP IIb-IIIa from M21-L/GP IIb cells with platelet GP IIb-IIIa suggests effective posttranslational glycosylation, since Duperray et al.

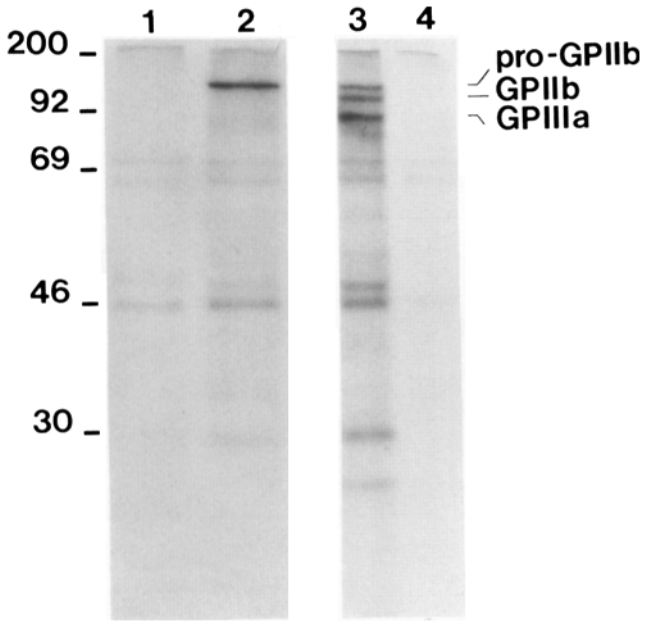


Figure 3. Immunoprecipitation of GP IIb from transfected COS/GP IIb cells or HEL-5J20 cells. Detergent extracts of [^{35}S]methionine and [^{35}S]cysteine labeled COS/GP IIb cells or HEL-5J20 cells were incubated with 10 μg of anti-GP IIb IgG (mAb S1). Immune complexes were precipitated with protein-A Sepharose and analyzed by SDS-PAGE under reducing conditions. Lanes 1 and 4: negative control; lanes *2* and *3*, immunoprecipitates obtained with mAb S1 from transfected COS/GPIIb cells (lane *2*) or HEL-5J20 cells (lane *3*).

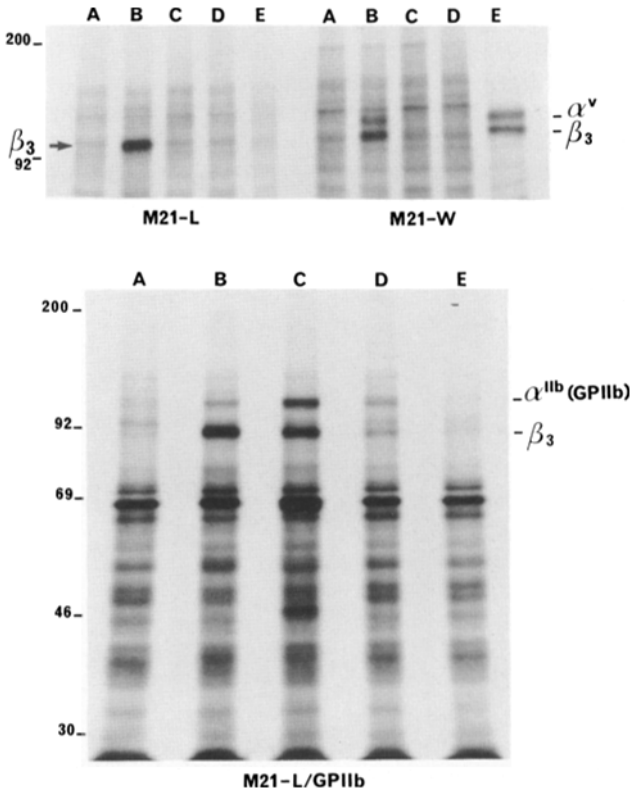


Figure 4. SDS-PAGE analysis of immunoprecipitates from M21-W, M21-L, and transfected M21-L/GP IIb cells. Detergent extracts of [^{35}S]methionine and [^{35}S]cysteine labeled cells were incubated with 10 μg of mAb-IgG. Immune complexes were precipitated with protein A-Sepharose and analyzed by SDS-PAGE under reducing conditions. Lane *A*, negative control; lane *B*, GP IIIa mAb SZ21; lane *C*, GP IIb-IIIa mAb A2A9; lane *D*, GP IIb mAb S1; lane *E*, VnR mAb LM609.

