Ancient Ubiquitous Protein 1 Binds to the Conserved Membrane-proximal Sequence of the Cytosplasmic Tail of the Integrin α Subunits That Plays a Crucial Role in the Inside-out Signaling of αIibβ3*

Modification of the cytoplasmic tails of the integrin αIibβ3 plays an important role in the signal transduction in platelets. We searched for proteins that bind to the αIib subunit using the yeast two-hybrid assay with a cDNA library of the megakaryocyte-derived cell line and identified a protein, ancient ubiquitous protein 1 (Aup1), that is ubiquitously expressed in human cells. Observation of UT7/TPO cells expressing a red fluorescent protein-tagged Aup1 indicated its localization in the cytoplasm. Immunoprecipitation of UT7/TPO cells with a cDNA library of the megakaryocyte-derived cell line revealed specific binding of Aup1 to the membrane-proximal sequence (KVGFFKR) that is conserved among the integrin α subunits and plays a crucial role in the αIibβ3 inside-out signaling. Aup1 possesses domains related to signal transduction, these results suggest involvement of Aup1 in the integrin signaling.

Integrin αIibβ3 (GPIIb-IIIa) is one of the receptors on the cellular surface of platelets and megakaryocytes. It binds to various adhesive proteins including fibrinogen, von Willebrand factor, vitronectin, and fibronectin that contain a core amino acid sequence of arginine-glycine-aspartic acids (RGD). Binding of fibrinogen to αIibβ3 leads to platelet aggregation and finally to thrombus formation at the injured vascular sites. A pivotal role of αIibβ3 in hemostasis is supported by the clinical observation that the congenital deficiency of αIibβ3, Glanzmann’s thrombasthenia, results in lifelong bleeding tendency (1). Whereas αIibβ3 on resting platelets does not bind soluble fibrinogen, once platelets are activated, conformation of the extracellular domains of the αIibβ3 is altered and its ligand-binding affinity is increased (affinity modulation) (2). This process of the inside-out signaling is considered to be mediated by modification of the short cytoplasmic tails of αIib and β3 subunits; however, the mechanism remains to be elucidated.

The nuclear magnetic resonance structural analysis of the αIib cytoplasmic tail revealed a closed conformation where the highly conserved N-terminal membrane-proximal region forms an α-helix followed by a turn, and the acidic C-terminal loop interacts with the N-terminal helix (3). Deletion of almost the entire αIib-cytosolic tail and mutations in its N-terminal sequence (GFFKR) conserved among the integrin α subunits enhanced the affinity of αIibβ3 for ligands (4–6). The cytoplasmic tail of the β3 subunit also has an amino acid sequence that is conserved among integrin β subunits: a stretch of 8 amino acids (KLFITHHDD) adjacent to the transmembrane domain. In a similar fashion to the αIib subunit, deletion or mutation in this conserved region induces activation of αIibβ3 (6, 7). These observations suggest that membrane-proximal regions of the cytoplasmic domains of both subunits exert a negative regulatory function and lock αIibβ3 in a low affinity state. Negative regulation may be mediated by the interaction between αIib and β3 cytoplasmic tails, possibly through a salt bridge between Arg-995 in αIib and Asp-723 in β3 (6), or binding of intracellular proteins to αIib and/or β3 subunits. Two candidates for the modulator proteins have been reported: calcium- and integrin-binding protein (CIB)1 (8) and β3-endonexin (9, 10), which bind to αIib and β3 cytoplasmic tails, respectively. Although CIB is unlikely to have a regulatory effect on αIibβ3 ligand binding function (11), β3-endonexin fused to GST protein induces the conformational change of αIibβ3 and activates it when co-transfected with αIib and β3 subunits in Chinese hamster ovary cells. Another mechanism of modification has been recently suggested: an interaction between cytoplasmic tails of αIibβ3 and the actin cytoskeleton. αIibβ3 and the actin cytoskeleton are physically linked by binding of talin to the β3 cytoplasmic tail (12), and αIibβ3 in resting platelets may be constrained in a low affinity state by the actin cytoskeleton (13). An increase in the cytosolic calcium evoked by agonist stimulation initiates actin filament turnover and may lead to relief of the cytoskeletal

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§ To whom correspondence should be addressed. Tel.: 81-3-3813-3111; Fax: 81-3-3813-0841; E-mail: atkato@med.juntendo.ac.jp.
Aup1 Binds to the Integrin α Cytoplasmic Tails

constraints on α6β4, resulting in a high affinity state of α6β4. Among these possible modification mechanisms, which one works in platelets remains to be determined.

