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Complementary DNA Cloning of the Alternatively Expressed Endothelial Cell Glycoprotein Ib β (GPIb β) and Localization of the GPIb β Gene to Chromosome 22

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Abstract

Glycoprotein Ib β (GPIb β) exists in platelets disulfide-linked to glycoprotein Ib α (GPIb α), a major receptor for von Willebrand factor. Both GPIb α and GPIb β are expressed in endothelial cells (EC). While the GPIb α mRNA and protein appear similar in platelets and EC, EC GPIb β mRNA is larger than platelet GPIb β and encodes a larger protein. We have cloned and sequenced EC GPIb β cDNA and report a 2793-nucleotide sequence which contains a 411-amino acid open reading frame. The EC sequence contains all of the platelet cDNA sequence and all but three amino acids of the primary translation product. Like the genes encoding GPIb α , GPIX, and GPV, the GPIb β gene appears simple in structure. Using human hamster hybrids, we have localized the GPIb β gene to chromosome 22pter \rightarrow 22q11.2. When we examined poly (A)⁺ RNA from several human tissues for GPIb β mRNA expression, we found that GPIb β mRNA was expressed in a variety of tissues but was most abundant in heart and brain, while GPIb α and GPIX mRNA expression was found only in lung and placenta at very low levels. The broad distribution of GPIb β mRNA suggests that it may be playing a role different than or additional to its function in platelets. (*J. Clin. Invest.* 1994; 93:2417–2424.)
Key words: platelet membrane glycoproteins • vascular endothelium • alternative splicing • von Willebrand factor • adhesiveness

Introduction

Glycoprotein Ib is a transmembrane heterodimer in platelets which is comprised of a 143-kD alpha chain (GPIb α) disulfide-linked to a smaller 24-kD beta chain (GPIb β). GPIb α contains binding sites for von Willebrand factor and thrombin in its amino-terminal extracytoplasmic region (1). Through von Willebrand factor binding, GPIb plays a critical role in platelet adhesion to the vascular subendothelium, especially under conditions of high shear flow (2). In nonactivated platelets, GPIb is complexed to actin-binding protein and thereby to the cytoskeleton, via the intracytoplasmic segment of the alpha chain (3). The role of GPIb β is unknown, although it is the

major platelet membrane protein to be phosphorylated when resting platelets are incubated with [³²P]phosphate (4). This phosphorylation, thought to occur at Ser166 in the mature platelet protein, is present under conditions that increase intracellular cAMP and may contribute to the inhibitory actions of cAMP by inhibiting collagen-induced polymerization of actin (5).

In the platelet membrane, GPIb exists in a 1:1 noncovalent complex with GPIX and is more loosely associated with GPV (1, 6). In the inherited bleeding disorder Bernard-Soulier syndrome, GPIb α , GPIb β , GPIX, and GPV are all missing or affected (1).

The cDNAs for both GPIb subunits and for GPIX have been cloned from a phorbol-stimulated human erythroleukemia (HEL)¹ cell cDNA library and are products of different genes (7–9). HEL cells express numerous megakaryocytic proteins, especially when stimulated with phorbol esters or DMSO (10). Platelet RNA contains the same GPIb α , GPIb β , and GPIX species as RNA from HEL cells (11). Lopez et al. (12) have expressed HEL cell-derived cDNAs for the GPIb complex in Chinese hamster ovary cells and have found that GPIb α , GPIb β , and GPIX must be expressed together for efficient functional expression of GPIb α on the cell surface. cDNA and genomic cloning of GPV has been reported recently (13, 14); its role in the function of the GPIb-IX complex in platelets remains to be elucidated.

Earlier studies by Sprandio et al. (15) and independently by Asch et al. (16) demonstrated that human umbilical vein endothelial cells (HUVEC) synthesize a protein immunologically related to and similar in size to platelet GPIb α . Later studies demonstrated the presence of GPIb α mRNA in these cells and showed that expression of this mRNA and its corresponding protein was markedly increased by exposure to TNF- α (17, 18). This effect of TNF on GPIb α mRNA expression was also seen in HEL cells. Based on Northern blotting and partial cDNA sequence analysis, the endothelial GPIb α mRNA species appears to be identical to that in HEL cells and platelets (17).

In this paper we show that the GPIb β mRNA and protein species are larger in endothelial cells (ECs) than in platelets and HEL cells, report the cDNA sequence of endothelial GPIb β , and evaluate GPIb β mRNA expression in different tissues. We also report chromosomal localization of the GPIb β gene.

Methods

Cell culture. HUVEC were propagated from pooled primary cultures of human umbilical veins as described previously (15). Cells were grown on 0.2% gelatin (GIBCO BRL, Gaithersburg, MD) coated tissue cul-

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1. *Abbreviations used in this paper:* EC, endothelial cell; EST, expressed sequence tag; HEL, human erythroleukemia; HUVEC, human umbilical vein endothelial cell; nt, nucleotide; UT, untranslated.

ture flasks (Corning Medical, Corning, NY) in Hepes-buffered M199 (GIBCO BRL) with 10% FBS (Hyclone Laboratories, Logan, UT) in a 5% CO₂ humidified atmosphere at 37°C. Culture media were supplemented with crude EC growth factor (100 µg/ml) and heparin (100 µg/ml). In a given experiment, all cells were grown in the same lot of FBS. HUVEC were passaged as a 1:4 split, fed every 2–3 d, and used at 2nd to 4th passage.

HEL cells were obtained from the American Type Culture Collection (Rockville, MD) (ATCC TIB 180, HEL 92.1.7) and propagated in RPMI 1640 (GIBCO BRL) with 10% FBS. CHRF-288 cells, kindly provided by M. Lieberman (Children's Hospital Medical Center, Cincinnati, OH), were propagated in Fischer's medium (GIBCO BRL) with 20% horse serum (GIBCO BRL). All cultures were maintained in a 5% CO₂ humidified atmosphere at 37°C.

RNA and DNA analysis. Total cellular RNA was isolated by immediate solubilization of the cells in guanidine hydrochloride, as described previously (19). Poly (A)⁺ RNA was isolated either by (a) incubation of total cellular RNA with oligo-dT cellulose (Collaborative Research Inc., Bedford, MA) followed by elution as described (20) or by (b) direct isolation from cells using the Mini-Ribosep kit (Collaborative Research Inc.). A Northern blot containing human megakaryocyte RNA was a kind gift of Dr. Paul Schick of the Cardeza Foundation. A Northern blot containing poly (A)⁺ RNA from various human tissues was purchased from Clontech Laboratories, Inc. (Palo Alto, CA).

