β_3-Endonexin, a Novel Polypeptide That Interacts Specifically with the Cytoplasmic Tail of the Integrin β_3 Subunit

Sanford J. Shattil,* Timothy O'Toole,‡ Martin Eigenthaler,‡ Vicki Thon,§ Michael Williams,‡ Bernard M. Babior,§ and Mark H. Ginsberg‡

*Departments of Medicine and Pathology and Laboratory Medicine, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104; and the ‡Department of Vascular Biology and §Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037

Abstract. The adhesive and signaling functions of integrins are regulated through their cytoplasmic domains. We identified a novel 111 residue polypeptide, designated β_3-endonexin, that interacted with the cytoplasmic tail of the β_3 integrin subunit in a yeast two-hybrid system. This interaction is structurally specific, since it was reduced by 64% by a point mutation in the β_3 cytoplasmic tail ($S^{752}\rightarrow P$) that disrupts integrin signaling. Moreover, this interaction is integrin subunit specific since it was not observed with the cytoplasmic tails of the α_{IIb}, β_1, or β_2 subunits. β_3-Endonexin fusion proteins bound selectively to detergent-solubilized β_3 from platelets and human umbilical vein endothelial cells, and β_3-endonexin mRNA and protein were detected in platelets and other tissues. A related mRNA encoded a larger polypeptide that failed to bind to β integrin tails. The apparent specificity of β_3-endonexin for the β_3 integrin subunit suggests potential mechanisms for selective modulation of integrin functions.

ADHESION receptors of the integrin superfamily are heterodimers composed of α and β type I transmembrane subunits (40). Each subunit consists of a relatively large extracellular domain that participates in ligand binding, a single transmembrane domain, and a short cytoplasmic tail that in most cases contains 20–70 amino acids. The β_3 integrins include $\alpha_{IIb}\beta_3$, which is specific for the megakaryocytic lineage, and $\alpha_v\beta_3$, which is also expressed on endothelial cells, vascular smooth muscle cells, monocytes, macrophages, osteoclasts, and certain subpopulations of lymphocytes (7, 17, 20, 57, 66). $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ recognize several Arg-Gly-Asp-containing adhesive ligands in a divalent cation-dependent manner and these interactions are crucial for hemostasis, wound healing and angiogenesis (28, 63).

In addition to their adhesive functions, it is now apparent that integrins interact with the intracellular signaling machinery of cells. The affinity or avidity of many integrins for their cognate ligands is regulated by cellular agonists and antagonists in a process called inside-out signaling. For example, $\alpha_{IIb}\beta_3$ in platelets is converted to a high affinity state following cellular activation, and this process can be prevented or reversed by compounds that increase platelet adenylyl cyclase or guanylyl cyclase (63). Affinity modulation may be controlled through an interaction of intracellular signaling molecules with integrin cytoplasmic tails, since deletions or mutations of the tails exert profound effects on receptor affinity and cell adhesion (37, 42, 53, 65).

Integrins also function to transduce extracellular cues into the cell, a process called outside-in signaling. In this case, adhesive ligands induce receptor clustering, activation of protein tyrosine kinases, such as $pp125^{FAK}$, dramatic rearrangements of the actin cytoskeleton, and changes in gene expression that affect cell growth, differentiation and programmed death (8, 12, 41). As is the case with inside-out signaling, integrin cytoplasmic tails may play a significant role. For example, when human integrin subunits are expressed in rodent cells, partial deletion of the α_{IIb} tail (Shattil, S., L. Leong, C. Abrams, M. Cunningham, T. Parsons, T. O'Toole, and M. Ginsberg. 1994. *Circulation.* 90:I–86) or overexpression of a chimeric β_1 tail (2, 49) results in anchorage-independent phosphorylation of $pp125^{FAK}$ on tyrosine residues.

Several studies have begun to identify proteins that bind to integrin cytoplasmic tails in vitro. For example, calreticulin binds to α-tails (48), and α-actinin, talin and $pp125^{FAK}$ bind to β tails (39, 54) (Otey, C. A., Schaller, M., and Parsons, J. T. 1993. *Mol. Biol. Cell* 4:347a). The functional relevance of these interactions in vivo remains to be defined. Nonetheless, given the strong circumstantial evidence that β cytoplasmic tails modulate integrin function, the present study was carried out to identify direct, binary interactions between a prototypic β tail (β_3) and intracel-

Address all correspondence to S. J. Shattil, Department of Vascular Biology, The Scripps Research Institute, 10666 N. Torrey Pines Rd., VB-5 La Jolla, CA. Tel.: (619) 554-7148. Fax: (619) 554-6451.

lular proteins. We describe here a novel polypeptide named β_3-endonexin, initially identified using a yeast two-hybrid approach (18, 31), that interacts with the β_3 integrin cytoplasmic tail in a structurally specific manner.

Materials and Methods

DNA Constructions

The cytoplasmic tails of the integrin subunits studied are shown in Table I. These tails were amplified from existing pCDM8 expression constructs (15, 53) using sense primers containing BamHI or EcoRI restriction sites and antisense primers containing PstI or BamHI sites. Gel-purified PCR products were digested with the appropriate restriction enzymes and directionally cloned into the yeast expression vector, pGBT9 (Clontech Laboratories, Inc., Palo Alto, CA) (6). This resulted in the in-frame fusion of each cytoplasmic tail to the 3′ end of the GAL4$_{(1\text{-}147)}$ DNA-binding domain. All DNA sequences were confirmed by sequencing both strands, either with Sequenase Version 2.0 (7-deaza-dGTP kit; US Biochemical Corp., Cleveland, OH) or by automated sequencing in the Scripps Research Institute DNA Core Facility.

Library Screening

A human cDNA library in a lambda vector (λACT) was derived from EBV-transformed peripheral blood B lymphocytes and was the kind gift of Stephen Elledge (Baylor College of Medicine, Houston, TX). This library, containing 3×10^6 independent clones, had been ligated into the vector at a XhoI site, resulting in fusion to the 3′ end of the GAL4$_{(768\text{-}881)}$ activation domain (25, 27). Before use, λACT was converted from phage λ to plasmid DNA (pACT) (27).