(1989) have shown that tunicamycin-treated megakaryocyte GP IIb-IIIa has an increased electrophoretic mobility as compared with normal GP IIb-IIIa.

To exclude the possibility that our selection procedure allowed the isolation of a revertant M21-L cell expressing the VnR, and to further document biosynthesis of GP IIb-IIIa, immunoprecipitation experiments were performed using steady-state metabolically labeled M21-L/GP IIb cells. The result is shown in Fig. 4. No VnR could be immunoprecipitated from M21-L/GP IIb cells, whereas mAb A2A9, S1, and SZ21 all precipitated two bands corresponding to GP IIb-IIIa. Immunoprecipitations performed on M21-W and M21-L cells confirmed their typical phenotype, i.e., the presence of the VnR in M21-W cells and the free β_3 subunit in M21-L cells.

Functional Properties of GP IIb-IIIa in M21-L/GP IIb Cells, HEL Cells, and Platelets

(a) GP IIb-IIIa Expressed in M21-L Cells Mediates Cell Attachment to Fibrinogen But Not to Vitronectin, von Willebrand Factor, or Fibronectin. To study the functional properties of GP IIb-IIIa and the VnR individually expressed in M21 melanoma cells, a cell adhesion assay was performed on microtiter plates coated with various adhesive proteins (Fig. 5). In accordance with previous results (Cheresh and Spiro, 1987), M21 cells expressing the VnR attached to fibrinogen, vitronectin, von Willebrand factor, and fibronectin, whereas mock transfected M21-L cells, which express $\alpha^5\beta_1$, only attached to fibronectin. When M21-L/GP IIb transfectants were tested, they attached to fibrinogen as well as fibronectin, however, they did not attach to vitronectin or von Willebrand factor, nor could adhesion to vitronectin or von Willebrand factor be induced by PMA (result not shown). To determine whether the M21-L/GP IIb cell interaction with fibrinogen or fibronectin was GP IIb-IIIa dependent, experiments were performed in the presence of mAb known to inhibit this interaction or a peptide containing the RGD sequence. The results are shown in Fig. 6. Pretreatment of M21-L/GP IIb cells with mAb A2A9 inhibited their attachment to fibrinogen. This inhibition was specific since neither the FnR MoAb BIIG2 nor the VnR MoAb LM609 had an inhibitory effect. Attachment was also inhibited by RGDS but not by RGES at a final concentration of 50 μM. When plated on fibronectin, MoAb A2A9 had no effect whereas the MoAb BIIG2 inhibited M21-L/GP IIb cell adhesion to a similar extent as RGD. Conversely, pretreatment of mock-transfected M21-L cells with BIIG2 inhibited their attachment to fibronectin, whereas mAb LM609 or A2A9 had no effect (results not shown).

(b) Properties of GP IIb-IIIa and the Vitronectin Receptor Constitutively Expressed in Nucleated Cells. To determine whether the functional properties of the GP IIb-IIIa in M21-L/GP IIb cells corresponded to those of native GP IIb-IIIa constitutively expressed in nucleated cells, we compared those of a subclone of the HEL cell line selected for high expression of GP IIb-IIIa and low expression of the VnR. As shown in Fig. 7, the subclone HEL-5J20 is characterized by

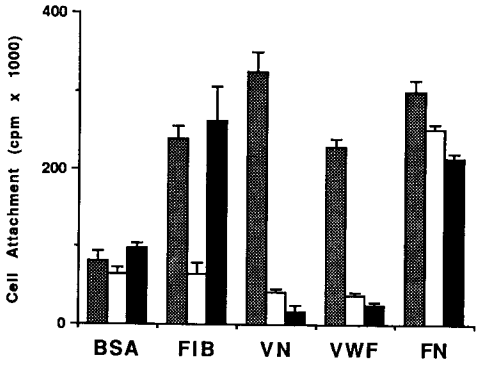
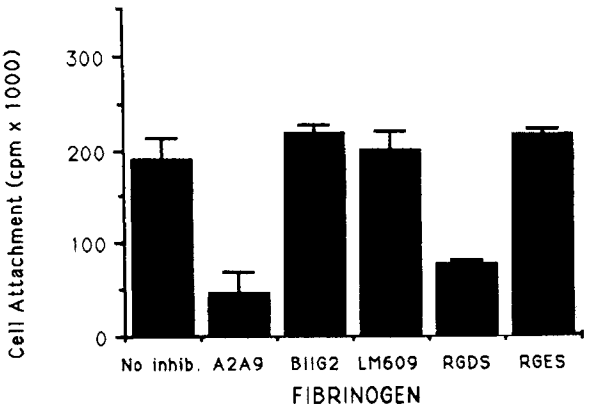