RNA was analyzed by Northern blotting. The RNA was fixed to the membrane with ultraviolet irradiation, prehybridized in 1 M NaCl, 0.1% SDS, 1.5 mg/ml herring sperm DNA, and 10% dextran for 3 h at 65°C, and hybridized at 65°C for 12–24 h in the prehybridization solution with the addition of 1.5 mg/ml sonicated herring sperm DNA and the appropriate radiolabeled probe. Probes used included (a) HEL cell-derived GPIbα cDNA, GPIb2.4 (7), (b) HEL cell-derived GPIbβ cDNA (8), (c) HEL cell-derived GPIX cDNA (9), and (d) the endothelial GPIbβ clone, ECβ4. cDNA inserts were radiolabeled directly in low melting agarose by random hexamer priming (21). The blots were washed to high stringency (0.1 × SSC, 0.1% SDS, 1 mM EDTA, 10 mM sodium phosphate [pH 6.8], at 65°C) and analyzed by autoradiography. Individual blots were rehybridized with a second radiolabeled probe after incubation in 5 mM Tris/HCl (pH 7.5), 2 mM EDTA, 0.1 × Denhardt's at 68°C for 3–4 h to remove the original probe. Successful "stripping" was confirmed by autoradiography. The Clontech multi-tissue blot was hybridized, washed, stripped of radiolabeled probe, and rehybridized according to the manufacturer's directions, except that it was washed to the stringency noted above.

Human genomic DNA was isolated from white blood cells and analyzed by Southern blotting as described previously (22), with hybridization and washing conditions identical to those described above. The PCR was performed on 250 ng of human genomic DNA with 500 ng of each primer using the Stoffel fragment of Taq DNA polymerase and the buffer provided by the manufacturer (Perkin-Elmer Corp., Norwalk, CT) except that 10% DMSO (final concentration) was added. Cycle times were 1 min at 94°C, 1 min at 48°C, and 1 min at 72°C (plus an additional 3 s per cycle) for 30 cycles. All reactions were completed with 10 min at 72°C. Restriction enzyme digests were performed on 5 µl of the PCR products using the buffer supplied by the enzyme manufacturer (Promega Corp., Madison, WI). Reaction products and digests were checked by electrophoresis on a 2% agarose gel. The genomic fragment (EC cDNA nucleotides [nt] 961–1446) was subcloned into the vector pCRII (Invitrogen, San Diego, CA), and DNA sequence analysis was performed.

Isolation and sequence analysis of cDNA clones. The HUVEC lambda gt11 random-primed cDNA library was kindly provided by Dr. David Ginsburg (University of Michigan, Ann Arbor, MI) and has been reported previously (22). Greater than 5 × 10⁶ recombinant phage were screened with ³²P-GPIbβ HEL cell-derived cDNA as described by Benton and Davis (23). Positive bacteriophage were plaque purified through two to three additional rescreening steps. Phage DNA preparation was performed as described (20), and after digestion with EcoRI the inserts were purified from agarose gels by electroelution

(20) and subcloned into M13mp18 and M13mp19 and either pGEM-7Zf (Promega Corp.) or pBluescript II KS (Stratagene Cloning Systems, La Jolla, CA). Sequence analysis was performed using both single-stranded and denatured double-stranded template by the dideoxy chain termination method using modified T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH) and M13 forward and reverse sequencing primers or synthetic primers complementary to determined sequence within the clones. Sequences within clone ECβ1 were also determined from nested deletions prepared using exonuclease III (Erase-a-Base; Promega Corp.). Sequence compressions were resolved by sequencing the areas in question with Sequenase using dITP instead of dGTP, increasing the temperature for template extension in the sequencing reactions to 45°C, or by modifying the Sequenase protocol by adding 0.5 µg of single-stranded binding protein (United States Biochemical Corp.) during the labeling reaction. After addition of the stop solution, 0.1 µg of proteinase K was added, and the reaction was heated to 65°C for 30 min before loading on the gel. All clones were sequenced on both strands in their entirety at least once. Sequences were stored and analyzed using the IBI-Pustell software package (International Biotechnologies, Inc., New Haven, CT).

Western blotting of platelet and HUVEC proteins. Platelets were prepared as described previously (24). After the final wash, the platelet pellet was solubilized in 1% SDS with 10% β-mercaptoethanol. Confluent second passage HUVEC were detached by incubation for 20 min at 37°C in a buffer composed of 150 mM NaCl, 2 mM Tris, 10 mM EDTA, 1 mM PMSF, 5 mM benzamidine, 200 kU/ml aprotinin, and 200 µg/ml leupeptin, pH 7.4. The cells were centrifuged at 1,100 g for 10 min, and an equal volume of 2% SDS with 10% β-mercaptoethanol was added to solubilize the pellet. The equivalent of 1 × 10⁵ HUVEC or 1 × 10⁷ platelets was added per lane and subjected to SDS-PAGE using a 4–20% linear gradient of acrylamide.

To concentrate EC GPIbβ, ~ 8 × 10⁸ HUVEC were lysed in PBS containing 0.1% RIA-grade BSA, 10 mM Hepes, 0.5% Triton X-100, 10 mM EDTA, 5 mM EGTA, 0.5 mg/ml DNaseI, 80 µg/ml leupeptin, 20 KIU/ml aprotinin, 10 mM benzamidine, and 2 mM PMSF. The Triton-soluble fraction was subjected to wheat germ agglutinin Sepharose (Sigma Immunochemicals, St. Louis, MO) chromatography followed by ion exchange chromatography using Mono Q Superose (HR5/5) (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The GPIbα-positive fraction was electrophoresed in the presence and absence of 5% 2-mercaptoethanol using a 4–20% gradient gel. Proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA), incubated with a polyclonal rabbit antibody to human platelet GPIbβ (a generous gift of Dr. Gerald Roth, University of Washington, Seattle, WA), and developed using a horseradish peroxidase-conjugated goat antibody to rabbit IgG supplied with the Bio-Rad Western blotting kit.