To screen for proteins that bind to the β_3 cytoplasmic tail, the yeast two-hybrid system was employed as described by Fields and co-workers (6, 18, 30, 31). 1×10^8 competent cells of the yeast strain Y190 (*MATa, leu2-3,112, ura3-52, trp1-901, his3-Δ200, ade2-101, gal4Δ, gal80Δ, URA3::GAL→lacZ, LYS2::GAL→HIS3 cyclohexamide^R*) were transformed with 5 μg of pGBT9/β_3 according to Schiestl and co-workers (61). Transformants were grown for three days at 30°C on agar plates containing SD synthetic media without tryptophan. Then a colony was selected and grown overnight in 5 ml of SD without tryptophan to seed a 1 litre culture in YPD medium, which was grown to a density of $OD_{600} = 0.3$. Cells were made competent in 30 ml of 0.1 M LiAc and TE (1 mM EDTA and 0.01 M Tris-Hcl, pH 7.5) and then transformed by adding 250 μg of pACT/B cell library DNA, 1 mg of denatured salmon sperm DNA (Oncor, Inc., Gaithersburg, MD), and 140 ml of LiAc/TE containing 40% PEG 4000 (Sigma Chemical Co., St. Louis, MO). After 30 min at 22°C, DMSO was added to a final concentration of 10% (vol/vol), the cells were heat-shocked for 6 min at 42°C, and then washed and resuspended in 10 ml of TE. 100 μl aliquots were plated in 100 × 15 mm plastic petri dishes containing SD agar with 25 mM 3-aminotriazole but no tryptophan, leucine or histidine. Transformants were grown for 8 d at 30°C. The Y190 host strain contains two reporter genes, *HIS3* and *lacZ*, under the control of GAL4. In principle, these genes should be transactivated by library fusion proteins that interact with the β_3 integrin cytoplasmic tail to reconstitute functional GAL4.

β-Galactosidase activity of individual colonies was determined qualitatively in a filter-lift assay (10). Positive colonies were restreaked on SD plates lacking leucine and tryptophan and retested. Positives were grown overnight in 5 ml SD without leucine and plasmid DNA was obtained (38), and used to transform competent HB101 cells by heat shock according to the supplier's protocol (GIBCO BRL, Gaithersburg, MD). Transformants were grown at 37°C for 1–2 d on M9 minimal agar without leucine in the presence of 50 μg/ml ampicillin. Colonies containing library DNA inserts were identified by digestion with XhoI and the DNA was purified by $CsCl_2$ ultracentrifugation (59). DNA was then re-introduced into yeast to confirm transactivation of *HIS3* and *lacZ* and to exclude false positives. Two additional expression constructs (Clontech) were used to exclude false positive reactions: pLAM 5′, which encodes a human lamin C/GAL4 DNA-binding domain hybrid in pGBT9, and pTD1, which encodes an SV-40 large T-antigen/GAL4 activation domain hybrid in pGAD3F. As described in Results, this selection process yielded a "true positive" clone (No. 28) which was then characterized in detail.

Cloning of Full-length cDNAs

Sequence analyses of the 719-bp clone 28 insert suggested it was a partial cDNA clone. To obtain the 5′ end of the cDNA, a human testes 5′-RACE–ready cDNA library (Clontech) was subjected to two-step 5′-RACE PCR (26). The first reaction contained 2 μl of template cDNA, an anchor sense primer (5′-CTGGTTCGGCCCACCTCTGAAGGTTC-CAGAATCGATAG-3′), and a clone 28–specific antisense primer (5′-TAGACATGCACCTGCCAACTGCTACGAG-3′) in a final vol of 50 μl. The second reaction contained 2 μl of a 1:10 dilution of the primary PCR product, the anchor primer and a nested clone 28-specific antisense primer (5′-CATTATCTCCATGATTTCTTCTGAC-3′). Reactions were "hot started" followed by incubation for 5 min at 94°C, 30 PCR cycles (62°C for 1 min; 72°C for 2.5 min; 94°C for 1 min), and a final 5-min incubation at 72°C. A 600-bp PCR product was gel-purified, subcloned into pCR (Invitrogen, San Diego, CA) and sequenced on both strands.

To identify potential alternate forms of clone 28-related mRNA, the B-cell cDNA library in pACT was subjected to PCR using clone 28-specific primers (sense: 5′-GTAGTATACAGTGACAAAAGTG-3′; antisense: 5′-TAGACATGCACCTGCCAACTGCTACGAG-3′). Hot-start reactions used 50 ng of cDNA, 50 pmol of each primer and 5 U of Taq polymerase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) in 50 μl for 30 cycles (55°C for 1.5 min; 72°C for 2.5 min; 94°C for 1 min). After a final incubation for 5 min at 72°C, 5 μl were electrophoresed on a 2.5% agarose gel and stained with ethidium bromide. PCR products were subcloned into pCR and sequenced.

Comparative Binding Studies of Integrin Cytoplasmic Tails

The two-hybrid system was also used to quantify the extent of binary interactions between clone 28–related polypeptides and the integrin cytoplasmic tails shown in Table I. After simultaneous transformation of Y190 with 5 μg of pGBT9/integrin tail DNA and 5 μg of pACT/clone 28 DNA, transformants were grown on SD agar without tryptophan and leucine. After 3 d, four independent colonies from each transformation were picked and grown overnight at 30°C in 3 ml of SD media without tryptophan and leucine. Then 0.5 ml of each were processed for quantitation of β-galactosidase activity, which was expressed in units (6). The results were taken as a measure of the strength of the interaction between a given integrin tail and clone 28–related polypeptides.

To examine interactions between the polypeptide expressed by clone 28 and a natural β_3 or β_1 subunit in vitro, the polypeptide was expressed as a GST fusion protein. PCR was used to introduce 5′ BamHI and 3′ XhoI restriction sites at either end of a 285-bp putative coding region in clone 28. The PCR product was digested, ligated into these sites in pGEX-5X-1 (Pharmacia Biotech, Inc., Piscataway, NJ), and then used to transform DH5α. DNA sequencing confirmed that the clone 28 insert was now fused in-frame to GST at a factor Xa recognition site. The mass of HPLC-puri-

Table I. Amino Acid Sequences of Integrin Cytoplasmic Tails Studied in the Yeast Two-hybrid System

Integrin tail	Residues*	Sequence‡
β_3	716-762	KLLITIHDRKEFAKFEEERARAKWFTANNPLYKEATSTFTNITYRGT
β_3 (S752→P)	716-762	KLLITIHDRKEFAKFEEERARAKWDTANNPLYKEATPTFTNITYRGT
β_3/β_1 Chimera	β1: 740-775	KLLITIHDRKEFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK
β_2	704-749	KALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES
α_{IIb}	989-1008	KVGFFKRNRPPLEEDDEEGE

*The residue numbers are derived from published sequences of the full-length integrin subunits, starting at the amino terminus (4,33,43,47).
‡The amino-terminal lysine in each tail is assumed to represent the exit point from the plasma membrane (71). In β_3 (S^{752}→P), the mutated residue is underlined. In the β_3/β_1 chimera, the β_3 sequence is underlined.

fied GST/clone 28 fusion protein as determined by electrospray mass spectrometry was that expected for the authentic protein (observed = 37,323 ± 2.7 D; calculated average isotopic composition = 37,322.9 D). Additional bacteria were transformed with pGEX-5X-1 as a source of GST.

