Structure of Platelet Glycoprotein IIIa

A Common Subunit for Two Different Membrane Receptors

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Abstract

The platelet membrane glycoprotein IIb/IIIa complex is a member of a family of α/β heterodimers that function as receptors for adhesive proteins. In this report we describe the structure of the human β subunit GPIIIa deduced from an analysis of 4.0 kb of overlapping cDNA sequences isolated from a human erythroleukemia (HEL) cell cDNA expression library. A continuous open reading frame encoding all 788 amino acids for GPIIIa was present. The deduced amino acid sequence included a 26-residue amino-terminal signal peptide, a 29-residue transmembrane domain near the carboxy terminus, and four tandemly repeated cysteine-rich domains of 33–38 residues. An exact correspondence of 128 amino acids from seven human platelet GPIIIa fragments with HEL GPIIIa indicates that HEL and platelet GPIIIa are the same gene product. The HEL GPIIIa sequence was compared with the sequences of the β subunit for the human LFA-1/Mac-1/p150,95 complex and human endothelial cell GPIIIa, revealing a 38% similarity with the former and virtual identity with the latter. Northern blot analysis using RNA from both HEL and endothelial cells revealed two GPIIIa transcripts of 5.9 and 4.1 kb. However, HEL RNA, but not endothelial cell RNA, contained a transcript for GPIIb. This indicates that the GPIIIa-containing heterodimers in platelets and endothelial cells are not identical structures, but are members of a subfamily within the human family of adhesion protein receptors sharing an identical β subunit.

Introduction

The platelet membrane glycoprotein IIb/IIIa (GPIIb/IIIa)[1] complex is a calcium-dependent heterodimer containing binding sites for fibrinogen, vWf, fibronectin, and vitronectin that are exposed by platelet activation (1–4). GPIIb/IIIa is also a member of a family of adhesive protein receptors that includes chick fibroblast integrin, the LFA-1/Mac-1/p150,95 complexes of leukocytes, the VLA complexes present on stimulated T lymphocytes, and the fibronectin and vitronectin receptors found on a variety of cells (5). Biochemical examination indicates that they are α/β heterodimers with α subunits analogous to GPIIb, and β subunits analogous to GPIIIa (5) and many interact with ligands containing the Arg-Gly-Asp sequence (4, 6). Recently, the amino acid sequences for several α and β subunits have been determined by analysis of cDNA and have demonstrated homology among the various α and among the various β subunits (7–12).

We report here the structure of GPIIIa deduced from an analysis of cDNA from a human erythroleukemia (HEL) cell expression library. The HEL cell line originated in a patient with erythroleukemia and has megakaryocyte-like properties, constitutively expressing a number of platelet proteins such as platelet factor 4, β-thromboglobulin, vWf, GPIb, and GPIIb/IIIa (13, 14). Recently, we reported the isolation of cDNA for platelet factor 4, a megakaryocyte-specific protein, and for GPIIb from this HEL library (7, 15). Comparison of the deduced sequence of HEL GPIIb with known partial amino acid sequences of platelet GPIIb demonstrated that HEL GPIIb is identical to platelet GPIIb (16, 17). The results described in the present paper indicate that HEL GPIIIa and platelet GPIIIa also appear to be products of the same gene and are essentially identical to the GPIIIa expressed by endothelial cells. In addition, we demonstrate that endothelial cells do not express a gene for GPIIb. These findings indicate that the adhesive protein receptors expressed by platelets and endothelial cells are distinct structures, but share a common β subunit.

Methods

Isolation of cDNA clones for HEL cell GPIIIa. Total cellular RNA was obtained from DMSO-stimulated HEL cells using the method of Chirgwin et al. (18). Poly $(A)^+$ RNA was isolated by chromatography on oligo(dT)-cellulose (19). A cDNA expression library in the vector λgt11 was used as previously described (7). Phages containing GPIIIa cDNA were identified by screening a lawn of transformed *Escherichia coli* Y1090 with monospecific polyclonal anti-GPIIIa IgG. This IgG was prepared by immunizing New Zealand white rabbits with purified, reduced, and alkylated platelet GPIIIa, prepared as previously described (20). Anti-GPIIIa IgG was prepared from immune sera by affinity chromatography on Protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). The IgG was incubated with thrombasthenic platelets and purified IIb to remove non-GPIIIa reacting contaminants. Plaques reacting with the anti-GPIIIa IgG were identified using biotinylated goat anti-rabbit antiserum and an avidin-horseradish peroxidase complex to detect the secondary antibody (Vector Laboratories, Inc., Burlingame, CA) (15).