In contrast, binding of fibrinogen to α6β4 leads to platelet shape change, release of granules, and platelet aggregation. These sequential biological phenomena are mediated by the outside-in signaling of α6β4, calcium mobilization, increase in the cytoplasmic pH, thromboxane A2 generation, and tyrosine phosphorylation of intracellular proteins including focal adhesion kinase and members of the Src family proteins (1). These signaling proteins are complexed with the actin cytoskeleton and are recruited to the focal contacts. In this process, the β3 cytoplasmic tail (residues 740-762) binds to the adaptor proteins She and Grb2 when tyrosine residues (Tyr-747 and Tyr-759) are phosphorylated (14). In addition, the β3 cytoplasmic tail is involved in clot retraction by transmitting the contractile force evoked by the rearrangement of the cytoskeletal proteins to the extracellular matrix (15). Thus, several proteins that bind to and modify the α6β4 cytoplasmic tail.

In this study, we searched for proteins that bind to the α6β4 cytoplasmic tail in the thrombopoietin-dependent acute megakaryocytic leukemia-derived cell line, UT7/TPO (16), by the yeast-two-hybrid assay and identified a protein, Aup1, that binds to the conserved membrane-proximal sequence of the cytoplasmic tail of the integrin α subunits.

EXPERIMENTAL PROCEDURES

Cell Lines—UT7/TPO cell line (16) was maintained with Iscove’s modified Dulbecco’s medium supplemented with 20% fetal calf serum (FCS) (Prairie Farm Bureau Bank, DM, USA) and thrombopoietin (Thrombopoietin BP, KVGFFKR) (Stallion, Iwaki, Japan). Cell lines including HL 60, K562, U937, Jurkat, Raji, and CMK (17), established from acute myelocytic leukemia, Burkitt (B cell) lymphoma, and acute megakaryocytic leukemia, respectively, were maintained with RPMI medium supplemented with 10% FCS. Other cell lines including 293, MCF7, A549, HeLa, and HepG2, established from embryonic kidney, breast carcinoma, lung carcinoma, epithelial cervical carcinoma, and hepatocarcinoma, respectively, were maintained with Dulbecco’s modified Eagle’s medium supplemented with 10% FCS.

Amplification of the cDNA Sequence for the Cytoplasmic Domain of Integrin α and β Subunits by PCR—The cDNA sequences for the cytoplasmic domains of various integrin α and β subunits were amplified by reverse transcription-PCR from RNA extracted from UT7/TPO, α1, α2, α5, and β3, HepG2 for α1, Raji for α5, K562 for α2, and HL60 for β1 and β2, respectively. The cDNA sequences for a mutant α6β4 (P992A) and for membrane-proximal (α6β4 MP, KVGFFKR) and membrane-distal (α6β4 MD, NRPPPLEDEEGEE) segments of the α6β4 cytoplasmic tail were amplified using the normal α6β4 cytoplasmic tail cDNA. The cDNA sequence of each cDNA fragment amplified by PCR was confirmed using the ABI Prism dRhodamine terminator cycle sequencing ready reaction kit (Applied Biosysyms Japan, Tokyo, Japan).