Glutathione Sepharose affinity matrices, containing either GST/clone 28 fusion protein or GST alone, were prepared as described by Frangione and Neel (34). Preliminary immunoblotting experiments with an anti-GST antiserum (Pharmacia) showed that approximately equal amounts of fusion protein or GST had bound to the matrix. Human platelets and human umbilical vein endothelial cells (passage 3) were used as sources of β_3. Platelets were washed (64) and resuspended to 2×10^9 cells/ml in a lysis buffer containing 1% Triton X-100, 0.05 M Tris, pH 7.4, 1 mM PMSF, 0.5 mM leupeptin, 100 U/ml aprotinin, and either 1 mM $CaCl_2$ to maintain the $\alpha_{IIb}\beta_3$ complex or 1 mM EDTA to dissociate it (9). After 30 min at 22°C, the detergent-soluble fraction was obtained by centrifugation at 14,000 rpm for 30 min at 4°C. The detergent-soluble fraction of sub-confluent endothelial cells was obtained in the same manner. After pre-equilibration of the glutathione Sepharose affinity matrices in lysis buffer, platelet or endothelial cell lysates were diluted eightfold in lysis buffer with inhibitors and 0.5 ml aliquots were added to 100 μl batches of affinity matrix for 12 h at 4°C with gentle shaking. The matrices were then washed with 10 bed volumes of lysis buffer and proteins were eluted by boiling for 5 min in 30 μl of SDS sample buffer under non-reducing conditions (46). 20 μl of each sample were electrophoresed in 7.5% SDS-polyacrylamide gels (46), transferred to a 0.45 μm nitrocellulose membrane (Millipore Corp., Bedford, MA) (68), and immunoblotted with a monoclonal antibody specific for β_3 (SSA6, 10 μg/ml) (1), a goat antiserum against human β_1 (1:500; a gift from Martin Hemler, Dana Farber Cancer Institute, Boston, MA), or a monoclonal antibody against P-selectin (S12, 10 μg/ml; a gift from Rodger McEver, Oklahoma Medical Research Foundation, Oklahoma City, OK) (51). Immunoreactivity was determined using affinity-isolated, peroxidase-conjugated goat anti–rabbit Ig (1:3,000) (Tago, Inc., Burlingame, CA) and the ECL chemiluminescence reaction (Amersham Corp., Arlington Heights, IL) (36).

To examine interactions between natural β_1 and β_3 integrin subunits and β_3-endonexin, the latter polypeptide was bacterially-expressed with a histidine (6) tag fused to the amino terminus (pET His Tag System; Novagen, Inc, Madison, WI). The protein was purified by HPLC and its mass verifed by mass spectometry. An affinity matrix was prepared by binding 2 mg of his-tagged β_3-endonexin to 1.5 ml of His Bind metal chelation resin according to the supplier's instructions (Novagen). After washing with platelet lysis buffer, 2 ml of platelet lysate (6.6 mg protein) was incubated with the matrix for 1 h at 4°C. The matrix was then loaded into a column and washed with lysis buffer (twice with 2 ml, and then thrice with 3 ml), and then eluted with 2 ml of a buffer containing 200 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9. Samples were analyzed by SDS-PAGE on 4-20% gels and by immunoblotting as described in the legend to Fig. 7.

Detection of Clone 28-related mRNAs in Cells and Tissues

Northern blots were performed using a Human MTN Blot II (Clontech) as a source of poly A^+ RNA according to the supplier's instructions. The 719-bp insert from clone 28 was obtained by XhoI digestion, labeled with (α-^{32}P)dATP using a commercial kit (Prime-It II Random Primer Labeling Kit; Stratagene, La Jolla, CA), and used as a probe.

Primer extension reactions were carried out to confirm the 5′ ends of mRNAs encoding clone 28-related proteins (59). First strand cDNA synthesis was accomplished using washed platelets as a source of total RNA and either of two clone 28-specific antisense oligonucleotides that had been 5′-labeled with ^{32}P (5′-AACCCAGCAACTTCCGAAAACAGAAA-ATCCGCC-3′ or 5′-CAGAAAATCCGCCAAAGGAAACCACGAAT-TCAC-3′). Control studies showed that no PCR product was obtained when first strand synthesis was omitted.

Several human cDNA libraries as well as reverse-transcribed platelet mRNA were subjected to PCR as described above to screen for the presence of a reaction product specific for β_3-endonexin. The primers are described in the legend to Fig. 4 *B*. The following libraries were examined: B cell in pACT, and placenta and brain in pGAD424 (the latter two from Clontech).

Detection of Proteins

Expression of GAL4 DNA-binding domain fusion proteins containing either the α_{IIb}, β_2, or β_3 tails was confirmed by immunoblotting with rabbit antisera (1:500) specific for these tails (53). Individual yeast transformants were grown overnight at 30°C in 5 ml of SD without tryptophan. Cells were washed, resuspended in SDS sample buffer containing 1 mM PMSF, 0.5 mM leupeptin and 100 KIU/ml aprotinin, and lysed by vortexing for 30 s × 3 in the presence of 0.5 vol of 500 μ acid-washed glass beads (Sigma) and then boiling for 10 min. Supernatants were analyzed for protein content (BCA; Pierce Chemical Co., Rockford, IL) and 35 μg aliquots were electrophoresed under non-reducing conditions and immunoblotted as described above.

β_3-Endonexin expression was analyzed in fresh human platelets and in a mononuclear fraction of peripheral blood leukocytes (5) by immunoblotting. Cells were washed and lysed for 10 min in boiling SDS sample buffer containing 5 mM EDTA, 0.5 mM leupeptin, 4 mM Pefabloc (Boehringer-Mannheim), 10 μg/ml pepstatin A (Sigma), and 2 mM N-methyl maleimide. Platelet and mononuclear leukocyte lysates (40 μg/lane) were electrophoresed under reducing conditions, and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) as described (36), except that the transfer solution was buffered with CAPS, pH 11. Blots were probed with two different rabbit antisera (1:1000) reactive with β_3-endonexin. The first (antiserum 0834) was obtained using a thyroglobulin-conjugated synthetic peptide consisting of the predicted carboxy-terminal 17 residues of β_3-endonexin. The second (antiserum 0835) was raised against the GST/clone 28 fusion protein described above. Both antisera reacted on immunoblots with this fusion protein, with clone 28 polypeptide obtained by factor Xa cleavage of the fusion protein, and with His-tagged β_3-endonexin; neither antibody reacted with GST.