Characterization of GPIIIa clones. Phage DNA from two clones was purified and digested with Eco R1 (7). Because the cDNA contained an internal Eco R1 site, two cDNA fragments were obtained

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1. *Abbreviations used in this paper:* GP, glycoprotein; HEL, human erythroleukemia.

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from each clone. The fragments were isolated by electroelution from agarose gels, subcloned separately into M13mp18 phage (21), and transfected into JM107 bacteria to prepare single-stranded DNA in both orientations. cDNA insert orientation and homology were determined by annealing single-stranded DNA from different isolates and digesting the hybrids with S1 nuclease (Bethesda Research Laboratories, Gaithersburg, MD) (22).

The nucleotide sequences of the cDNA in M13mp18 were obtained using the dideoxy chain termination technique of Sanger et al. (23) and the universal M13 primer (Bethesda Research Laboratories) (24). The complete sequence of each cDNA was obtained by preparing a series of overlapping deletion mutants using the enzyme Bal 31 (22). Full-length cDNA sequences were obtained in both orientations.

Computer analysis of the GPIIIa cDNA sequence. Computer analysis was performed using the Bionet system (Intelligenetics, Inc., Palo Alto, CA). The hydropathic profile of GPIIIa was calculated as described by Kyte and Doolittle using segment lengths of five amino acids (25). Comparison of the amino acid sequence of GPIIIa with those of chick fibroblast integrin band 3 and the β subunit of LFA-1 was accomplished by initially aligning the sequences using the FASTP program and optimizing the alignments manually.

Northern blot analysis of HEL and endothelial cell mRNA. Total cellular RNA from HEL cells and from cultured human umbilical vein endothelial cells (a gift from Dr. Douglas Cines, University of Pennsylvania, Philadelphia, PA) was isolated as described above. In addition, RNA was obtained from K562 cells stimulated with 10^{-8} M phorbol-12-myristate-13-acetate to serve as a positive control for GPIIIa and a negative control for GPIIb (20). Northern blotting was performed as described by Thomas (26).

Results

Isolation of cDNA for GPIIIa. An initial screen of 2×10^5 recombinant λgt11 with anti–GPIIIa IgG identified at least nine positive clones. Two clones were selected, plaque-purified, and digested with Eco R1, releasing cDNA inserts ~ 3.8 kb long. Because each insert contained an internal Eco R1 site, two fragments ~ 1.6 and 2.2 kb in size were released after digestion. The cDNA fragments were then subcloned into Eco R1–linearized M13mp18 phage in both orientations.

HEL GPIIIa cDNA sequence. Complete nucleotide sequences for the 1.6- and 2.2-kb cDNA were determined in both orientations. The cDNAs contained a total of 3,997 bp and encode a continuous open reading frame for 788 amino acids (Fig. 1). Charo, Fitzgerald, and co-workers have directly determined the sequence of the 19 amino-terminal amino acids of platelet GPIIIa (12, 16). These amino acids are encoded in our sequence beginning at residue 27. Amino acids 1–26 are predominantly hydrophobic and begin with a methionine. This suggests that they constitute an amino-terminal signal peptide and that the methionine at residue 1 is the translation initiation site. Fitzgerald et al. have recently reported the partial amino acid sequences of four cyanogen-bromide cleavage products of platelet GPIIIa (12), and Hiraiwa et al. have published partial amino acid sequences for platelet GPIIIa fragments generated by the enzyme lysyl endopeptidase (17). These amino acid sequences, representing 128 residues, are present in our GPIIIa sequence beginning at residues 361, 413, 594, 727, and residues 152, 218, and 439, respectively. The exact correspondence of multiple amino acid sequences from fragments of human platelet GPIIIa with deduced amino acid sequences encoded by HEL GPIIIa cDNA is strong evidence that HEL and platelet GPIIIa are the same gene product.