Yeast Two-hybrid Assays—A cDNA library was constructed by ligating cDNA synthesized from UT7/TPO RNA to a pAD-Gal4 vector (Stratagene, La Jolla, CA). The cDNA sequence for the cytoplasmic domain of the integrin α6β4 subunit was ligated in-frame to a pBD-Gal4 vector (Stratagene). Procedures for screening and the filter lift assay to confirm interactions between the bait and target proteins were according to the manufacturer’s instructions. Briefly, yeast YRG-2 cells were transformed with a pAD-Gal4 plasmid encoding the bait, then yeast cells were plated on selective SD agar plates without leucine, tryptophan, and histidine (Leu “Trp” “His” ). Colonies grown on the selective plates, indicating interactions between target and bait proteins, were subjected to the filter lift assay to examine the β-galactosidase activity to confirm the interaction. Positive yeast colonies were transferred to Whatman filter papers, frozen in the liquid nitrogen, thawed, and incubated with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (0.3 mg/ml) in Z buffer (per liter, 16.1 g of NaH2PO4/7H2O, 5.5 g of Na2HPO4/7H2O, 0.75 g of KCl, 0.246 g of MgSO4/7H2O, and 2.7 ml of 2-mercaptoethanol; pH 7.0). Colonies that produced blue color were picked up for the subsequent experiments.

Quantitative β-Galactosidase Assay—To compare interactions between the target protein and the cytoplasmic domains of various integrin subunits, the quantitative β-galactosidase assay (18) was performed. Briefly, yeast cells grown in 5 ml of medium at 30 °C until the near log phase (O.D.600 = 1.0) were resuspended in Z buffer (200 µl) and incubated at 25 °C for 1 h. After one cycle of freeze-thawing, 1 ml of 0.1 M sodium phosphate and acid-washed glass beads were added to the samples, followed by vigorous vortexing. Then, 50 µl of o-nitrophenyl-β-D-galactoside (4 mg/ml, Sigma-Aldrich Japan, Tokyo, Japan) was added to the supernatants and the samples were incubated at 30 °C until a yellow color developed. After addition of 120 µl of Na2CO3 (1 N), the O.D.420 of each sample was measured. Assays were normalized to the yeast concentration (O.D.600) of each sample, and the β-galactosidase activity was calculated as follows: β-galactosidase units = 1,000 × O.D.420 × V × O.D.600 where t is time of incubation in minutes, V = volume of culture added to Z buffer in (ml).

Northern Blot Analysis—Approximately 30 µg of the total RNA extracted from UT7/TPO cells was electrophoresed in 1.5% agarose/formaldehyde gels, transferred to the nylon membranes (Hybond-N+, Amersham Biosciences, Buckingham, UK). The membranes were then hybridized with a full-length Aup1 cDNA fragment labeled with [α-32P]dCTP using a random-primed DNA labeling kit (Roche Molecular Biochemicals). To compare the expression of Aup1 transcripts among different human tissues, the Human 12-lane MTN Blot (CLONTECH Japan, Tokyo, Japan) was hybridized with the same probe.

Preparation of the Synthetic Peptides and Antibody Production—Peptides for Aup1 (RITLPDKAESHMRQRHHRPRL) (Fig.1, A and B), α6β4 and β3 cytoplasmic tails, to which a cytoine residue was added at the N terminus for the antibody production, were synthesized using PSSM-8 (Shimadzu, Kyoto, Japan). Each peptide was coupled to the keyhole limpet hemocyanin (Sigma-Aldrich) and injected subcutaneously to rabbits for immunization.

Immunoblot Analysis—Platelets were isolated from the platelet-rich plasma of the normal peripheral blood, and leukocytes were isolated from theuffy coat after removal of erythrocytes by hypotonic lysis in 0.14 M NaCl, 20 mM Tris (pH 7.2) at 37 °C. Microscopic observation revealed that more than 90% of the prepared leukocytes were neutrophils. To extract proteins, platelets, leukocytes, UT7/TPO, and other cell lines, including CMK, HL60, K562, U937, Jurkat, Raji, 293, HepG2, HeLa, MCF7, and A547 were resuspended in the cell lysis buffer (0.15 M NaCl, 10 mM Tris (pH 7.4), 1 mM PMSF, 1.8 µg/ml aprotinin, 100 µg/ml leupeptin, and 1% Triton X-100). Samples were incubated for 30 min on ice with occasional vortexing, and the cell lysates were subjected to 10% SDS-PAGE, transferred to the nitrocellulose membranes (Trans-blot transfer medium, Nippon Bio-Rad Laboratories, Tokyo, Japan), blocked with 5% skimmed milk in Tris-buffered saline buffer (10 mM Tris (pH 7.4), 150 mM NaCl) for 1 h, membranes were incubated with a preimmune rabbit serum or the rabbit antiserum for the Aup1 peptide (Aup1-2) for 1 h, and then with the horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (Dako Japan, Kyoto, Japan). Signals on membranes were detected with the ECL system (Amersham Biosciences).