Rodent-human hybrids. Hybrid DNAs were from previously described rodent-human hybrid cell lines (25) or from the Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ). Hybrids retaining partial chromosome 22 have also been described (26). Hybrid DNAs were tested for the presence of specific human EcoRI or SstI restriction fragments detected by the radiolabeled GPIbβ probe using standard Southern hybridization methods.

Results

GPIbβ mRNA expression in EC, HEL cells, megakaryocytes, and the megakaryoblastic cell line CHRF-288. Poly (A)⁺ RNA from HUVEC was analyzed for GPIbβ mRNA expression (Fig. 1 A). Using a "full length" HEL cell-derived GPIbβ cDNA as the probe (8), Northern blot analysis showed an ~ 3.5-kb mRNA species in HUVEC RNA, while total cellular RNA from unstimulated HEL cells contained only the 1.1-kb species that has been reported previously by Lopez et al. (8) (Fig. 1 A). Platelet RNA contained only the 1.1-kb species (11). We evalu-

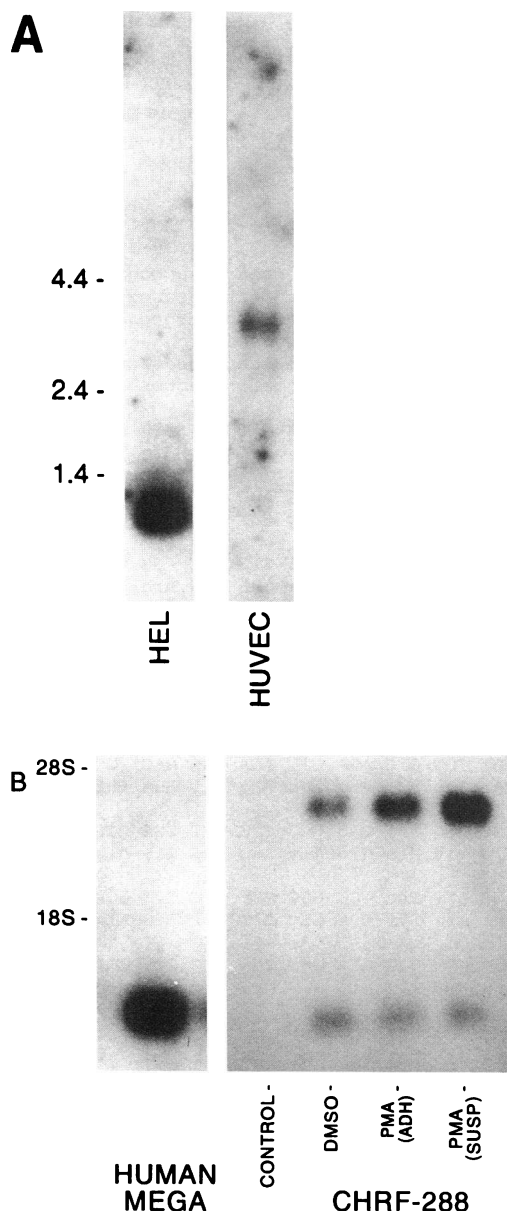


Figure 1. Northern blot analysis of GPIIb/IIIa mRNA. Northern blot analysis was performed on (A) 5 μ g of total cellular HEL cell RNA and 4 μ g of poly (A)⁺ HUVEC RNA, and in B on 3 μ g of human megakaryocyte and 10 μ g of CHRF-288 total cellular RNA using the HEL cell-derived GPIIb/IIIa as the probe. The CHRF-288 cells were untreated (CONTROL) or treated with DMSO or PMA for 4 d. RNA was harvested from PMA-treated cells that were adherent (ADH) or from cells that remained in suspension (SUSP). The positions of RNA size markers are indicated. For B, the positions of the 28S (4.8 kb) and 18S (1.7 kb) ribosomal bands are indicated.

ated human megakaryocyte RNA and also found only the 1.1-kb mRNA species (Fig. 1 B). The megakaryoblastic cell line CHRF-288 cells contained no detectable GPIIb/IIIa mRNA when untreated, but when treated with either DMSO (1.25% final concentration) or the phorbol ester PMA (100 nM final concentration) for 4 d, GPIIb/IIIa mRNA expression was induced. As shown in Fig. 1 B, both the 3.5- and the 1.1-kb mRNA species were detected in CHRF-288 cells, using the HEL cell-derived GPIIb/IIIa cDNA as a probe.

GPIIb/IIIa protein in HUVEC and platelets. To determine whether the larger HUVEC GPIIb/IIIa mRNA species encodes a

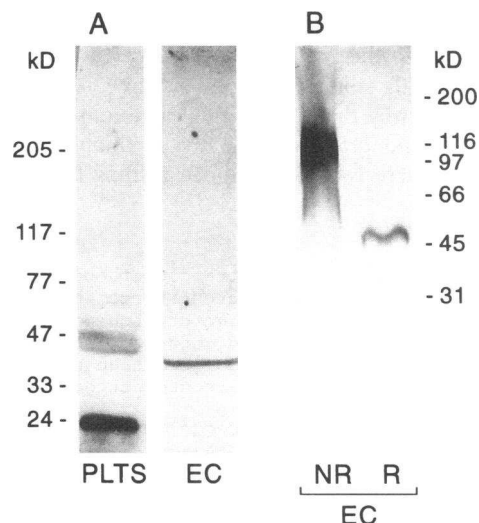


Figure 2. Western blot analysis of platelet and HUVEC GPIIb/IIIa. (A) Triton X-100 extracts of washed platelets (PLTS) and HUVEC (EC) were subjected to SDS-PAGE under reducing conditions, or (B) EC proteins selected by wheat germ agglutinin and ion exchange chromatography were subjected to SDS-PAGE under reducing (R) or nonreducing (NR) conditions, transferred to nitrocellulose, incubated with a polyclonal anti-GPIIb/IIIa antibody, and developed with peroxidase-labeled goat anti-rabbit IgG. The locations of molecular mass size markers in kD are shown on the side of each blot.

larger protein, HUVEC protein was evaluated by Western blotting. Using a polyclonal anti-human platelet GPIIb/IIIa antibody, an \sim 45-kD protein was found in HUVEC versus the \sim 24-kD protein seen in platelet extracts (Fig. 2 A) when evaluated under reducing conditions. Partially purified EC GPIIb/IIIa (see Methods) was subject to Western blot analysis under reducing and nonreducing conditions (Fig. 2 B). A broad band of \sim 116 kD was observed under nonreducing conditions. Although intact GPIIb/IIIa is \sim 143 kD, little of this is isolated from EC, even in the presence of protease inhibitors (18). Thus the cross-reacting EC band probably represents GPIIb/IIIa linked to GPIIb/IIIa degradation products.