The hydropathic profile of GPIIIa confirms the hydrophobic character of the first 26 amino acids of the GPIIIa sequence and identifies a second stretch of 29 hydrophobic amino acids near the carboxy terminus of the sequence that meets criteria for a transmembrane domain (27) (data not shown). The putative transmembrane domain is followed by 41 predominantly hydrophilic residues that may represent a short cytoplasmic segment. This segment contains a tyrosine in a region homologous to the region surrounding the major autophosphorylation site of the epidermal growth factor receptor (28), and may be a site for phosphorylation by tyrosine kinases. Seven potential sites for N-linked glycosylation are present in the sequence, six in that portion of GPIIIa likely to be extracellular. The HEL GPIIIa sequence also contains four tandemly repeated domains of 33–38 amino acids, each containing either seven (first repeat) or eight cysteines (repeats 2–4) and a core sequence of CxCxxCxC. The cysteine missing in the first repeat is the first cysteine of this core sequence. The coding region of our GPIIIa cDNA is followed by 1,614 bp of untranslated nucleotides that do not contain either a polyadenylation consensus sequence or a poly(A) tail, suggesting that our cDNAs do not account for the entire GPIIIa message.

Comparison of the HEL GPIIIa sequence with the known sequences of other human β subunits. The amino acid sequences for the β subunit of the human LFA-1/Mac-1/p150,95 complex and for human endothelial cell GPIIIa have recently been reported (9, 12). We compared the amino acid sequences of these proteins with the sequence of HEL GPIIIa. When compared with HEL GPIIIa, the β subunit of LFA-1/Mac-1/p150,95 contains 38% identical amino acids, another 39% conservative amino acid changes, and the positions of the 56 cysteine residues common to the two proteins are conserved (9). The amino acid sequences for HEL GPIIIa and for endothelial GPIIIa are essentially identical, except for an alanine to valine substitution at residue 12 in the signal peptide of endothelial GPIIIa (12). Furthermore, when the nucleotide sequences of the cDNA for each GPIIIa are compared, there are two silent nucleotide changes in the coding region and three nucleotide substitutions in the 3′ untranslated region. This represents a 0.5% variation and is consistent with the HEL and endothelial GPIIIa genes being alleles or with errors in DNA sequence determination.

Northern blot analysis using HEL and endothelial cell RNA. Both endothelial and HEL cells express GPIIb/IIIa complexes with identical mature GPIIIa subunits. To examine the GPIIb and GPIIIa gene products from these cells, we performed Northern blotting using RNA isolated from HEL cells and from human umbilical vein endothelial cells. RNA from K562 cells stimulated with phorbol-12-myristate-13-acetate was used as a control, because these cells express GPIIIa but not GPIIb (20). We used a 3.2-kb cDNA probe for GPIIb (7) and a 2.2-kb cDNA probe for GPIIIa. The GPIIIa probe hybridized with mRNAs of 5.9 and 4.1 kb in HEL, endothelial, and K562 cells (Fig. 2). Under more stringent washing conditions (68°C and 0.1× sodium chloride sodium citrate buffer) both bands persisted (data not shown), suggesting that GPIIIa mRNA in these cells consists of several species, the result of either alternative splicing, stable nuclear intermediates, or cytoplasmic degradation products of the GPIIIa transcript. As we reported previously, the GPIIb cDNA hybridized with a single mRNA species of ~ 3.7 kb present in HEL cell RNA, but not