Subcellular Localization of Aup1—To study the subcellular localization of Aup1, a full-length Aup1 cDNA was ligated into the pDsRed1-N1 vector that codes a red fluorescent protein (RFP) (CLONTECH). Then, UT7/TPO cells were transfected with a control vector and a plasmid encoding the Aup1-RFP fusion protein by electroporation using the Gene Pulser II (Bio-Rad). After selection with neomycin, stable cell lines that express RFP (UT7/TPO.VR4-4) and Aup1-RFP fusion protein (UT7/TPO.Aup1 R23-1) were established. To examine the subcellular localization of Aup1, UT7/TPO.VR4-4 and UT7/TPO.Aup1R23-1 cells were applied to glass coverslips and observed using the LSM 510 laser scanning microscope (Carl Zeiss Microscopy, Jena, Germany) with appropriate filters. For the nuclear staining, cells were treated with 3.7% formaldehyde in phosphate-buffered saline and mounted with a mount containing containing 4,6-diamidino-2-phenyldene (Vector Laboratories, Burlingame, CA).

Immunoprecipitation—UT7/TPO cell extracts (600 µg of protein) with the cell lysate buffer containing 1% digitonin and 1 mM Ca2+ were incubated with a preimmune rabbit serum or the Aup1-2 antiserum (40 µl) for 1 h, and then with protein-G-Sepharose beads (30 µl) (Amersham Biosciences) for 3 h at 4 °C with gentle shaking. After washing with the
cell lysis buffer, the beads were resuspended in the SDS sample buffer and boiled, and the supernatants were subjected to SDS-PAGE, followed by immunoblot analysis using mouse monoclonal antibodies for the αIIb (SZ22, Cosmo Bio, Tokyo, Japan) and β3 subunits (SZ21, Cosmo Bio). Signals were detected with the ECL system after incubation with the horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins. To identify the αIIb sequence to which Aup1 binds, the GST-Aup1 fusion protein (125–28936 residues) was incubated with the immobilized GST-Sepharose beads (50 μl) for 30 min at room temperature, and the supernatants (20 μl) were subjected to 15% SDS-PAGE, followed by immunoblot analysis using the PVDF membrane and the rabbit antiserum for the cytoplasmic tails of αIIb. The resulting immunoblots of the αIIb cytoplasmic tail were quantified by densitometry, and the affinity of Aup1 for binding to the αIIb cytoplasmic tail was calculated by Scatchard analysis (19).

**RESULTS**

**Identification of a Protein That Binds to the Integrin αIIb Cytoplasmic Tail**—To search for proteins that bind to the cytoplasmic tail of the integrin αIIb subunit, we constructed a cDNA library from a megakaryocyte-derived cell line, UT7/TPO. UT7/TPO cells constitutively express αIIbβ3 on the cellular surface, as confirmed by fluorescence-activated cell sorting analysis (data not shown). Screening of the cDNA library by the yeast two-hybrid assay with a bait of the αIIb cytoplasmic tail identified a 800-bp cDNA fragment that encodes a partial C-terminal peptide composed of 173 amino acids (48L21) (Fig. 1A). To obtain a cDNA sequence for the N-terminal region, we performed PCR using UT7/TPO cDNA and primers from the 5′-terminal sequence of the cloning site of pAD-Gal4 (5′-AGG-GATGTTAATACCACTAC-3′) and the 5′-terminal sequence of 48L21 (5′-48L21) was amplified, and the nucleotide sequencing revealed the sequence was continuous to the 5′-terminus of 48L21. For the final cloning of a full-length cDNA, PCR with UT7/TPO cDNA and primers from the 5′-terminal sequence of 48L21 (5′-GGCTCTGGGTGCA-3′) and 3′-terminal sequence of 48L21 (5′-GGCTCTGGGTGC- CATCCTG-3′) was performed. Nucleotide sequencing of the amplified PCR product (1.3-kb cDNA fragment) revealed that the protein was composed of 410 amino acids. Subsequent data