Results

Detection of a Polypeptide That Binds to the Cytoplasmic Tail of the Integrin β_3 Subunit

A yeast two-hybrid system was used to screen for proteins that interact with the β_3 cytoplasmic tail. Nucleotides encoding the entire 47–amino acid cytoplasmic tail of β_3 (Ta-

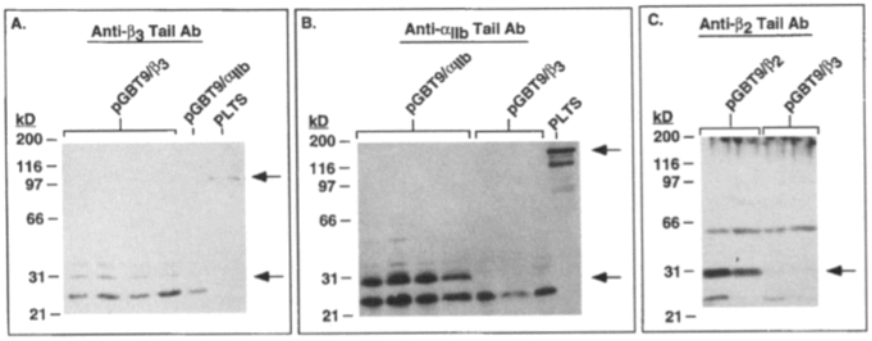

Figure 1. Expression of the cytoplasmic tails of the β_3, α_{IIb}, and β_2 integrin subunits as GAL4 fusion proteins in yeast. As described in Materials and Methods, one to four independent colonies from each transformation were grown in liquid culture, lysed, and subjected to SDS-PAGE and immunoblotting. The primary antibody used in each panel is shown. The lane on the far right in panels A and B was loaded with 20 μg of platelet lysate as a source of full length β_3 or α_{IIb}, respectively (*upper arrows*). The lower arrows indicate the position of the GAL4 DNA-binding domain fusion proteins. Other bands in the yeast samples are non-specific.

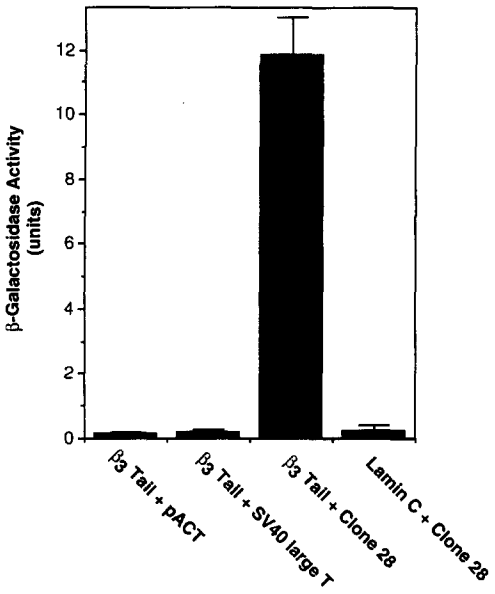

Figure 2. The fusion protein expressed by clone 28 binds to the cytoplasmic tail of β_3 in yeast. Yeast were co-transformed as indicated with two of the following plasmids: pGBT9/β_3, pACT, pGAD3F/SV-40 large T antigen, pACT/clone 28, and pGBT9/lamin C. Transformants were grown in liquid culture and assayed for β-galactosidase activity as described in Materials and Methods. Data bars represent the mean ± SD of six separate experiments, each performed on four independent colonies.

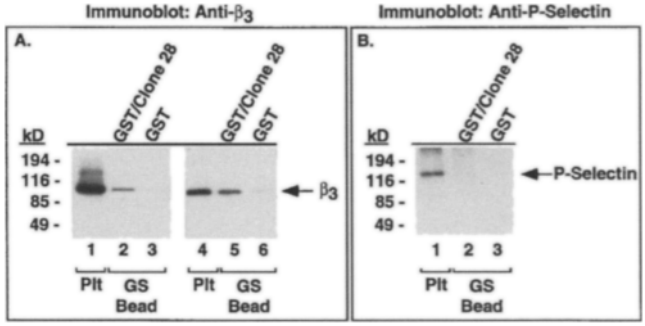

Figure 3. Specific interaction in vitro between the polypeptide expressed by clone 28 as a GST fusion protein and the β_3 integrin subunit. As described in Materials and Methods, affinity matrices were prepared containing either GST or GST fused to the 95–amino acid polypeptide expressed by clone 28. Platelet lysate was then incubated with the matrices for 12 h at 4°C, followed by SDS-PAGE and immunoblotting of bound proteins with monoclonal antibodies specific for β_3 (*A*) or P-selectin (*B*). As positive immunoblot controls, lanes *1* and *4* were loaded with 20 μg of platelet lysate. Lanes *1–3* represent platelet lysate prepared in the presence of 1 mM EDTA, while lanes *4–6* represent lysate prepared with 1 mM $CaCl_2$. This experiment is representative of three so performed.

ble I) were fused in frame to the DNA-binding domain of GAL4 in the yeast plasmid, pGBT9. Western blots of yeast lysates transformed with pGBT9/β_3 confirmed that the β_3 tail fusion protein was being expressed (Fig. 1 *A*). However, fusion protein expression did not cause transactivation of the two GAL4 reporter genes present in the yeast host strain (*HIS3* and *lacZ*). Therefore, the β_3 tail was used as a ligand to screen for binding partners in an EBV-transformed B lymphocyte cDNA library which had been fused to the DNA activation domain of GAL4 (25, 27).

Of 1.7 million co-transformants plated, 90 colonies grew in the absence of histidine. Seven also expressed β-galactosidase activity in a filter-lift assay, suggesting that a library–derived fusion protein was interacting with the β_3 cytoplasmic tail. Five of these colonies contained an identical 719-bp library insert and were considered to be "true positives". Their plasmid DNA caused transactivation of *HIS3* and *lacZ* when reintroduced into yeast expressing the β_3 cytoplasmic tail, but no such activation was observed in yeast expressing an unrelated fusion protein (lamin C). Fig. 2 shows the results of quantitative β-galactosidase assays for one of the positive clones (No. 28), which was subsequently characterized in detail.

To determine whether the polypeptide expressed by clone 28 could interact with the β_3 cytoplasmic tail in a context outside of yeast, the cloned DNA insert was sequenced and a 285-bp coding region was identified. This sequence was used to construct and express the corresponding 95–amino acid polypeptide as a soluble GST fusion protein. When the fusion protein was attached to a glutathione Sepharose matrix and incubated with a detergent extract from platelets (a source rich in $\alpha_{IIb}\beta_3$), the β_3 subunit was retained by the affinity matrix. In contrast, another highly expressed platelet protein, P-selectin, was not retained by this matrix, nor was β_3 retained by a control GST matrix (Fig. 3). The retention of β_3 on the clone 28 affinity matrix was observed independent of whether platelets had been solubilized in the presence of 1 mM $CaCl_2$ to maintain the integrity of the $\alpha_{IIb}\beta_3$ complex or in 1 mM EDTA to dissociate the integrin subunits (9). Although not shown, similar results were obtained with β_3 from human umbilical vein endothelial cells, while β_1 from endothelial cells and platelets was not retained on the clone 28 affinity matrix. Taken together with the binding experiments in yeast, these data indicate that a 95–amino acid polypeptide encoded by clone 28 binds directly to the cytoplasmic tail of integrin β_3 in a specific manner.