Figure 5. Adhesion of M21-W, M21-L, and M21-L/GP IIb cells to extracellular matrix proteins. ^{35}S-metabolically labeled M21-W cells (*shaded bar*), M21-L cells (*white bar*), or M21-L/GP IIb cells (*black bar*) were allowed to attach to adhesive protein-coated microtiter wells for 3 h at 37°C as described in Materials and Methods. After adhesion, unattached cells were washed away and the remaining cells lysed with 2% SDS. The radioactivity from each well was counted in a β scintillation counter. Cell attachment was expressed as the number of cells (counts per minute) attached per well. Each bar represents the mean ± SD of three replicates.



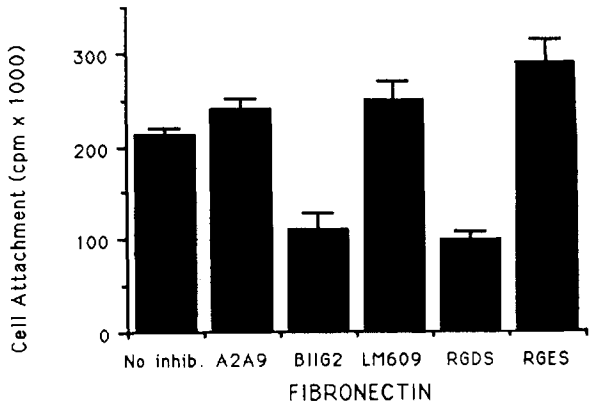


Figure 6. Inhibition of M21-L/GP IIb cell attachment to fibrinogen or fibronectin. Metabolically labeled M21-L/GP IIb cells (5×10^4) were allowed to attach to microtiter wells coated with 10 μg/ml of fibrinogen or fibronectin. Before addition to the protein-coated wells, the cells were allowed to react with mAb IgG (10 μg) or the synthetic peptides RGDS and RGES (final concentration 50 μM). After 3 h at 37°C, unattached cells were washed off and the remaining radioactivity counted. Cell attachment is expressed as the number of cells (counts per minute) attached per well. Each bar represents the mean ± SD of three replicates.