**A**

| MELPSGPGERLFDSHRLPGDFLLVLVLLYAPVGCFILLRFLFLGHIVF | 1 |
| LVSCFLPSVLRRVVRMCAPVGLVQARQDGSLRHDVRLVISNHVTP | 100 |
| DHHINULCTCSTPLSNPSSWCWREFGEMENRGELVESLKRFCASRT | 201 |
| LPTPTLLLPPFEEATNREGG111RFFSPPSQDVQPLTLTQVRPRVLVST | 301 |
| VSDDAVWSELLWSFVPFTYQQVRWLPAPRQGEANEFAVLRQVQLVK | 401 |

**B**

lipid attachment site  
PlsC  
Aup1-2  
CUE

**Fig. 1. Amino acid sequence and a schematic representation of Aup1.** A, the arrowhead indicates the position where 66 amino acids are inserted in the reported long isoform (Ref. 21; GenBank™ accession no. AF100754). The solid line indicates the amino acid sequence, against which the rabbit antisera (Aup1–2) was raised. The broken line indicates the sequence encoded by 48L21 that was identified by the yeast two-hybrid assay. B, the shaded area and the arrowhead represent a putative signal sequence and a signal cleavage site, respectively. Aup1–2, the peptide used for antibody production; PlsC, phosphate acyltransferase domain; CUE, coupling of ubiquitin conjugation to the endoplasmic reticulum degradation domain.
Fig. 2. Expression of the Aup1 transcripts in human cells and tissues. A, total RNA of UT7/TPO was probed with a ^32P-labeled Aup1 cDNA fragment. B, mRNA on the Human 12-lane MTN Blot (CLONTECH) was hybridized with the same probe; leukocytes (lane 1), lung (lane 2), placenta (lane 3), small intestine (lane 4), liver (lane 5), kidney (lane 6), spleen (lane 7), thymus (lane 8), colon (lane 9), skeletal muscle (lane 10), heart (lane 11), and brain (lane 12). Arrows indicate an Aup1 transcript (~1.7 kb), and the arrowhead (~2.1 kb) (B) may represent an alternatively spliced transcript. Molecular size markers are presented (28 and 18 S; ribosomal RNA, 2.4 and 1.35 [RNA size in kb]). β2MG, filters were reprobed with a ^32P-labeled β2-microglobulin cDNA.

base searches indicated that the sequence was identical with the short isoform of Aup1 (Refs. 20 and 21; GenBank™ accession no. AF100753) (Fig. 1, A and B).

Aup1 Is Ubiquitously Expressed in Human Cells and Tissues—Northern blot analysis with the total RNA extracted from UT7/TPO cells using a full-length Aup1 cDNA probe revealed a transcript of ~1.7 kb (Fig. 2A). Because it was reported that Aup1 is expressed in all mouse tissues (20), we examined the expression of Aup1 transcripts in various human tissues. In concordance with the mouse tissues, Aup1 was expressed in all human tissues examined (Fig. 2B). To examine the expression of Aup1 protein in UT7-TPO cells, platelets, leukocytes, and other cell lines, a rabbit antiserum was raised against a synthetic peptide for Aup1 (Fig. 1, A and B). Immunoblot analysis using this antiserum (Aup1–2) revealed duplicate bands of ~40 kDa at the reducing as well as non-reducing conditions in UT7/TPO cells (Fig. 3A). These bands were observed in other cell lines including CMK, HL60, K562, U937, Jurkat, Raji, HepG2, 293, HeLa, MCF7, and A547 (Fig. 3B). Treatment with the protein phosphatases did not change the intensity of these two bands (data not shown). In contrast, only a smaller band was detected in platelets and leukocytes (Fig. 3C).