Isolation of endothelial GPIIb/IIIa cDNA. To explore the differences between platelet and EC GPIIb/IIIa, EC GPIIb/IIIa cDNA was cloned from a random-primed HUVEC cDNA library. Greater than 5×10^6 recombinant phage were screened using radiolabeled HEL cell-derived GPIIb/IIIa cDNA (8) as the probe. Three clones were plaque purified, and their DNA sequence was determined.

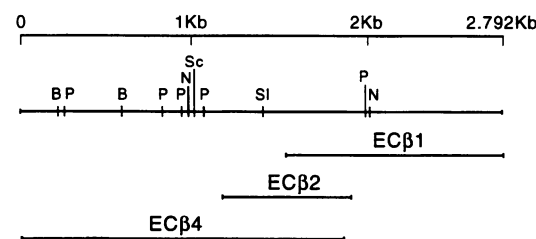


Figure 3. EC GPIIb/IIIa cDNA restriction map. Restriction sites are included for: B, BamHI; P, PstI; N, NcoI; Sc, SacI; SI, SalI. The relative positions of clones ECβ1, ECβ2, and ECβ4 are shown. The figure is drawn from 5' to 3' (left to right) with respect to the coding sequence, and the size, in kb, is indicated at the top.

The three isolated clones were sequenced in their entirety and are illustrated in Fig. 3. The complete sequence and putative translation product is illustrated in Fig. 4. There is a long 5' untranslated region with stop codons in all three reading frames. This is followed by three "in frame" methionines at nucleotides 637, 658, and 715. Using the first methionine as the initiation codon, there is a 1233-nt open reading frame.

The HEL cell cDNA sequence, as reported by Lopez et al. (8), is contained entirely within the EC sequence, as illustrated in bold type in Fig. 4. Our DNA sequence of this region was identical to that reported by Lopez et al. (8) except for nt 1275 (HEL cell sequence nt 46) which is a "C" in our clones and a "G" in the HEL cell sequence and does not change the amino acid encoded.