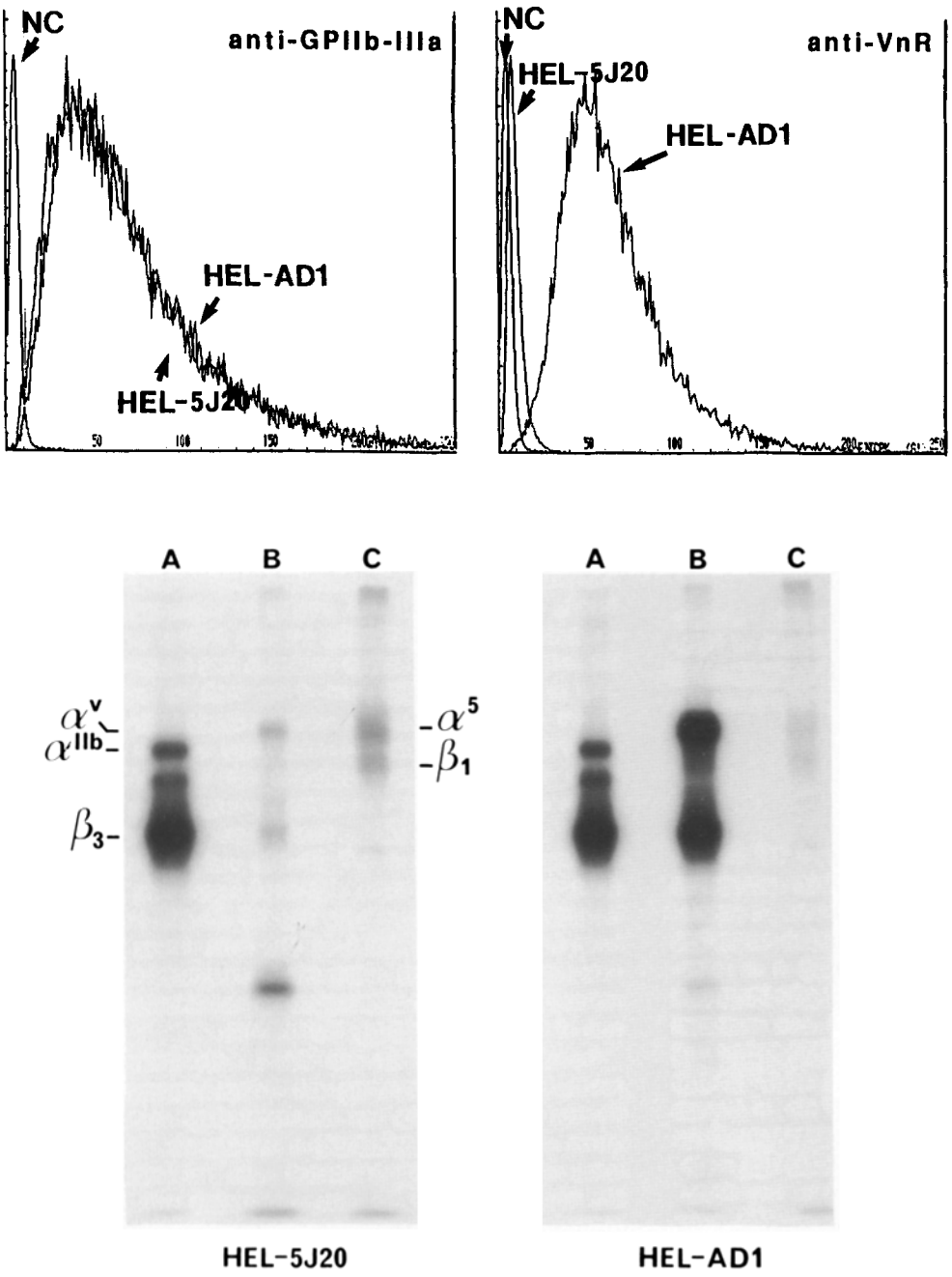


Figure 7. (*Top*) β_3-integrin phenotype of HEL-5J20 and HEL-AD1 cells. Washed unfixed cells were stained for indirect immunofluorescence and flow cytometry analysis using the GP IIb-IIIa mAb A2A9 or the vitronectin receptor mAb LM609. Nonspecific fluorescence (negative control, *NC*) was determined by incubating the cells with the second fluorescein isothiocyanate antibody alone. (*Abscissa*, channel number of fluorescence intensity; *ordinate*, frequency of cells). (*Bottom*) Quantitative immunoprecipitation of GP IIb-IIIa, the VnR and the fibronectin receptor from ^{125}I-surface labeled HEL-5J20 and HEL-AD1 cells. Detergent extracts (600 μg protein) of HEL-5J20 cells and HEL-AD1 cells were incubated with 10 μg of GP IIb-IIIa mAb A2A9 (*A*), VnR mAb LM609 (*B*), or the FnR mAb BIIG2, and the precipitated immune complexes analyzed by SDS-PAGE under reducing conditions.

a high expression of GP IIb-IIIa and only background expression of the VnR, whereas the subclone HEL-AD1 expresses similar amounts of both GP IIb-IIIa and the VnR. When the two HEL clones were studied for their adhesion properties, HEL-5J20 cells attached to fibrinogen but not to vitronectin or von Willebrand factor, whereas HEL-AD1 cells attached to all ligands (Fig. 8). Interestingly, similar to M21-L/GP IIb cells, adhesion to vitronectin or von Willebrand factor could not be induced through activation of the protein kinase C pathway by PMA (data not shown). Together, these results provide evidence that in contrast to the VnR, GP IIb-IIIa expressed in nucleated cells mediates exclusively attachment to fibrinogen.

(c) Resting Platelet GP IIb-IIIa Mediates Platelet Adhesion to Immobilized Fibrinogen. GP IIb-IIIa-mediated platelet adhesion to immobilized adhesive proteins is known to be activation dependent and adhesion assays are most commonly performed with thrombin-activated platelets (Haverstick et al., 1985; Santoro and Cowan, 1986; Cheresh et al., 1989). To determine whether GP IIb-IIIa in its resting inactive form was able to mediate platelet attachment to immobilized fibrinogen, we compared the adhesive properties of platelets isolated in the presence of PGI_2 with those of platelets activated with thrombin or PMA. As shown in Fig. 9, resting PGI_2-treated platelets attached to fibrinogen but not to vitronectin or von Willebrand factor, whereas thrombin-