Alternate Forms of Clone 28-related mRNA Encode Polypeptides That Bind Differentially to the β_3 Cytoplasmic Tail

The 719-bp library insert from clone 28 was used to probe Northern blots of eight human tissues. A band of reactivity was observed at approximately 1.1 kb in all tissues, with greatest reactivity in testes and colon (Fig. 4 *A*). In an attempt to obtain full-length cDNA clones representing this mRNA, 5′-RACE PCR was performed using a testes cDNA library as template. To complement this analysis, PCR reactions were carried out using the original B lymphocyte cDNA library as a template and clone 28–specific oligonucleotides as primers. With this combined approach, the 5′-untranslated and coding regions and most of the 3′-untranslated region were characterized, and two closely related mRNAs were identified: a "shorter" form containing 897 bp and a "longer" form containing 1041 bp (Fig. 4 *B*). The apparent transcription start site for both mRNAs at residue 1 in Fig. 5 *A* was confirmed by primer extension analysis of platelet mRNA (not shown). A putative start

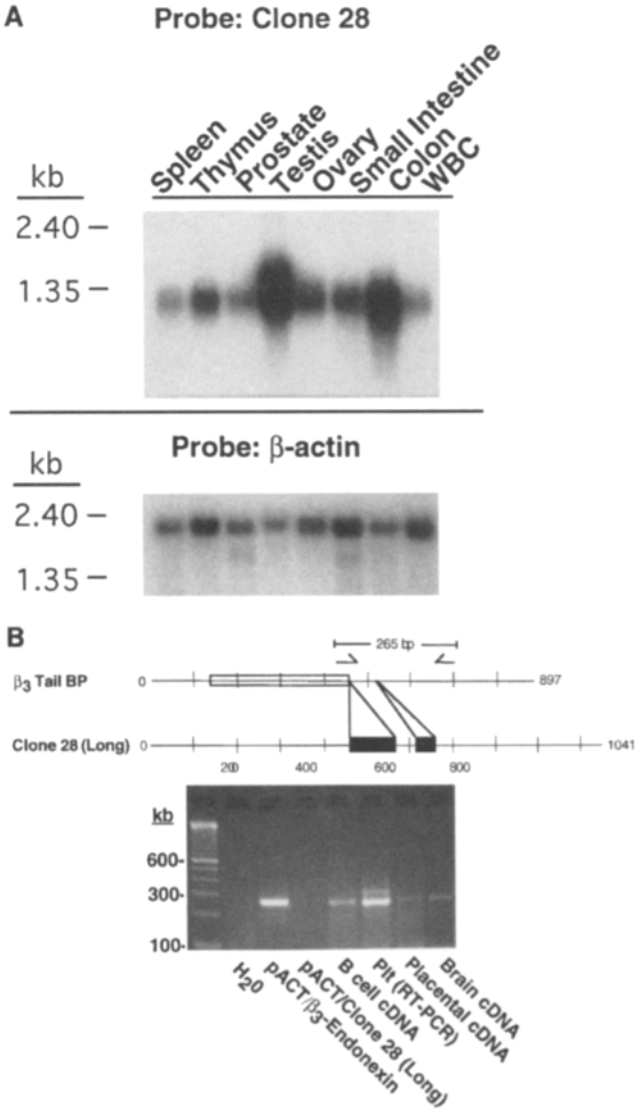

Figure 4. (*A*) Northern blot demonstrating the presence of clone 28–related mRNA in human tissues. A β-actin probe was used as a control. (*B*) PCR detection of β_3-endonexin cDNA from various human tissues. As shown schematically on the top, a primer pair was selected that would prime β_3-endonexin but not the longer mRNA species (*clone 28 Long*). The sense and antisense primers were 5′-GCAAAATTTAAGTAGTATACAGTGAC-3 and 5′-CTCGTAGCAGTTGGCAGGTGCATGTCTA-3′, respectively. As shown on the bottom, the predicted 265-bp β_3-endonexin PCR product was observed when B cell, placenta and brain cDNA libraries and reverse-transcribed platelet mRNA were used as templates. As controls, this band was also observed when β_3-endonexin in pACT was used as a template, but not when pACT/Clone 28 Long was used.

codon at bases 131–133 is in a suitable environment for translation initiation (44), and it is in-frame with downstream sequences that encode the clone 28 fusion protein identified in the two-hybrid screen. An open reading frame for the shorter mRNA species would encode a 111 amino acid, 12.6-kD polypeptide. The carboxy-terminal 90 amino acids are identical to corresponding amino acids in clone 28. The longer mRNA species differs from the shorter one due to insertions of 93 and 50 bp, as indicated

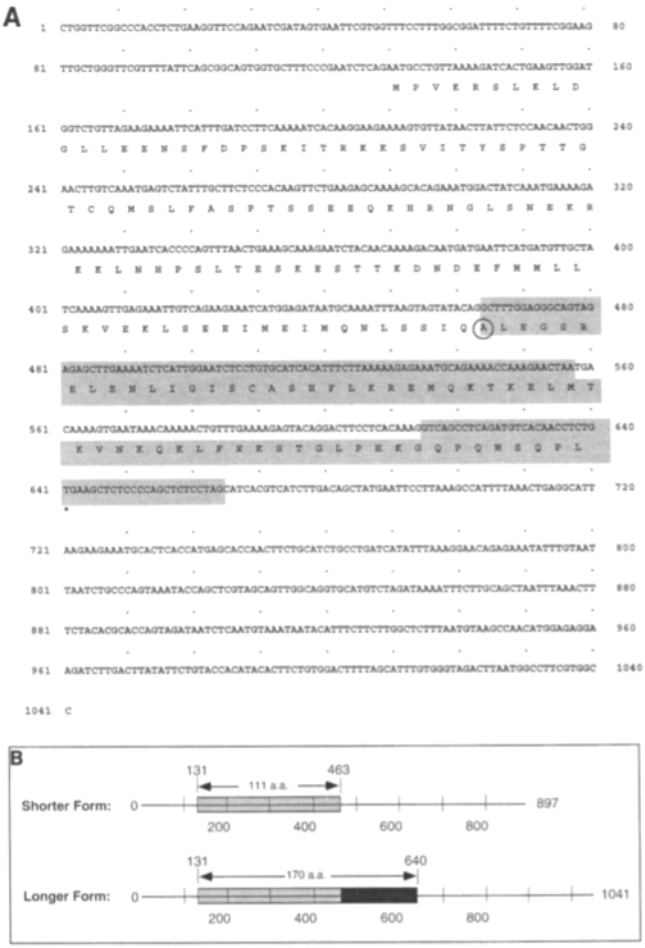

Figure 5. Sequences of clone 28–related cDNAs. Panel A shows the nucleotide sequence and deduced amino acid sequence of cDNA derived from the longer of the two clone 28–related mRNAs described in the text. A start codon is at nucleotides 131-133. The shaded areas show which nucleotides and amino acids are deleted to form the shorter mRNA and its deduced polypeptide (β_3-endonexin). The circled alanine is encoded by a GCT in the longer mRNA but this is replaced by a stop codon in β_3-endonexin. *B* compares these mRNAs diagrammatically. The coding region for β_3-endonexin is represented by the shaded rectangle (nucleotides 131-463), and the dark rectangle in the longer mRNA represents nucleotides encoding an additional 59 amino acids. These sequence data are available from EMBL/GenBank/DDBJ under accession number U37139.

in Fig. 5 *A*. In this case, the open reading frame would encode a 170 amino acid, 19.2-kD polypeptide that differs from the shorter polypeptide due to an additional 59 amino acids at the carboxy terminus (Fig. 5 *B*).