Aup1 Is Present in Cytoplasm—To examine the subcellular localization of Aup1, stable UT7/TPO cell lines that express the Aup1-RFP fusion protein (UT7/TPO.Aup1R23-1) and the control RFP (UT7/TPO.VR4-4) were established. Overexpression of Aup1 did not affect the expression of αHβ3 on the cellular surface, as confirmed by fluorescence-activated cell sorting analysis of UT7/TPO, UT7/TPO.VR4-4, and UT7/TPO.Aup1R23-1 cells (data not shown). Observation of UT7/TPO.VR4-4 and UT7/TPO.Aup1R23-1 cells by confocal microscopy revealed that RFP was distributed evenly throughout the cell; however, the Aup1-RFP fusion protein was observed in the cytoplasm, but not in the nucleus (Fig. 4). Because it was reported that the N terminus of mouse Aup1 resembles the signal peptide of secreted protein, followed by a putative signal cleavage site, we examined whether Aup1 is secreted from cells. Immunoblot analysis with the culture supernatant of UT7/TPO cells using the Aup1–2 antiserum revealed that Aup1 could not be detected in the concentrated (10-fold) culture supernatant (data not shown). These results indicate that Aup1 is a cytoplasmic protein.

Approximately 40% of the αHb Subunit Is Complexed with Aup1 in UT7/TPO Cells—To examine whether Aup1 is associated with the αHb cytoplasmic tail in the eukaryotic cells, UT7-TPO cell lysate was immunoprecipitated with the Aup1–2 antiserum. Immunoblot analysis with the precipitates using anti-αHb and -β3 antibodies indicated that Aup1 bound to the
αIIIb, but not to the β3 subunit (Fig. 5A). We then measured how much of the cellular αIIIb subunit is complexed with Aup1 by the immunodepletion assay. Densitometric analysis of the resulting αIIIb bands with the supernatants of the UT7/TPO cell lysate after immunoprecipitation with the Aup1 and the control serum revealed that 41.7 ± 3.2% (mean ± S.D., results from three independent experiments) of the αIIIb subunit was complexed with Aup1 (data not shown). Binding of Aup1 to the αIIIb cytoplasmic tail was confirmed by the GST pull-down assay, revealing that GST-tagged Aup1 binds to the αIIIb, but not to the β3 subunit (Fig. 5B).

Aup1 Interacts with the αIIIb Cytoplasmic Tail with a Low Affinity—We next studied interaction between the synthetic peptide for the αIIIb cytoplasmic tail and immobilized GST-Aup1 fusion protein to measure the affinity of interaction. The Kd value calculated from the Scatchard plot analysis was 90 μM, suggesting a relatively weak affinity of interaction between Aup1 and the αIIIb cytoplasmic tail (Fig. 6).

Aup1 Binds to Cytoplasmic Tails of Various Integrin α Subunits—As Aup1 is expressed ubiquitously in human cells and tissues, we examined whether Aup1 binds to cytoplasmic tails of other integrin α as well as β subunits by the yeast two-hybrid assays. Yeast cells were co-transformed with plasmids encoding Aup1 (48L21) and cytoplasmic tails of αIIIb, αV, αM, β3, β2, and β1. In selective Leu- Trp- His- plates, only colonies co-transformed with Aup1 and α subunits grew. These colonies were positive for both of the filter lift assay and the quantitative β-galactosidase assay (Fig. 7). These results indicate that Aup1 binds to cytoplasmic tails of various integrin α subunits.

Aup1 Binds to the Conserved Membrane-proximal Sequence of the Cytoplasmic Tail of the Integrin α Subunits—The amino acid sequence of the membrane-proximal region of the cytoplasmic tail is highly conserved among the integrin α subunits and plays a crucial role in the inside-out signaling of αIIIbβ3 (4–6).

As the results from the yeast two-hybrid assay suggested binding of Aup1 to this conserved sequence, we examined interaction between the purified Aup1 and immobilized GST fusion proteins of the αIIIb cytoplasmic tail, including the normal and a mutant (F992A) αIIIb that leads to the high affinity state of αIIIb.
Aup1 Binds to the Integrin α Cytoplasmic Tails