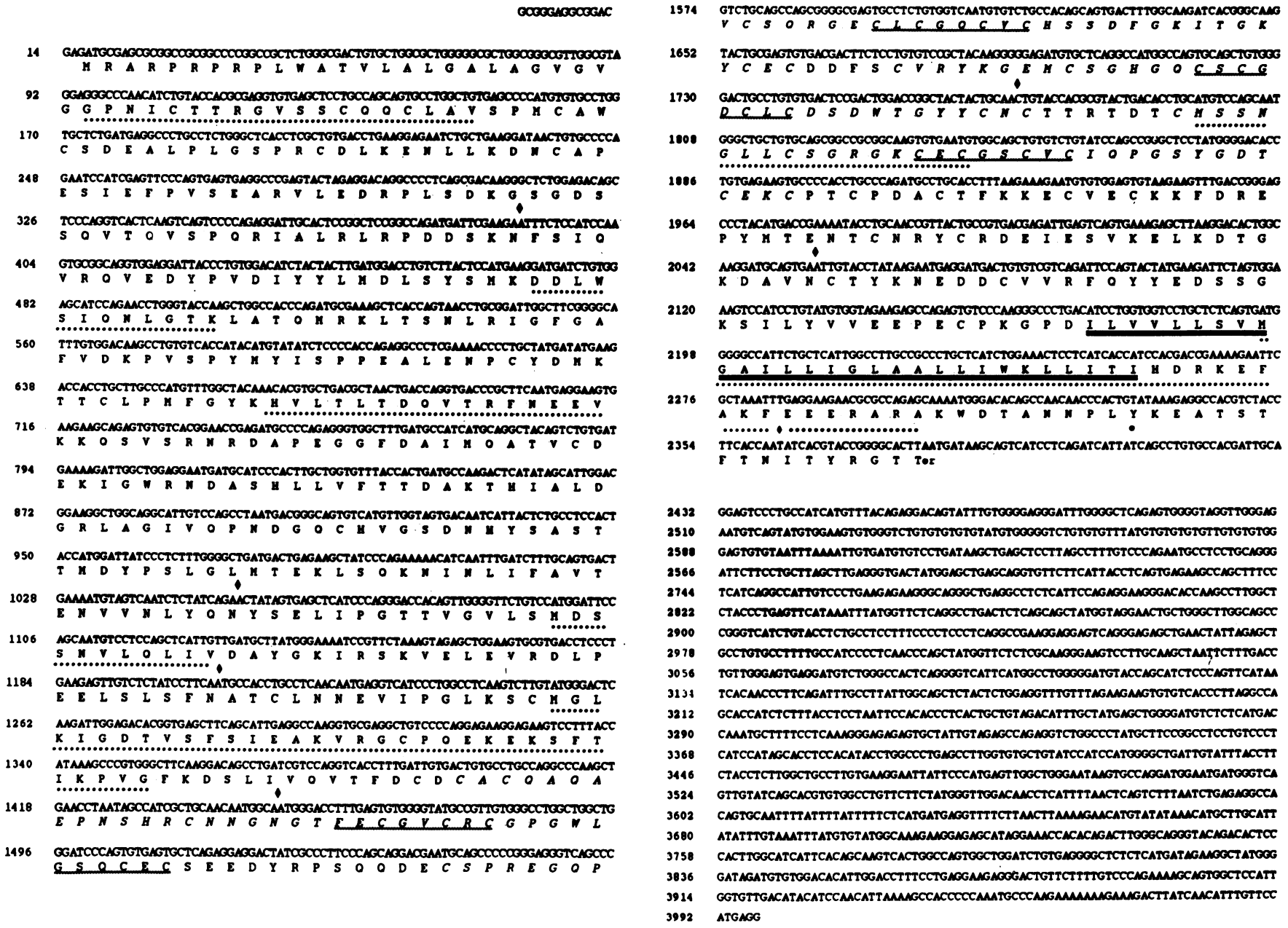

Figure 1. DNA sequence for GPIIIa. The 3,997-bp sequence encodes a continuous reading frame for 788 amino acids. (Amino acids are indicated by the single letter code under the nucleotide sequence.) A potential transmembrane domain is underlined by a heavy solid line. Previously reported human platelet GPIIIa amino acid sequences are underlined by dotted lines. The amino acids of the cysteine-rich tandem repeats are shown in italics and the positions of their core sequence (CxCxxCxC) are underlined by a wavy line. The solid diamonds indicate potential sites for N-linked glycosylation. The solid circle indicates a potential site for phosphorylation by tyrosine kinase.