5'ctttattctcagcaccacccctccaggtcattgtgtctgtttccgaggggctggaacgtagccccgccagctggccctctctgaccttgggggatc	101
aggagcgaagttggcgaggacttcagagatccgctcccttacccttccccgcccgagcgtcacagcaccacacccagggccctgctctggcaggcag	204
gcaaaagctaggcagaagaggattccagagatccctgggtctgttccctgccccagtagctgcagaacggacttgggagccctccttggctgctcccggggtc	307
accagcgagtgctgagaccatttctgtcgaggcgggccgagcttcccttatccccagacgcctagcgggaggggttgggtggaatcaaatgggagccc	410
tcagacataaggaggccagaggtcgaaggagcggggtcgtgaccgcttacacccttctccacagcccgccgacctggaggggccccccggggcactgg	513
gcggtagccacctcctggcaactctcggtgcgctccctcgctcgaggcctcttctccccagaccgctgtggtgtgccgggactcctgagcctaggc	616
ctcccgatgttccacccgc	636
ATG ATC CCT TCC CGC CAC ACG ATG CTC CGT TTT CTT CCC GTT GTG AAT GCC GCG TCC TGT CCT GGT GAC AGG AGA ACA	714
Met Ile Pro Ser Arg His Thr Met Leu Arg Phe Leu Pro Val Val Asn Ala Ala Ser Cys Pro Gly Asp Arg Arg Thr	26
ATG TTG GTG AAC GTC GCA GCG GGT GTC CGA GTG CTC CGT GTG CCC CTG AGA GCG GGT GGG AGC GGA AGC CTG AGC GGC	792
Met Leu Val Asn Val Ala Ala Gly Val Arg Val Leu Arg Val Pro Leu Arg Ala Gly Gly Ser Gly Ser Leu Ser Gly	52
CTG CGG CCT CCG GCG ATA GTG TGC TAT CTG CCG CTG CAG CGC GCG TCC GCG GCC TCT GGG CTA TTT CTG GCC AGG CCG	870
Leu Arg Pro Pro Ala Ile Val Cys Tyr Leu Pro Leu Gln Arg Ala Ser Ala Ala Ser Gly Leu Phe Leu Ala Arg Pro	78
CAG CAC TGT GGT CGG TGC GGG CGT GGC AGG GGC GGG GCG GCC TTA TCG CTC GGC TCT CCC GCC TAC GCC TCC CGC TGC	948
Gln His Cys Gly Arg Cys Gly Arg Gly Arg Gly Ala Ala Leu Ser Leu Gly Ser Pro Ala Tyr Ala Ser Arg Cys	104
AGA GTA AGC CCG GCT GCC GTC TTC TCG CCA TGG GCT CCG GTG AGT CTG GAG TCC GGT CGG GCC CCC GGC TGC TCC CTA	1026
Arg Val Ser Arg Ala Ala Val Phe Ser Pro Trp Ala Pro Val Ser Leu Glu Ser Gly Arg Ala Pro Gly Cys Ser Leu	130
GGC CGA CCC GGG TTG AGA GGA GCT CTG GTC GTT TGG CTG CAG CTG GGA GAG ACT TGG GTC AGA CTT AGA GGG GAC TTC	1104
Gly Arg Pro Gly Leu Arg Gly Ala Leu Val Val Trp Leu Gln Leu Gly Glu Thr Trp Val Arg Leu Arg Gly Asp Phe	156
CAG CCG GCG TGC GGG GTG GTC AGG GTG GAG AGG CTG GCG GGC TAC CGG GAC GCC GGG CAT CAG GGG CTG GAT GGA GCC	1182
Gln Pro Ala Cys Gly Val Val Arg Val Glu Arg Leu Ala Gly Tyr Arg Asp Ala Gly His Gln Gly Leu Asp Gly Ala	182
GGG CCG GCA GTC TGG GTA CTC AGA GAT GTC GCC CAG GTG CCC GCC GAC CGC TCG GCT TAC TGC GGC GCT TCC CTT GCA	1260
Gly Pro Ala Val Trp Val Leu Arg Asp Val Ala Gln Val Pro Ala Asp Arg Ser Ala Tyr Cys Gly Ala Ser Leu Ala	208
GGG CCG CGC GGG GCC CTG AGC TTA CTG CTC CTG CTG CTG GCC CCG CCG AGC CGC CCG GCC GCA GGT TGC CCG GCG CCC	1338
Gly Pro Arg Gly Ala Leu Ser Leu Leu Leu Leu Leu Leu Ala Pro Pro Ser Arg Pro Ala Ala Gly Cys Pro Ala Pro	234
TGT AGC TGC GCG GGG ACG CTC GTG GAC TGC GGG CGC CGC GGG CTG ACT TGG GCC TCG CTG CCG ACC GCC TTC CTT GTC	1416
Cys Ser Cys Ala Gly Thr Leu Val Asp Cys Gly Arg Arg Gly Leu Thr Trp Ala Ser Leu Pro Thr Ala Phe Pro Val	260
GAC ACA ACC GAG CTG GTG CTG ACC GGC AAC AAC CTG ACG GCG CTG CCG CCG GGG CTG CTG GAC GCG CTG CCC GCG CTG	1494
Asp Thr Thr Glu Leu Val Leu Thr Gly Asn Asn Leu Thr Ala Leu Pro Pro Gly Leu Leu Asp Ala Leu Pro Ala Leu	286
CGC ACC GCA CAC CTG GGC GCC AAC CCC TGG CGC TGC GAC TGC CGC CTT GTG CCG CTG CGC GCC TGG CTG GCC GGC CGC	1572
Arg Thr Ala His Leu Gly Ala Asn Pro Trp Arg Cys Asp Cys Arg Leu Val Pro Leu Arg Ala Trp Leu Ala Gly Arg	312
CCC GAG CGT GCG CCC TAC CGC GAC CTG CGT TGC GTG GCG CCC CCA GCG CTG CGC GGC CGC CTG CTG CCC TAT CTG GCC	1650
Pro Glu Arg Ala Pro Tyr Arg Asp Leu Arg Cys Val Ala Pro Pro Ala Leu Arg Gly Arg Leu Leu Pro Tyr Leu Ala	338
GAG GAC GAG CTG CGC GCC GCT TGC GCT CCC GGC CCG CTC TGC TGG GGG GCG CTG GCG GCG CAG CTT GCG CTG CTG GGC	1728
Glu Asp Glu Leu Arg Ala Ala Cys Ala Pro Gly Pro Leu Cys Trp Gly Ala Leu Ala Ala Gln Leu Ala Leu Leu Gly	364
CTT GGG CTG CTG CAC GCG TTG CTG CTG GTG CTG CTG CTG TGC CGC CTG CCG AGG CTG CCG GCC CCG GCC CGC GCT CGC	1806
Leu Gly Leu Leu His Ala Leu Leu Leu Val Leu Leu Leu Cys Arg Leu Arg Arg Leu Arg Ala Arg Ala Arg Ala Arg	390
GCC GCA GCC CCG CTG TCG CTG ACC GAC CCG CTG GTG GCC GAG CGA GCC GGA ACC GAC GAG TCC tgaggagagaacccggtgcg	1888
Ala Ala Ala Arg Leu Ser Leu Thr Asp Pro Leu Val Ala Glu Arg Ala Gly Thr Asp Glu Ser	411
tcctgaggagagaacccggcgctgggcaacacgggctgcaaaactgcagaggacctgcccagggggccctcgcccaacctggacgggtccccgcctctccg	1991
ctgcccactctctcagaccaccccccacctgcagggccagaccagctgggacagaaactcctgcccacctaccocaggggaggogaacccgcacttccaggtt	2094
gggagggaccatggggcacaatcggtgtccagaccctgctgctgctcccttccaaactctggtgctgaataaaccttctgatctggtcttctctcgcacgactga	2197
cctggaaattccctcgccaaactcaggaagggcccgaggagcggttcaaggggacacaaacacatcctcacatgccaccctaacctcaggggcagacag	2300
gagtcagtggtgacctctcccttccagacacactcccaaaaggccacatgggaagggcatgagagagatgctaagtataacgtggggggcagtgaggacgt	2403
tgtggggacactgaggaccctgtggggcgagtgaggatgctgtggggatgctgtggggacagtaaggacactgggggcagtgaggacgctgtggacactgcgg	2506
acgctatggggacaatgacgctgtggacagtgaggacgctgtggggcggtgaggacactgtggagtgaggacactgggggcagtgaggacgctgtggacagt	2609
ggggcgctgtggggcggtgaggacgctgtaggggcggtgaggacgctgtggacggtgagaacgctgtggggacactgaggatgctgtgaggacgctgtggg	2712
ggcagtgaggacgctgtggaccgtgaggacgctgtggggcgagtgaggacgctgtggaccgtgaggacgctgtggggatgg 3'	2793

Figure 4. EC GPIIb cDNA sequence. The cDNA sequence is shown. Nucleotide sequence is indicated at the right at the end of each line, and amino acid residue numbers, beginning with the putative initiator methionine at nt 637, are indicated at the right immediately below the nucleotide number. Nucleotides and amino acids highlighted in bold are identical to the reported HEL cell sequence. The HEL cell signal peptide cleavage point is indicated by Δ . The putative transmembrane domain for the EC and HEL peptides (8) is underlined. The putative phosphorylation site (5) is indicated by *. The sequence has been submitted to GenBank, accession number 20860.

The first 32 nt of the HEL cell sequence correspond to EC sequence nt 956–987, however the EC sequence is not in the same reading frame as in the HEL cell sequence. In the EC sequence these 32 nt are followed by 274 nt not found in the HEL cell cDNA (nt 988–1261), and then the sequence is again identical, resuming with HEL cell nt 33 (EC nt 1262) in the same reading frame as found in the HEL cell sequence.

Using the first in frame methionine as the initiation codon, the EC sequence gives a predicted translation product of 411 amino acids with a calculated molecular mass of 43 kD. From amino acid 209 through the carboxy terminus of the EC protein, it is identical to the HEL cell translation product, amino acids 4–206. The HEL cell translation product contains a 25-amino acid signal peptide at its NH₂ terminus, which is cleaved to produce the mature protein. There are no sequences suggestive of a signal peptide at the 5' end of the EC protein (27). Much of the signal peptide sequence of the HEL cell protein is retained within the EC sequence and may serve as an internal signal peptide. Computer assisted analysis (28, 29) predicts only one transmembrane region, identical to the one in the HEL cell sequence.