The capacity of these two different clone 28-related mRNAs to express polypeptides that bind to the β_3 cytoplasmic tail was tested in the two-hybrid system. The shorter 111–amino acid polypeptide bound to the β_3 cytoplasmic tail as well as the original clone 28 fusion protein (Fig. 6). In light of its ability to bind to a portion of an integrin subunit normally located inside the cell, the polypeptide was named β_3-endonexin (from the roots, "endon" or within, and "nexus" or connection). In contrast, the 170–amino acid polypeptide, hereafter referred to simply as "clone 28-long", failed to bind to the β_3 tail (Fig. 6). Not

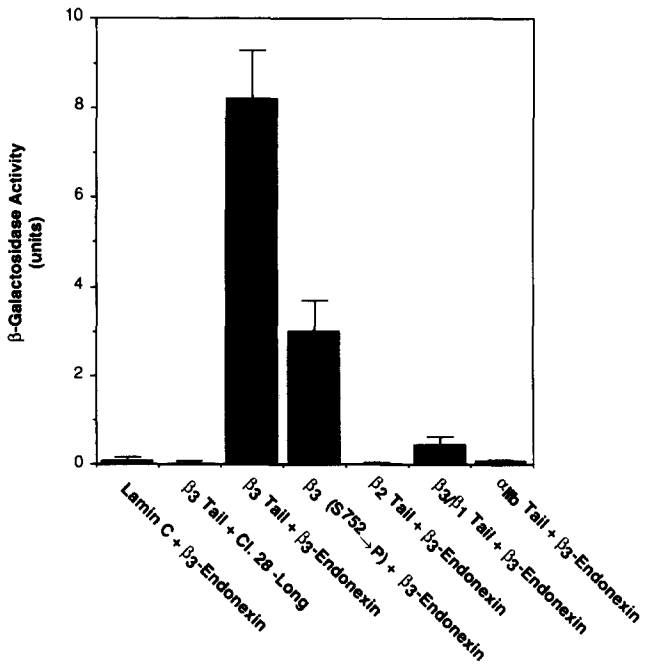

Figure 6. Structural specificity of binding of β_3-endonexin to the cytoplasmic tail of β_3. Binary interactions between pairs of fusion proteins were studied in yeast using a liquid β-galactosidase assay as described in Materials and Methods and in the legend to Fig. 2. All GAL4 DNA-binding domain fusions were in pGBT9. All clone 28-related fusions were in pACT. Data bars represent the mean ± SD of five separate experiments, each performed on four independent colonies.

shown is the fact that the longer polypeptide also failed to bind to the cytoplasmic tails of the β_1 or β_2 integrin subunits.

β_3-Endonexin nucleotide sequences were not represented in either GenBank or EMBL nucleotide databases as of May 18, 1995. Searches of these databases through the National Center for Biotechnology Information using either the BLASTN (3) or FASTN (56) algorithms failed to disclose complete identities. A 395-bp expressed sequence tag derived from mouse testis (MUSBO48A) exhibited 78% identity over a 232-nucleotide stretch. Other than this, no other similarities were identified. Thus, β_3-endonexin is a novel protein.

The 111–amino acid β_3-endonexin sequence was analyzed with the University of Wisconsin Genetics Computer group package (23). This polypeptide has a predicted molecular mass of 12,624 and a predicted pI of 8.3. Its amino acid composition is unremarkable, being comprised of 31% charged and 23% hydrophobic residues. The presence of a single cysteine suggests that the mature protein could contain a free sulfhydryl group. The protein contains 17 Ser and 9 Thr residues suggesting that it may be subject to modification by phosphorylation or glycosylation. Indeed, Ser^{28} is in an appropriate context for phosphorylation by protein kinase A (29, 35), and Ser^{6}, Thr^{24}, and Thr^{79} are in contexts favorable for protein kinase C (72). Analysis of hydrophilicity (45), averaged over a window of seven residues, revealed no long hydrophobic stretches consistent with the absence of both a signal peptide and transmembrane domains. Consequently, β_3-endonexin is predicted to be an intracellular protein.

Searches of non-redundant protein sequence databases through the National Center for Biotechnology Information using BLAST (3) and FASTA (56) revealed no homologies of obvious biological significance. Similarly, search of the PROSITE database with the MOTIFS program (23) failed to identify any rare sequence motifs within β_3-endonexin.

Structural Specificity of the β_3-endonexin/β_3 Cytoplasmic Tail Interaction

There are several highly conserved regions in the cytoplasmic tails of most β integrin subunits (e.g., see Table I) (60, 71). Despite this, the β_2 cytoplasmic tail did not interact with β_3-endonexin in the yeast two-hybrid system. Moreover, a chimeric β_3/β_1 cytoplasmic tail containing the membrane-proximal 11 residues of β_3 and the distal 36 residues of β_1 bound minimally to β_3-endonexin. Also, no interaction was observed between β_3-endonexin and the cytoplasmic tail of α_{IIb} (Fig. 6). This lack of interaction with integrin tails other than β_3 was not likely to be due to insufficient expression of these tails in yeast. Expression of the α_{IIb} and β_2 fusion proteins was confirmed by immunoblotting with tail-specific antibodies (Fig. 1, *B* and *C*). Altogether, these results indicate that β_3-endonexin binds selectively to the β_3 cytoplasmic tail, probably due to recognition of membrane-distal sequences unique to β_3.

Further support for this conclusion was obtained by studying a point mutant of the β_3 cytoplasmic tail, $S^{752}\rightarrow P$ (Table I). In human platelets, this mutation is associated with a bleeding disorder due to defective agonist-induced activation of and fibrinogen binding to $\alpha_{IIb}\beta_3$ (14). Moreover, CHO cells expressing this mutant exhibit markedly reduced spreading and focal adhesion formation following adhesion to fibrinogen (16). When the $S^{752}\rightarrow P$ cytoplasmic tail was tested in the two-hybrid system, it showed a 64% reduction in binding to β_3-endonexin compared to the wild-type β_3 tail ($P < 0.001$) (Fig. 6).