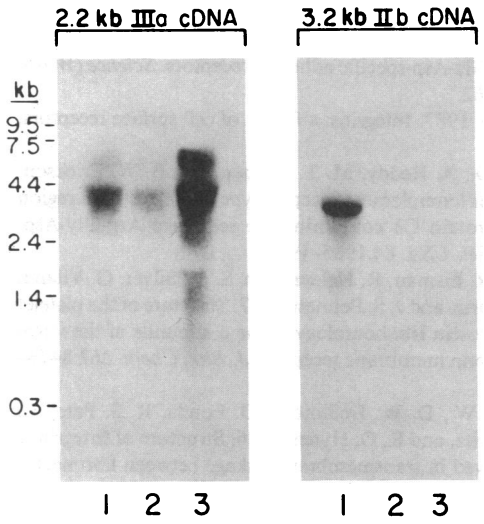

Figure 2. Northern blot analysis using HEL and endothelial cell RNA. 30 μg total cellular RNA from HEL cells, human umbilical vein endothelial cells, and K562 cells stimulated with 10^{-8} M phorbol-12-myristate-13-acetate for 48 h was denatured in the presence of glyoxal and electrophoresed on 1% agarose gels (26). RNA from the K562 cells was used as positive and negative controls for GPIIIa and GPIIb, respectively (20). After electrophoresis, the RNA was transferred to nylon paper and hybridized to cDNA probes for GPIIIa and GPIIb that had been labeled with ^{32}P. Lane *1*, RNA from HEL cells; lane *2*, RNA from endothelial cells; lane *3*, RNA from K562 cells.

in K562 cell RNA (7). Furthermore, we did not detect a transcript for GPIIb in the RNA from endothelial cells. These studies indicate that the GPIIb/IIIa-like complexes expressed by endothelial cells cannot be identical to those expressed by HEL cells and platelets, but must correspond to another member of the GPIIIa-containing adhesion protein receptor subfamily.

Discussion

To obtain the amino acid sequence for platelet GPIIIa, we isolated cDNA from an HEL cell expression library using anti-human platelet GPIIIa IgG. We chose this library because previous biochemical and immunologic evidence had suggested that this library would contain cDNA for a protein very similar, if not identical, to platelet GPIIIa (20). First, HEL cells express a GPIIIa-like protein whose mobility on unreduced and reduced SDS-polyacrylamide gels is identical to that of platelet GPIIIa. Second, HEL GPIIIa cross-reacts with both polyclonal and monoclonal antibodies raised against the platelet protein. Third, immunologic studies indicated that HEL GPIIIa resides as a heterodimer complex with a GPIIb-like protein in the HEL cell plasma membrane. Moreover, we recently cloned a GPIIb-like protein from our HEL cell expression library and found that this protein is identical to platelet GPIIb (7).

We found that the nucleotide sequences of the cDNAs identified using anti-human platelet GPIIIa IgG encode the full length of a GPIIIa-like protein. Partial peptide sequences have been published for eight human platelet GPIIIa fragments that are distributed throughout the molecule and that account for 16.8% of the GPIIIa residues (12, 17). When we compared our deduced amino acid sequence for HEL GPIIIa with the partial amino acid sequences of platelet GPIIIa, we found that the sequences were identical, strongly suggesting that HEL and platelet GPIIIa are products of the same gene.

Endothelial cells contain a protein immunologically related to platelet GPIIIa (16, 29). The amino acid sequence of the precursor for this protein has been deduced from the nucleotide sequences of cDNAs isolated from a human endothelial cell library and is virtually identical to the amino acid sequence we deduced for HEL GPIIIa (12). The HEL GPIIIa precursor, like endothelial cell GPIIIa, contains 762 amino acids, a 25-residue amino-terminal signal peptide, and a putative 29-residue hydrophobic residue domain. The location of this latter stretch of amino acids and of six of seven potential N-linked glycosylation sites defines the likely orientation of the GPIIIa in the plasma membrane, with the majority of the protein extracellular and with a short 41-residue hydrophilic carboxy-terminal cytoplasmic tail. Preceding the transmembrane domain are four homologous stretches of ~ 40 residues that are rich in cysteines and contain a core sequence of CxCxxCxC. Cysteine-rich repeats are also present in the LDL, insulin, and epidermal growth factor receptors where they are thought to be ligand binding sites (30).