Analysis of the GPIIb gene. The genes encoding GPIIb α , GPIIb β , and GPV are simple in structure, with the coding regions contained within a single exon (13, 14, 30, 31). To evaluate the GPIIb β gene, human genomic DNA was cut with the restriction enzymes BamHI, NcoI, and PstI, which all cut within the EC GPIIb β cDNA (see Fig. 3), and analyzed in duplicate by Southern blotting using both EC β 4 and the HEL cell-derived GPIIb β cDNA as probes. The size of the fragments predicted from the cDNA were detected in genomic DNA as illustrated in Fig. 5. We also amplified nt 715–1218 and 1276–2168 using PCR to amplify genomic DNA. The sizes predicted from the cDNA were found in genomic DNA and are illustrated in Fig. 5. To confirm that the amplified bands were GPIIb β , the PCR products were cut with the restriction enzymes SacI and SalI, respectively, and the appropriate-sized products were obtained. Taken together these data suggest that at least nt 266–2102 are contained within one exon.

To confirm that the cDNA sequence present in the endothelial transcripts, but apparently spliced out in megakaryocyte

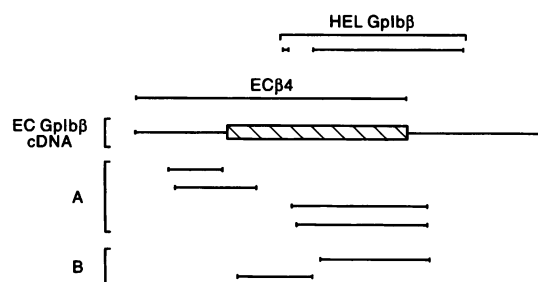


Figure 5. Evaluation of the GPIIb β gene using Southern blot analyses and PCR. 10 μ g of genomic DNA was cut with BamHI, NcoI, or PstI and analyzed in duplicate by Southern blotting using either the HEL cell-derived or the EC GPIIb β clone EC β 4 as the probe. A drawing of the EC GPIIb β cDNA is shown in the middle of the figure. Above this the areas included in the probes are indicated. The fragments predicted by restriction enzyme analysis of the cDNA sequence, which were the same size detected by Southern blot analysis, are designated in A. For B, PCR primers were devised from GPIIb β cDNA sequence and used to amplify genomic DNA. The size fragments predicted from the cDNA were found in genomic DNA and their locations are indicated.

and HEL cell transcripts, is present in genomic DNA, we amplified by PCR nt 961–1446 from genomic DNA prepared from peripheral blood white blood cells. DNA sequence analysis through both presumed intron–exon boundaries confirmed their presence in genomic DNA.

The GPIIb gene maps to chromosome region 22pter \rightarrow 22q11.2. The radiolabeled human cDNA probe EC β 1 detects one human EcoRI restriction fragment of \sim 25 kb. 14 rodent–human hybrid DNAs, each carrying a few or many human chromosomes so that most human chromosome regions were represented, were tested for the presence of the GPIIb β -specific human EcoRI fragment by filter hybridization to the radiolabeled cDNA probe. All hybrids carrying human chromosome 22 exhibited the strongly hybridizing 25-kb human EcoRI band, while all hybrids without human chromosome 22 were negative for the human fragment. Thus the human GPIIb β gene is located on chromosome 22 (data summarized in Fig. 6). A chromosome 22 hybrid mapping panel, in which each hybrid DNA carries a specific portion of chromosome 22 (26), was tested similarly for the presence of the GPIIb β fragment. Only hybrids that retained 22pter \rightarrow 22q11.2 tested positive for the GPIIb β gene; a drawing of the most relevant hybrids is shown in Fig. 7.

GPIIb mRNA expression in different tissues. To evaluate the pattern of GPIIb α , GPIIb β , and GPIX mRNA expression, we probed a Northern blot containing poly (A)⁺ RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas with cDNA probes for GPIIb α , GPIIb β , and GPIX as illustrated in Fig. 8. When probed with full length HEL cell-derived GPIX cDNA, a faint 1-kb mRNA species was seen only in the RNA from lung and placenta. When re-probed with full length HEL cell-derived GPIIb α cDNA, a 2.5-kb mRNA species was detected also in lung and placental RNA, and upon longer exposure, faintly in liver. However, when the blot was probed with full length HEL cell-derived GPIIb β cDNA (EC nt 956–988; 1262–2179), the 3.5- and the 1.1-kb mRNA species were seen in heart, and the 3.5-kb mRNA species was seen in brain, both at a much greater relative abundance than the levels of GPIIb α and GPIX mRNA detected in lung and placental RNA. The two GPIIb β mRNA species, in varying relative abundance, were also detected at lower levels in the other tissues. When the blot was re-probed with an EC-specific probe (the 367-bp BamHI fragment of EC β 4, nt 233–600), the 3.5-kb mRNA species and, in addition, an \sim 2.3-kb species were detected predominantly in heart and brain, but also at a lower relative abundance in other tissues (Fig. 8).

Discussion

GPIIb β is part of a four protein complex in platelets that, through the GPIIb α chain, serves as a receptor for von Willebrand factor and thrombin. This platelet complex plays a major role in platelet–subendothelial interactions. Patients lacking this complex have a moderate to severe bleeding disorder, the Bernard-Soulier syndrome. However, the expression of GPIIb α , GPIIb β , and GPIX is not limited to platelets and megakaryocytes. GPIIb α and GPIX mRNA and protein are expressed in EC and appear to be identical in EC and platelets (17, 18, and Konkle, B. A., F. J. Meloni, and S. S. Shapiro, unpublished observations). GPIIb β mRNA and protein species are different in EC from those found in platelets and HEL cells, as we have illustrated in this paper.