To determine whether β_3-endonexin exhibited a selective interaction with the β_3 integrin subunit in vitro, a histidine-tagged form of β_3-endonexin was expressed in bacteria and attached non-covalently to a metal chelation affinity resin. The β_3 integrin subunit from a detergent extract of platelets was retained and eluted from this β_3-endonexin affinity matrix, while the β_1 integrin subunit from the same cells was not (Fig. 7). Thus, studies in yeast and with recombinant β_3-endonexin in vitro indicate that the binding of this polypeptide to the β_3 integrin tail is structurally specific.

Tissue and Cellular Expression of β_3-Endonexin

In order to begin to assess the significance of β_3-endonexin expression in cells, PCR of several cDNA libraries was carried out using oligonucleotides specific for β_3-endonexin. A PCR product was detected in cDNA libraries from human brain, B lymphocytes and placenta as well as in cDNA obtained from platelets by RT-PCR (Fig. 4 *B*). The product from B lymphocytes was cloned and sequenced and its identity to β_3-endonexin was confirmed.

Next, platelets and a mononuclear fraction of peripheral blood leukocytes were examined by immunoblotting to

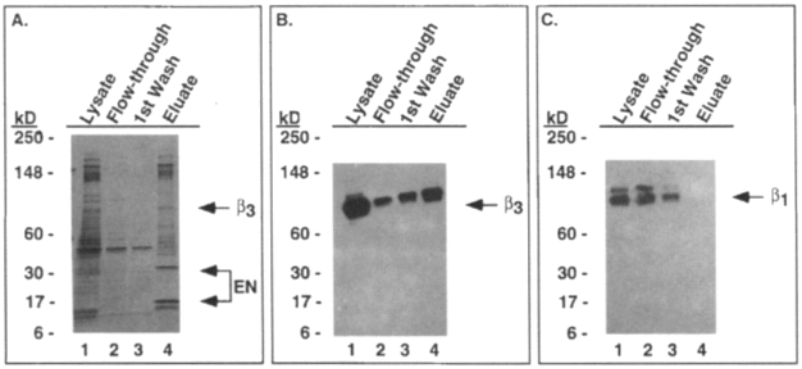

Figure 7. Specific interaction between recombinant β_3-endonexin and the β_3 integrin subunit. As described in Materials and Methods, an affinity matrix was prepared containing histidine-tagged β_3-endonexin bound non-covalently to a metal chelation resin. Platelet lysate (2 ml) was incubated with the affinity resin for 12 h at 4°C. After five washes, proteins were eluted from the resin in 2 ml of buffer containing 200 mM imidazole. Lysates (lane *1*; 12 μg protein), initial flow-through (lane *2*; 15 μl), first column wash (lane *3*; 15 μl), and resin eluate (15 μl) were then subjected to SDS-PAGE under non-reducing conditions and the gel was stained with Coomassie blue (*A*) or transferred to nitrocellulose and immunoblotted with monoclonal antibodies specific for β_3 (*B*) or β_1 (*C*). Some proteins were present in the first wash but none were detectable in the fifth wash (not shown). Note in *A* that a number of proteins in the platelet lysate were depleted in the flow-through and eluted from the column with imidazole. Some of these proteins, one of which migrated identically with the β_3 integrin subunit (*upper arrow*), may represent proteins that bind directly or indirectly to β_3-endonexin. Two faster migrating bands (*double arrows*) represent monomeric and dimeric forms of recombinant β_3-endonexin. Immunoblotting demonstrated that the β_3 integrin subunit was relatively depleted from the flow-through and eluted from the resin (*B*), while the β_1 integrin subunit was not. This experiment is representative of three so performed.

characterize β_3-endonexin expression at the protein level. Using a rabbit anti-peptide antiserum specific for the putative carboxy terminus of β_3-endonexin, an immunoreactive band migrating at approximately 13 kD was observed in platelets. This band was specific because it was not observed with pre-immune serum or when the immune serum had been pre-incubated with the immunizing peptide (Fig. 8). The same band was observed using a different rabbit antiserum raised against a GST/clone 28 fusion protein. A specific band was also detected in blood mononuclear leukocytes using the anti-peptide antiserum (Fig. 8).

Discussion

In the present study, a human cDNA has been identified that encodes a novel 12.6 kD, 111–amino acid polypeptide that binds to the cytoplasmic tail of the β_3 integrin subunit. Designated β_3-endonexin on the basis of its binding specificity, this polypeptide was first detected in a yeast two-hybrid screen of a B lymphocyte library. Several observations suggest that β_3-endonexin may be relevant to integrin biology in mammalian cells: (*a*) a selective interaction could be demonstrated using bacterially-expressed partial or full-length forms of β_3-endonexin and detergent-solubilized β_3 from platelets or human umbilical vein endothelial cells. (*b*) mRNA specific for β_3-endonexin could be detected in several human tissues, and the polypeptide was detected by immunoblotting in platelets and peripheral blood mononuclear leukocytes, both of which express β_3 integrins. (*c*) Binding of β_3-endonexin to the β_3 cytoplasmic tail was structurally specific: A Ser → Pro mutation at position 752 of the β_3 tail caused markedly reduced binding to β_3-endonexin, and the cytoplasmic tails of the β_1 and β_2 integrin subunits failed to bind. Moreover, a cDNA related to β_3-endonexin was cloned from B lymphocytes that encoded a larger polypeptide containing an extra 59 amino acids at the carboxy terminus of β_3-endonexin. When expressed in the yeast system, it failed to bind to the cytoplasmic tails of β_1, β_2, or β_3.

Expression of β_3-Endonexin in Mammalian Tissues and Cells

Northern blot analysis using a cDNA probe from the original positive yeast clone 28 demonstrated a ~1.1-kb message in eight human tissues of diverse origin. Expression appeared to be greatest in testes and colon, but this type of study can not identify the cells of origin of the mRNA. Similarly, analyses of cDNA libraries by PCR with primers specific for β_3-endonexin found evidence for this mRNA in B lymphocytes, brain and placenta, again suggesting a wide tissue distribution. In addition, β_3-endonexin mRNA