Comparison of the structure of the HEL GPIIIa precursor with the precursors of other adhesive protein receptor β subunits confirms biochemical evidence that the β subunits are related and suggests that they evolved from a common ancestral gene. Kishimoto and co-workers have compared the amino acid sequence of the 120,000-mol wt β subunit of chick integrin with the sequence of the β subunit of the human leukocyte LFA-1/Mac-1/p150,95 complex, finding 45% amino acid identity (9, 31). Similarly, Fitzgerald et al. compared the chick integrin β subunit with human endothelial cell GPIIIa and found 47% amino acid identity (12). In turn, we have compared HEL GPIIIa with the human leukocyte β subunit, finding only 38% sequence identity. Moreover, although the positions of all 56 cysteines in these two proteins have been conserved, GPIIIa contains 19 more amino acids and one more potential site for N-linked glycosylation (9). These results suggest that human GPIIIa and the human LFA-1/Mac-1/p150,95 β subunit may be more closely related to the β subunit of chick integrin than to each other and that they may have evolved from a common β subunit precursor, perhaps from the β subunit of a primordial fibronectin binding complex.

Heterodimer complexes similar to platelet IIb/IIIa have been isolated from both human and bovine endothelial cells using antibodies raised against the platelet proteins (16, 32). The smaller protein of the endothelial cell complex is biochemically similar, if not identical, to platelet GPIIIa, with the same apparent molecular weight and the same isoelectric point (16), and as we noted above, the cDNA sequences of GPIIIa precursors for platelets and endothelial cells are virtually identical. On the other hand, the GPIIb-like component of the endothelial cell heterodimer has an apparent molecular weight slightly greater than platelet GPIIb and a more acidic pI, 5.2–5.3 as compared with 5.5 (16). Furthermore, Ginsberg et al. recently demonstrated that this endothelial cell protein and the α subunit of the vitronectin receptor obtained from endothelial cells comigrate on SDS-polyacrylamide gels and that

there is only 33% identity between the amino-terminal 12 amino acids of platelet GPIIb and the vitronectin receptor α subunit (33). We were unable to detect a transcript for GPIIb in Northern blots of endothelial cell RNA, whereas a transcript for GPIIIa was detected. These data indicate that the endothelial cell and platelet heterodimers are not identical, containing different α subunits and identical β subunits. These findings also suggest that the phenotypic expression of abnormalities in GPIIb synthesis may be limited to platelets, perhaps manifested as Glanzmann's thrombasthenia. In contrast, abnormalities of GPIIIa synthesis might involve at least endothelial cells as well as platelets, and have a more widespread phenotypic effect.

Hynes proposed that there are three subfamilies within the human family of adhesion protein receptor heterodimers based upon the number of different β subunits (5). Our results support this proposal. The platelet and endothelial cell heterodimers use GPIIIa, the leukocyte heterodimers contain a 95,000-mol wt β subunit that is homologous to GPIIIa but is clearly a different protein (9), and the fibronectin receptors contain a β subunit that appears to be analogous to band 3 of integrin (4, 34). The genetic factors responsible for the tissue-specific expression of each β subunit and for the distribution of α subunits remain to be determined.

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Note added in proof. Since the submission of this manuscript, we have become aware of two errors in our GPIIIa sequence. Correction of these errors results in a frame shift changing five amino acids at positions 623–627 (EPYMT → GALHD) as first noted by Rosa et al. (Rosa, J.-P., P. F. Bray, O. Gayet, G. I. Johnston, R. G. Cook, M. A. Shuman, and R. P. McEver. 1987. Cloning of glycoprotein IIIa cDNA from human erythroleukemia (HEL) cells: sequence polymorphism and colocalization of the glycoprotein IIb and glycoprotein IIIa genes to chromosome 17. *Blood.* 70(Suppl. 1):359*a*. (Abstr.) We wish to thank Dr. J.-P. Rosa, Dr. R. P. McEver, and Dr. M. A. Shuman for bringing these changes in the HE GPIIIa sequence to our attention. An additional error was also present in the published sequence for endothelial cell GPIIIa (L. Fitzgerald, personal communication). Correction of this error replaces the valine at residue 12 of the signal peptide of endothelial GPIIIa with an alanine such that the sequences of HEL and endothelial cell GPIIIa are identical.

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