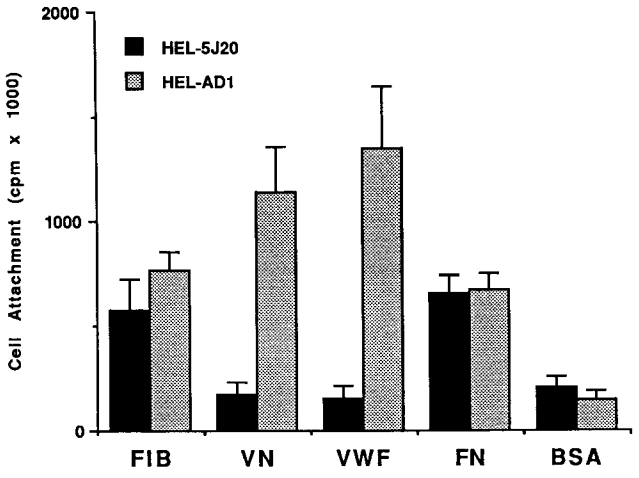


Figure 8. Adhesion of HEL-5J20 and HEL-AD1 cells to purified extracellular matrix proteins. The adhesion assay was performed as described in the legend to Fig. 5.

or PMA-activated platelets attached to fibrinogen, vitronectin, and von Willebrand factor. Also, attachment of PGI_2-treated as well as PMA-treated platelets to fibrinogen could be inhibited by mAb A2A9 as well as the RGDS peptide (results not shown).

Discussion

This study compares the adhesive activities of GP IIb-IIIa and the VnR on control, inactivated cells. Three approaches were used to characterize these properties. First, an expression system was established so that the adhesive properties of GP IIb-IIIa and the VnR could be compared in an otherwise identical cellular environment. As previously described (Cheresh and Spiro, 1987), the VnR in M21 cells mediated cellular adhesion to fibrinogen, von Willebrand factor and vitronectin. To measure the adhesive activity of GP IIb-IIIa in these same cells we have expressed GP IIb in human M21-L cells which synthesize β_3 but lack expression of the VnR. GP IIb-IIIa in these cells, termed M21-L/GP IIb, mediated cellular adhesion to fibrinogen but not to von Willebrand factor or vitronectin. Second, variants of HEL cells were selected so that the adhesive activities of GP IIb-IIIa and the VnR could be compared in another cell type. It was found that while the VnR mediated cellular adhesion to fibrinogen, von Willebrand factor, and vitronectin, GP IIb-IIIa selectively mediated cellular adhesion to fibrinogen. The third approach examines the ability of GP IIb-IIIa to mediate the adhesion of unstimulated platelets to adhesive proteins. Although GP IIb-IIIa on activated platelets binds the soluble form of the three adhesive proteins examined (reviewed in Plow et al., 1986), GP IIb-IIIa on control platelets only mediated platelet adhesion to fibrinogen. We conclude that the adhesive properties of GP IIb-IIIa on unstimulated platelets and nucleated cells is similar, selectively mediating cellular adhesions to fibrinogen, and distinct from those of the VnR which mediate cellular adhesions in addition to von Willebrand factor and vitronectin.

The present study was facilitated by the stable transfection of full-length cDNA coding GP IIb into M21-L cells. The GP IIb-IIIa expressed on the surface of these cells after transfection of GP IIb used the endogenous β_3 (GPIIIa) that these cells synthesized. Immunomagnetic bead selection rather than FACS was used for the initial isolation of transfectants expressing GP IIb-IIIa. This was particularly useful since the beads provide a quick, inexpensive method that allowed easy isolation of the GP IIb-IIIa positive cells and convenient visualization of the morphologic changes that occurred when the selected cells were cultured on fibrinogen-coated plates. Although the beads remained bound to the cells for several days, they apparently did not interfere with cell attachment and spreading, cell growth, or cell division. This immunomagnetic bead selection procedure might be useful for other adhesive protein receptor expression systems and represents an alternative method for the selection of stable transfectants. Immunological criteria were also used to show that the expressed protein in M21-L/GP IIb cells does indeed correspond to platelet GP IIb-IIIa rather than a revertant endogenous VnR. Neither M21-L cells nor M21-L/GP IIb cells bound the mAb LM609, which recognizes a complex-dependent epitope of the VnR on M21-wild-type cells. In addition, mAb LM609 was unable to immunoprecipitate a complex from metabolically labeled M21-L or M21-L/GP IIb cells, providing further evidence that these cells are devoid of an intracellular pool of the VnR. In contrast, mAb A2A9, which recognizes a complex-dependent determinant on GP IIb-IIIa and does not cross-react with the VnR, exclusively reacted with the transfected M21-L/GP IIb cells from which it precipitated a complex corresponding to GP IIb-IIIa. Finally, mAb SZ21, which reacts with the individual β_3 subunit common to GP IIb-IIIa and the VnR, precipitated the VnR only from M21-wild type cells, the free β_3 subunit from M21-L cells and the GP IIb-IIIa complex only from the transfected M21-L/GP IIb cells. These results provide evidence that our initial immunomagnetic bead cell enrichment method allowed successful identification and isolation of GP IIb-expressing M21-L cells.