**DISCUSSION**

One of the obstacles to elucidate the integrin αIIbβ3 signaling is the absence of appropriate cell lines that exhibit similar properties to platelets including expression of αIIbβ3 and intracellular signaling molecules, and the response to platelet physiological agonists. Because the αIIb subunit is exclusively expressed in platelets and megakaryocytes, it is suggested that a megakaryocyte-derived cell line would be suitable to search for proteins that bind to the αIIb cytoplasmic tail. In this study, we performed the yeast two-hybrid assay and screened the cDNA library from UT7/TPO cells using the αIIb, cytoplasmic tail as bait. Binding of Aup1 to the αIIb cytoplasmic tail was demonstrated by the following results. First, Aup1 bound to the αIIb cytoplasmic tail in the yeast two-hybrid assay. Second, Aup1 is a cytoplasmic protein, as indicated by the observation with the confocal microscopy of UT7/TPO cells that express an RFP-tagged Aup1. Third, the αIIb subunit was present in the immunoprecipitate of the UT7-TPO cell lysate by the antisera for Aup1. Fourth, GST-tagged Aup1 bound to the αIIb subunit in the UT7-TPO cells.

The N terminus of Aup1 is hydrophobic and resembles the signal sequence of secreted proteins, followed by an 11-amino acid sequence with similarity to a prokaryotic lipid attachment site (20) (Fig. 1B). These characteristic amino acid sequences and the present observations with confocal microscopy and immunoblot analysis with the culture supernatant of UT7/TPO cells suggest that Aup1 is a cytoplasmic protein in possible association with the plasma membrane. Immunoblot analysis with the antisera for an Aup1 peptide revealed duplicate bands of ~40 kDa with an estimated molecular mass difference of ~3–4 kDa in UT7/TPO and other cell lines. In contrast, only a smaller band was detected in platelets and leukocytes. Although only a single cDNA fragment encoding 410 amino acids was amplified by PCR with UT7/TPO cDNA in the present study, a long isoform of Aup1 composed of 476 amino acids has been reported to be produced by alternative splicing of the Aup1 gene (21). However, considering the difference in the number of amino acids (66) between these two isoforms, it is unlikely that duplicate bands observed in the immunoblot analysis are produced by alternative splicing. Another possible examination is the posttranslational modification including glycosylation, phosphorylation, and cleavage. As the consensus amino acid sequences for the O- and N-linked glycosylation sites are absent in Aup1 and only a smaller band is observed in terminally differentiated platelets and leukocytes, it is hard to explain that the larger protein represents a glycosylated mature protein. With regard to phosphorylation, tyrosine, serine, and/or threonine residues are phosphorylated upon cellular stimulation as observed in a number of intracellular signaling proteins. However, modification by phosphorylation is unlikely because duplicate bands were constitutively expressed and did not exhibit any difference in their intensity after treatment with the protein phosphatase in the immunoblot analysis. On the other hand, the difference in the estimated molecular mass of these two bands (~3–4 kDa) is concordant with that of the postulated signal sequence composed of 37 amino acids. Accordingly, it is conceivable that a larger band represents a precursor protein subjected to cleavage to the smaller mature protein, followed by possible modification with lipid attachment including myristoylation, prenylation, and/or palmitoylation to be associated with the internal leaflet of the plasma membrane (22).

It was reported that mouse Aup1 also consists of 410 amino acid and is expressed in all mouse tissues. In addition, it exhibits an amino acid sequence similar to those of Caenorhabditis elegans and human Aup1 (20). Because of its evolutionary conservation of the amino acid sequence and ubiquitous expression, it appears that Aup1 plays an essential role in cell biology. It was unexpected that Aup1, a ubiquitously expressed protein in various tissues, binds to the cytoplasmic tail of the αIIb subunit that is exclusively expressed in platelets and megakaryocytes. Accordingly, we examined whether Aup1 binds to the cytoplasmic tails of other integrin α as well as β subunits. The yeast two-hybrid assays revealed that Aup1 binds to the cytoplasmic tails of the α1, α3, α5, α5, and αv subunits, but not to the β1, β2, and β3 subunits, indicating specific binding of Aup1 to the integrin α subunits. To confirm association between Aup1 and these α subunits, we performed immunoprecipitation using cell lines that express a relatively high level of these α subunits, including IMR32 treated by retinoic acid for α3, CCRF-CEM for α2, HeLa treated by IL-6 for α5, K562 for αM, and RAW264.7 for αv. However, we could co-precipitate Aup1 with these integrin α subunits neither by the Aup1-2 nor by various antisera for these subunits, probably because the expression level of these proteins is extremely low compared with the αIIb subunit in UT7/TPO cells (data not shown). On the other hand, subsequent GST pull-down assay indicated binding of Aup1 to the membrane-proximal amino acid sequence (KVGGFRK) that is conserved among the cytoplasmic tails of the integrin α subunits. Accordingly, it seems that one of the essential biological functions of Aup1 is to bind to the cytoplasmic tail of the integrin α subunits through the conserved membrane-proximal sequence.