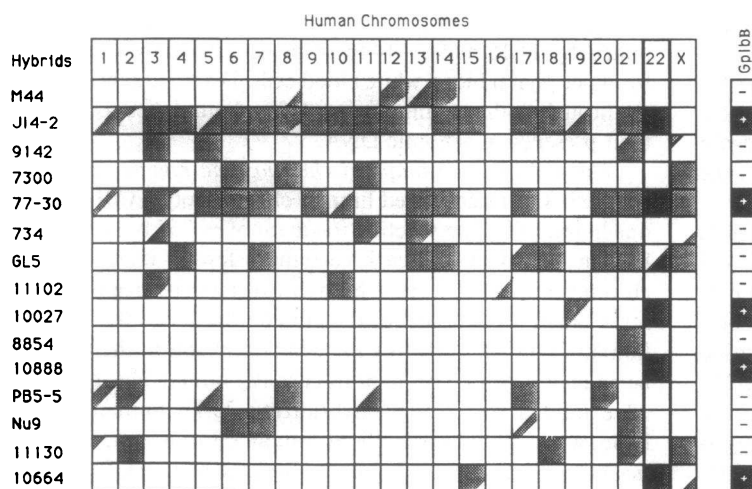


Figure 6. Presence of the GPIb β locus in a panel of 14 rodent-human hybrids. ■ indicates that the hybrid named in the left column contains the chromosome indicated in the upper row; ▨ indicates presence of the long arm of the chromosome (or part of the long arm represented by a smaller fraction of stippling); □ indicates presence of the short arm (or partial short arm) of the chromosome; □ indicates the absence of the chromosome listed above the column. The column for chromosome 22 is boldly outlined and stippled to highlight correlation of presence of this chromosome with presence of the GPIb β gene. The pattern of retention of the gene in the panel is shown to the right of the figure where presence of the locus in a hybrid is indicated by a stippled box with a plus sign, and absence of the locus is indicated by an open box enclosing a minus sign.

EC GPIb β cDNA is approximately three times the size of HEL cell and platelet species. Some of this difference is due to additional 5' and 3' untranslated (UT) regions not present in HEL cell cDNA. Our clones encompass 2.8 kb of cDNA, and thus additional 5' and 3' UT sequences are likely present in the full length cDNA. The EC coding region is approximately twice that of HEL cells. The predicted EC translation product of 43 kD fits well with the protein size found on Western blot analysis of HUVEC proteins.

We are not certain which of the three in frame methionines present at the beginning of the coding region serves as the translation initiation site. Translation does not initiate with the ATG used for the HEL cell sequence (located at EC nt 956), since, with the unique EC sequence that follows that ATG, there would be no long open reading frame. In addition, since a polyclonal anti-GPIb β antibody recognizes both the EC and platelet proteins, they must share a common region, and this homology can only occur if one of the upstream initiation sites is used. Studies are currently underway to obtain amino-terminal protein sequence to confirm the translation initiation site. We have used the first methionine as the assumed initiation site since this is true for 95% of mRNAs reviewed by Kozak (32). However, the second methionine at nt 658 fits the consensus sequence proposed by Kozak (33) for translation

initiation better than the first and may serve as the translation initiation site.

EC GPIb β contains an unusually long 5' UT region. cDNAs with long leader sequences and multiple ATG codons have been recognized most commonly in oncogenes, growth factors, transcription factors, signal transduction components, and receptor proteins (34, 35). These frequently have G-C-rich leader sequences (70–90% GC), implying considerable secondary structure. The EC GPIb β 5' UT contains 66% G + C. The entire cDNA for the HEL cell and the EC species is relatively G-C rich, containing 73 and 68% G + C, respectively.

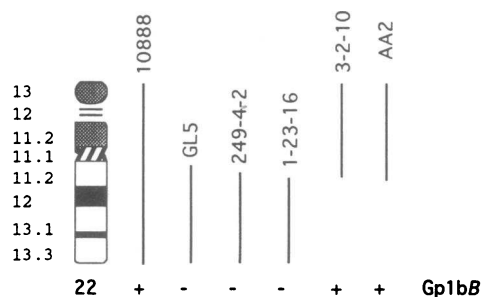


Figure 7. Regional localization of the GPIb β gene in rodent-human hybrids carrying partial chromosome 22. Hybrids carrying chromosome 22 or a region of 22 are illustrated to the right of the 22 ideogram, with results of filter hybridization to the GPIb β -specific probe indicated below the lines representing specific hybrids; the GPIb β locus is present in hybrids which retain the region 22pter → 22q11.2 in common. Hybrid GL5 retains a derivative 17 chromosome from a constitutional t(17;22)(p13;q11.2) translocation break. All these hybrids were described in detail previously (26).

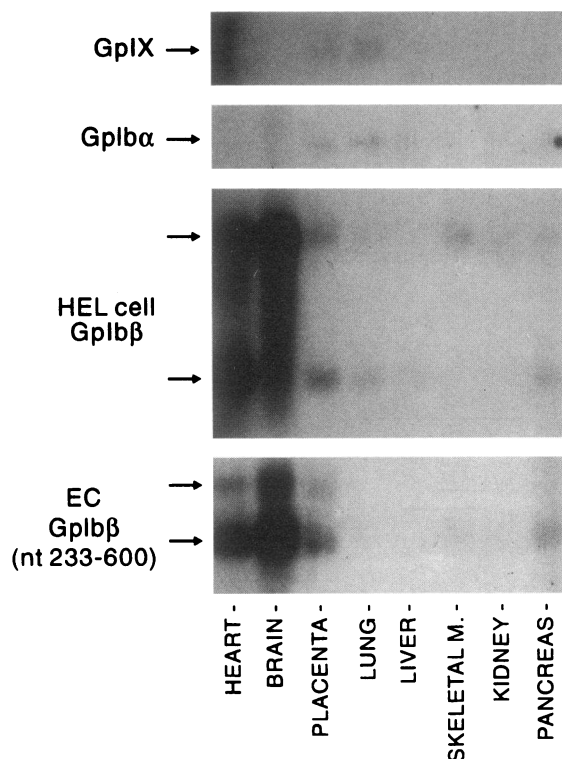


Figure 8. Northern blot analysis of various human tissues for GPIb α , GPI β , and GPIb β mRNA expression. A Northern blot containing 2 μ g of poly (A)⁺ RNA from the tissues indicated was probed, stripped of radiolabeled probe, then reprobated with the cDNAs indicated on the left side of the figure. For the EC-specific GPIb β probe, the BamHI fragment (nt 233–600) of EC β 4 was used.