Transfection of GP IIb into M21-L cells allowed complete posttranslational processing and surface expression of a GP IIb-IIIa complex. This was documented by immunoprecipitation experiments performed in parallel on ^{125}I-surface labeled or [^{35}S]methionine labeled cells. ^{125}I-labeled GP IIb-IIIa from M21-L/GP IIb cells comigrated with platelet GP IIb-IIIa under both nonreducing and reducing conditions providing evidence that only the fully processed receptor reached the cell surface. The inability of mAb S1 (which reacts with individual GP IIb as well as pro-GP IIb) to precipitate pro-GP IIb from steady-state [^{35}S]methionine-labeled M21-L/GP IIb cells demonstrates the absence of an intracellular pool of unprocessed pro-GP IIb and suggests that pro-GP IIb did associate with endogenous GP IIIa to form the GP IIb-IIIa complex. This is in contrast with our results obtained after transient expression of the same pBJ1-GP IIb construct in COS cells where only individual unprocessed pro-GP IIb could be immunoprecipitated with mAb S1. In HEL cells, mAb S1 precipitated pro-GP IIb as well as the processed GP IIb-IIIa complex (Fig. 3). Since COS cells express a dimeric complex immunologically related to the integrin β_3 subunit, as identified by Western blot analysis and immunoprecipitation experiments using polyclonal rabbit IgG directed against GP IIb-IIIa (Kieffer, N., and D. R. Phillips, unpublished results), these results