A data base search for the homologous domain structure revealed that Aup1 possesses two domains: CUE and PlsC domains (23). The yeast protein Cue1p is a prototype of CUE domain family and belongs to the integral endoplasmic reticulum membrane proteins. It exhibits a scaffolding activity and recruits the ubiquitin-conjugating enzymes Ubc7p and Ubc6p in the proximity of the translocon pore cytoplasmic exit to deliver proteins for ubiquitination and subsequent digestion by the proteasome (24). The CUE domain is also present in several eukaryotic cytoplasmic proteins. It was suggested that some of the CUE-containing proteins are not associated with endoplasmic reticulum and possess functions different from that of Cue1p (23). Recent studies identified two eukaryote proteins with the CUE domain, Toll-
Aup1 Binds to the Integrin α Cytoplasmic Tails

interacting protein (Tollip) and transforming growth factor β-activated kinase 1 (TAK1)-binding protein 2 (TAB2), that exhibit novel functions in the IL-1 signal transduction pathway. Tollip is present in a complex with the serine/threonine IL-1 receptor (IL-1R)-associated kinase (IRAK) and binding of IL-1 to IL-1R results in the rapid assembly of a membrane-proximal signaling complex that consists of IL-1R, an adaptor protein (myeloid differentiation protein; MyD88), IRAK, and Tollip. Because overexpression of Tollip results in impaired IL-1β-induced activation of the nuclear transcription factor c-Jun N-terminal kinase, it may inhibit IL-1 signaling by silencing components of the signaling cascade including IRAK (25). TAB2 is an adaptor protein that mediates activation of TAK1. IL-1 stimulates translocation of TAB2 from the membrane to the cytosol where it mediates association of TAK1 with the tumor necrosis factor receptor-associated factor 6 (TRAF6), leading to activation of TAK1 (26).

In addition to possessing the CUE domain, the amino acid sequence of Aup1 exhibits a significant similarity to taffazins that belong to the acyltransferase superfamily (27, 28), suggesting that Aup1 may exhibit an enzymatic activity. Taffazins are composed of a highly hydrophobic N terminus of 30 amino acids that may serve a membrane anchor and a central hydrophilic domain composed of 72 residues that may serve as an exposed loop interacting with other proteins. Mutations of a gene encoding taffazins (G4.5) lead to a severe inherited (X-linked) disorder, Barth syndrome, that is characterized by cardiac and skeletal myopathy, short stature, and neutropenia, indicating an essential biological function of taffazins (29). Acyltransferases of the taffazin superfamily all function in phospholipid synthesis and have either glycerophosphate (GPAT, EC 2.3.1.12), 1-acylglycerophosphate (AGPAT, EC 2.3.1.15), 2-acylglycerophosphate, or 2-acylglycerophosphoethanolamine acyltransferase activity (28). The initial step of phospholipid biosynthesis involves the acylation of glycerol-3-phosphate by GPAT to form lysophosphatidic acid (LPA), followed by acylation of LPA by AGPAT to form phosphatidic acid (PA). In addition to being the key enzyme involved in the acylation of glycerol-3-phosphate by AGPAT to form phosphatidic acid (PA), in platelets, thrombin stimulation leads to the rapid activation of tyrosine kinases, involving Syk, which is activated within seconds (35), and Src family kinases, and tyrosine phosphorylation of the signaling proteins (36). Considering the remarkable rapidity of this signaling process, Aup1 which binds to the α1b cytoplasmic tail reversibly as suggested by the relatively low affinity of interaction, may be suitable for one of the modulators in the α1bβ3 inside-out signaling. However, further studies are necessary to elucidate implication of Aup1 in the integrin signaling and other biological functions.

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