There are no sequences suggestive of a signal peptide at the 5' end of the GPIIb β coding sequence. Much of the coding sequence which serves as the signal peptide for platelet and HEL cell GPIIb β is present in the middle of the EC GPIIb β cDNA sequence. This region may serve as an internal signal peptide for EC GPIIb β . Internal signal peptides are common in proteins that have multiple transmembrane regions, but may also be seen in proteins with only one membrane-spanning region (36).

EC GPIIb β appears to have the same transmembrane region as that found in platelets and HEL cells. The cysteines external to the membrane in platelet GPIIb β , one of which is probably involved in the disulfide-mediated linking of GPIIb β to GPIIb α (8), are present in the EC sequence. Western blot analysis of unreduced HUVEC protein reveals a larger species than that obtained in reduced gels, and we have successfully purified EC GPIIb β from HUVEC protein using an affinity column containing the monoclonal antibody to GPIIb α , AP-1, suggesting that at least some GPIIb β is disulfide linked to GPIIb α in EC also.

GPIIb α , GPIIb β , GPIX, and GPV are known to be members of the leucine-rich glycoprotein family. GPIIb α contains 7 tandem LRG repeats of 24 amino acids each, while HEL cell GPIIb β and GPIX each contain 1, and GPV contains 15 LRG repeats (2, 13, 14). EC GPIIb β contains the one repeat common to the HEL cell species, but no additional ones in the unique amino-terminal region. The function of LRG repeats in these proteins as well as in the other proteins in which they are found is unknown, although they may play a role in adhesion (2).

When the EC cDNA sequence was compared with other sequences accessible through GenBank and EMBL databases, sequence similarity was found to other cDNAs encoding proteins with LRG repeats. These similarities were within the regions common to HEL cell and EC cDNA. Sequence similarity with the cDNA unique to EC was found with two previously reported expressed sequence tags (EST), both cloned from brain cDNA (37, 38). Both were obtained by rapid, single pass sequencing methods. One 248-bp clone (EST 01258) was obtained from a human hippocampal library (37) and is nearly identical to EC GPIIb β nt 2467–2714, located in the 3' UT region. The other clone (IB616) was obtained from an infant brain cDNA library (38), is 132 nt in length, and is nearly identical to EC GPIIb β nt 1174–1301. This is located within the EC coding sequence. These ESTs most likely represent the same transcript as the EC GPIIb β cDNA, as both were reported with a number of undetermined nucleotides (six each in EST 01258 and IB616), and the differences in sequence between the ESTs and EC GPIIb β cDNA (2/248 in EST 01258 and 5/132 in IB616) may represent sequencing errors or possibly polymorphisms.

The GPIIb β gene, with two or possibly three alternatively processed mRNA species, still appears to be quite simple in structure, as are genes for GPIIb, GPIX, and GPV. The GPIIb α , GPIX, and GPV genes have been cloned and, in each case, the coding region is contained within one exon (13, 14, 30, 31). The EC GPIIb β cDNA is also contained within one exon, and the HEL cell and platelet species are produced by splicing out the 274-nt EC sequence, nt 987–1261. This EC sequence has perfect splice acceptor and donor sequences at its 5' and 3' ends (39).

ECs appear to contain only the larger 3.5-kb GPIIb β mRNA species, while HEL cells and platelets contain only the smaller 1.1-kb mRNA species. We do not know whether the multiple mRNA species seen in the tissues reflect alternative expression

in different cells or within a single cell type. However, it is interesting that the megakaryoblastic cell line CHRF-288 expresses both mRNA species. CHRF-288 cells were derived from a solid tumor in an infant with megakaryoblastic leukemia and have retained some characteristics of the original solid tumor, including production of basic fibroblast growth factor and TGF- β (40). The larger mRNA species seen in these cells, but not in normal megakaryocytes, may be due either to this solid tumor phenotype or, possibly, to the malignant phenotype of these cells. Of note, HEL cells derived from a fluid phase leukemia (41) do not express the larger mRNA species.

When we probed the human tissue blot with the 5' end of the endothelial cDNA, we observed a third mRNA species of ~ 2.3 kb. This was not detected using the HEL cell-derived probe, even under conditions of low stringency, suggesting that the gene extends 5' from our sequence. Part or all of the lung 5' UT region of the EC GPIIb β cDNA may contain part of this mRNA species, although additional processing would be needed as there is not a long open reading frame in this area. We have not detected the 2.3-kb mRNA species in EC, HEL cell, or CHRF-288 cell RNA (data not shown).

In patients with the Bernard-Soulier syndrome, GPIIb α , GPIIb β , GPIX, and GPV are all missing or affected. We now know that they are products of separate genes. GPIIb α and GPIX are located on chromosomes 17 and 3, respectively (30, 31), and, as we have shown, GPIIb β is located on chromosome 22. The molecular defects present in patients with Bernard-Soulier syndrome are now being elucidated. Three different mutations in GPIIb α have been reported in three different patients (42–44), and a family with two mutations in GPIX producing a double heterozygous state has been reported (45). No mutations have been reported to date in GPIIb β .

It appears from our studies that GPIIb α and GPIX may not need to be present for GPIIb β expression, at least at the mRNA level, since we found relatively abundant GPIIb β mRNA expression in heart and brain (and less abundant expression in other tissues), while GPIIb α and GPIX mRNA species were detected only in placenta and lung. Considering that placenta and lung are the most vascular of those tested, this may represent EC expression. In situ hybridization and immunohistochemistry on tissue sections will be needed to confirm this. In collaboration with Asch and Nachman, we previously performed in situ hybridization and immunohistochemistry on tonsillar tissue and have found that GPIIb α appeared to be expressed only in the ECs in that tissue (17). While GPIIb β mRNA expression could represent endothelial expression in different vascular beds, this is unlikely given the pattern of distribution and the expression of both mRNA species.

The broad distribution of GPIIb β mRNA in different tissues suggests that it may be playing a role different than or additional to its function in platelets. Other than its presence being required for stable expression of GPIIb α , the function of GPIIb β in platelets is not known. GPIIb β protein is phosphorylated under conditions that increase cAMP and may play a role in mediating platelet shape change through GPIIb α which is linked to actin-binding protein (5). Whether GPIIb β serves as a signal transduction molecule for other proteins or binds additional ligands itself remains to be explored.

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