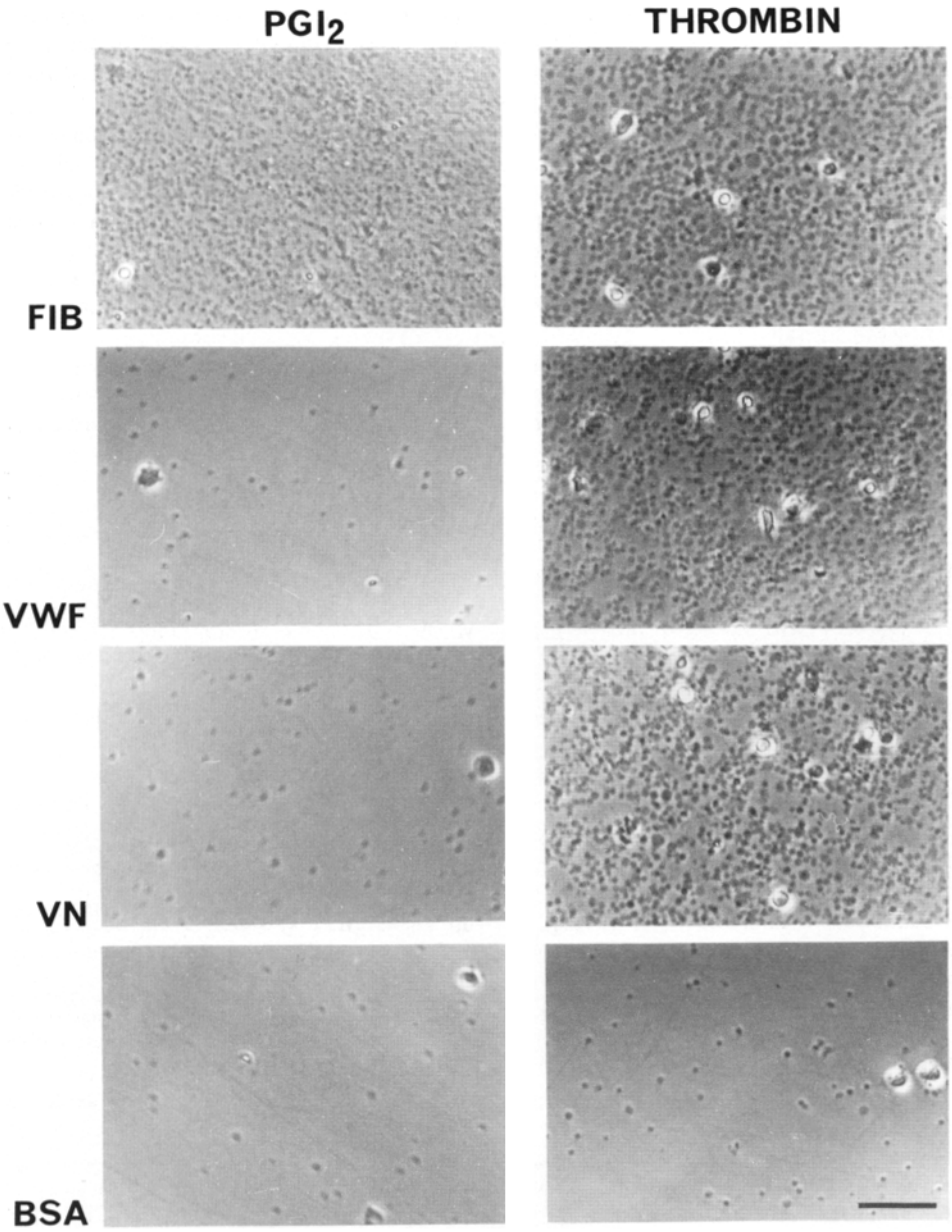


Figure 9. Phase-contrast micrographs of adhesion assays performed with PGI_2-treated resting platelets or thrombin-activated platelets. Platelets were allowed to attach to microtiter wells coated with fibrinogen (*FIB*), vitronectin (*VN*), von Willebrand factor (*VWF*), and BSA (*BSA*) as described in Materials and Methods. After adhesion, unattached platelets were washed off and the remaining cells were microphotographed. Bar, 50 μm.

suggest that human α^{IIb} does not associate with monkey β_3 unlike murine α^5 and avian β_1, which do (Solowska et al., 1989).

The successful stable expression of GP IIb-IIIa in M21-L cells allowed us to compare the functional properties of GPIIb-IIIa and the VnR individually expressed in M21 cells, and to compare these properties with those of native GP IIb-IIIa constitutively expressed in HEL cells or platelets. For this purpose, subclones of the HEL cell line were selected that express either GP IIb-IIIa (HEL-5J20) or GP IIb-IIIa and the vitronectin receptor (HEL-AD1). By using two distinct cell systems as well as human platelets, we have been able to demonstrate that GP IIb-IIIa in its inactive, resting form is capable of mediating cell attachment to immobilized fibrinogen, but not to other RGD-containing adhesive proteins such as vitronectin or von Willebrand factor. Evidence to support this conclusion is derived from the following experiments. First, M21-L/GP IIb cells as well as HEL cells expressing GP IIb-IIIa attached to fibrinogen but not to vitronectin or von Willebrand factor, whereas HEL-AD1 cells and M21 cells expressing the VnR did. Second, M21-L/GP IIb cell-, HEL cell- or platelet interaction with immobilized fibrinogen could be specifically inhibited with a complex dependent anti-GP IIb-IIIa mAb, demonstrating that the observed cell attachment to fibrinogen was GP IIb-IIIa dependent. Third, in contrast to thrombin or PMA-activated platelets, which attach to RGD-containing adhesive proteins such as fibrinogen, vitronectin, or von Willebrand factor, PGI_2-treated resting platelets attached exclusively to immobilized fibrinogen. Finally, that PMA did not induce M21-L/GP IIb or HEL cell attachment to vitronectin or von Willebrand factor suggests that in nucleated cells, GP IIb-IIIa is blocked in a functional state similar to that of resting platelets.

Since resting platelets and nucleated cells expressing GP IIb-IIIa attach to immobilized fibrinogen but are unable to

bind soluble fibrinogen (Coller, 1980; Marguerie et al., 1979; Thiagarajan et al., 1987; Kieffer, N., and D. R. Phillips, unpublished results), the molecular mechanism underlying soluble versus immobilized fibrinogen/GP IIb-IIIa interaction must be distinct. Cheresh et al. (1989) have recently shown that vitronectin receptor-dependent endothelial cell adhesion to immobilized fibrinogen is exclusively mediated through the RGD-containing site near the COOH terminus of the fibrinogen Aα chain, whereas GP IIb-IIIa-mediated adhesion of activated platelets to fibrinogen involves either RGD sequences as well as the COOH terminus portion of the γ chain. Our results suggest that in contrast to activated platelets, GP IIb-IIIa mediated attachment of resting platelets to immobilized fibrinogen might be mediated through the dodecapeptide sequence of the fibrinogen γ chain, since this sequence is unique to fibrinogen among the adhesive proteins, whereas GP IIb-IIIa-mediated adhesion to other adhesive proteins such as vitronectin or von Willebrand factor is dependent on the RGD recognition sites and requires the activated form of GP IIb-IIIa. Shiba et al. (Shiba, E., J. Lindon, M. Kloczawiak, J. Hawiger, G. Matsueda, B. Kudryk, and E. W. Salzman. 1988. *Circulation.* 78:II662.) have indeed shown that antibodies generated against the peptide 385–411 of the fibrinogen γ chain are effective in blocking the interaction of human platelets with fibrinogen adsorbed to a synthetic polymer surface. Alternatively, conformational changes of the RGD sequence in immobilized fibrinogen might increase its affinity for resting GPIIb-IIIa. Together with the data of Lindon et al. (1986), it is therefore tempting to speculate that changes in the conformation of immobilized fibrinogen lead to the exposure of recognition sites (γ chain dodecapeptide and/or a high-affinity RGD site) which are not accessible on soluble fibrinogen. Finally, due to the high density of immobilized fibrinogen molecules, several unidentified binding sites with modest affinities might cooperate to function as multivalent ligands for resting GP IIb-IIIa and allow a sufficiently high degree of adhesive specificity and affinity.

The expression of immunoreactive and functional GP IIb-IIIa on M21-L/GP IIb cells provides evidence that a transfected exogenous α^{IIb} subunit can form heterodimers with an existing endogenous VnR β_3 subunit and rescue a fully complexed $\alpha^{IIb}\beta_3$ receptor on the cell surface. This result is of particular interest with respect to patients with the genetic integrin deficiency Glanzmann's thrombasthenia, an autosomal recessive hemorrhagic disorder caused by a quantitative or qualitative defect of GP IIb-IIIa to bind fibrinogen (for a review see George et al., 1990). Although the molecular basis for GP IIb-IIIa deficiency in thrombasthenic patients is still unclear, the biosynthetic pathway of GP IIb-IIIa (Bray et al., 1986, Duperray et al., 1987, 1989; Rosa et al., 1989) suggests that a defect in only one of the glycoprotein subunits would prevent the formation of the complex and its insertion into the membrane. Indeed, in several thrombasthenic patients characterized by a complete absence of surface expression of the GP IIb-IIIa complex, trace amounts of GP IIb and GP IIIa or GP IIIa alone could be detected by immunoblotting (Nurden et al., 1985; Coller et al., 1987). Also, normal expression of the VnR ($\alpha^v\beta_3$) in endothelial cells of a patient lacking platelet GP IIb-IIIa suggest that this type of thrombasthenia is caused by defective synthesis of GP IIb (Giltay et al., 1987). M21-L cells, which have an intracellular pool of free GP IIIa, mimic the GP IIb-defective type of Glanzmann's thrombasthenia, and thus constitute an excellent human experimental cell model for integrin gene therapy in this disease.

Finally, our data provide a molecular model for the understanding of some physiological aspects of hemostasis and thrombosis. The interaction of resting platelet GP IIb-IIIa with immobilized fibrinogen constitutes a possible mechanism by which unactivated platelets are recruited into growing thrombi. Since this mechanism might also play a key role in platelet adhesion to artificial surfaces used for prosthetic arterial grafts or dialysis membranes (Lindon et al., 1986; McManama et al., 1986), the design of artificial surfaces resistant to fibrinogen adsorption appears to be a prerequisite for the development of nonthrombogenic materials.

We thank Dr. C. Damsky (Department of Stomatology, UCSF) for the gift of the VnR mAb BIIG2. We also wish to thank Bill Hyun (Laboratory for Cell Analysis, UCSF) for his expert technical assistance with FACS analysis and cell sorting.

This work was supported in part by National Institutes of Health grant HL-28947.

Received for publication 18 September 1990 and in revised form 28 December 1990.

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