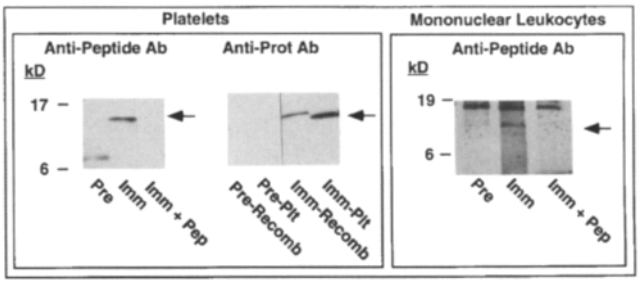

Figure 8. Detection of β_3-endonexin polypeptide in platelets and mononuclear leukocytes. These blood cell fractions were processed for immunoblotting as described in Materials and Methods. Some cell lysates were analyzed using an anti-peptide antiserum specific for the carboxy terminus of β_3-endonexin. Note that both platelets (100 μg protein, or 4×10^7 platelets/lane) and leukocytes (40 μg/lane) exhibited an immunoreactive band at approximately 13 kD (*arrows*) with the immune serum (*Imm*). In contrast, this band was not observed with pre-immune serum (*Pre*) or with immune serum that had been pre-incubated for 30 min with 15 μM of the immunizing peptide (*Imm* + Pep). Lysates from platelets (38 μg, or 1.5×10^7 platelets/lane) and a bacterially expressed recombinant form of β_3-endonexin (3 ng/lane) were also analyzed using antiserum specific for the GST/β_3-endonexin fusion protein described in the legend to Fig. 3 (*Anti-Prot Ab*). The mobility of the immunoreactive band from platelets was slightly greater than that of the recombinant protein.

was found in washed platelets by RT-PCR. Here again, however, the cellular origin of the mRNA is not entirely unambiguous since platelet preparations are always contaminated with some leukocytes. Since β_3 integrin expression appears relatively restricted (e.g., endothelial cells, platelets, monocytes/macrophages, osteoclasts and certain lymphocyte subsets (7, 17, 20, 57, 66), the apparent wide tissue distribution of β_3-endonexin mRNA suggests that the polypeptide may have some function unrelated to integrin binding. On the other hand, the presence of endothelial cells in virtually all tissues could also explain this result.

A hydropathy plot of β_3-endonexin is consistent with the interpretation that the polypeptide contains neither a signal sequence nor a transmembrane domain. Thus, it is probably an intracellular protein. β_3-endonexin polypeptide could be detected in platelets and in a mononuclear fraction of blood containing lymphocytes and monocytes by immunoblotting with two different specific polyclonal antisera. Based on the immunoreactivity of recombinant β_3-endonexin and β_3-endonexin from platelets (Fig. 8), we estimate that there are roughly 5,000–50,000 molecules of this polypeptide per platelet, similar to the number of β_3 integrin molecules per platelet (63). Preliminary attempts to examine whether β_3-endonexin co-immunoprecipitates with β_3 integrins from cellular lysates have been complicated by proteolysis of the polypeptide during various immunoprecipitation protocols, with a resultant loss of reactivity to the available antibodies. Thus, additional studies using new antibodies and other immunochemical and genetic approaches will be required to document the extent to which β_3-endonexin interacts with and modulates the functions of β_3 integrins within cells.

Structural Specificity of the Interaction between β_3-Endonexin and the β_3 Cytoplasmic Tail

Divalent cations are essential for adhesive ligand binding to β_3 integrins and for $\alpha\beta$ subunit association (9, 21). In the present study, a GST/β_3-endonexin–derived fusion protein bound specifically to detergent-solubilized β_3 from platelets, whether or not $CaCl_2$ was present to maintain the $\alpha_{IIb}\beta_3$ complex or EDTA was present to dissociate it. Thus, unlike extracellular integrin ligands, the binding of β_3-endonexin is independent of divalent cations and may not require a complex between α and β subunits. The binding studies in yeast further suggest that the interaction between β_3-endonexin and the β_3 tail is binary, although they do not formally exclude the possibility that one or more additional yeast proteins participate in or modulate the interaction.

Integrins exhibit an intimate but poorly understood relationship with the signaling machinery of cells. In many cases, their affinity for adhesive ligands can be influenced by the state of cellular activation (24, 40). Furthermore, integrin ligation and clustering can trigger biochemical reactions that affect the growth, differentiation and death programs of cells (8, 41, 52). Implicit in these observations is a requirement for regulated interactions between integrins, cytoskeletal and cytoplasmic proteins. Accordingly, there is currently intense interest in identifying the components of integrin signaling pathways as well signaling elements that link these pathways to others that transfer information from the cell surface to the nucleus (22, 69). Recent studies of a variety of cell types have begun to define such interactions (13, 19, 48, 55, 60, 62). In one such study in fibroblasts (70), immunoprecipitation was used to demonstrate that insulin stimulation promotes an association of $\alpha_v\beta_3$ and insulin receptor substrate-1, a protein that mediates insulin signaling by specifically binding to several intracellular targets. This interaction required the β_3 subunit but not α_v.

Other studies have begun to map sites within the β cytoplasmic tails that are involved in interactions with specific intracellular proteins. For example, using synthetic peptides derived from integrin tails, Otey and co-workers have identified two discontinuous sequences within the β_1 tail that bind α-actinin (54) and another membrane-proximal linear sequence in β_1 and β_3 that binds $pp125^{FAK}$ (Otey, C. A., M. Schaller, and J. T. Parsons. 1993. *Mol. Biol. Cell* 4:347a). This latter finding is supported by observations in CHO cells that have been transfected with $\alpha_{IIb}\beta_3$. Cells expressing wild-type $\alpha_{IIb}\beta_3$ adhered to fibrinogen, exhibited tyrosine phosphorylation of $pp125^{FAK}$ and underwent spreading. Cells expressing a truncated form of β_3 missing 35-carboxy-terminal residues still exhibited FAK phosphorylation during adhesion to fibrinogen, but they did not spread (Shattil, S., L. Leong, C. Abrams, M. Cunningham, T. Parsons, T. O'Toole, and M. Ginsberg. 1994. *Circulation.* 90:I–86). Other studies have identified an NPXY sequence in the β_1 or β_3 cytoplasmic tails as necessary for cellular regulation of affinity modulation or for assembly of focal adhesions (53, 58).

In contrast to the above studies, where more than one type of β subunit is involved in interactions with α-actinin or FAK, we found that β_3-endonexin bound only to the cytoplasmic tail of β_3. The specificity of this interaction is underscored by the inability of the larger clone 28-related polypeptide to interact with the β_3 tail (Fig. 6). Neither the β_2 tail nor a chimeric tail made up of the membrane–proximal 11 residues of β_3 and the distal 36 residues of β_1 interacted with β_3-endonexin. Since the sequences of the membrane-proximal portions of the three β subunits are very similar (Table I), residues unique to the distal portion of the β_3 tail must be responsible for binding. This notion is supported by two other observations. First, interaction of the β_3 tail with β_3-endonexin in the yeast system was markedly reduced when proline was substituted for serine at β_3 residue 752 (Fig. 6). Second, recent studies have demonstrated that the human β_3 integrin subunit expressed as an $\alpha_{IIb}\beta_3$ complex in CHO cells binds to a β_3-endonexin affinity column. In contrast, a β_3 subunit lacking the COOH-terminal 39 residues does not bind (Eigenthaler, M., S. J. Shattil, and M. H. Ginsberg, unpublished observations).

Both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ are involved in integrin-mediated signaling (63). Of note in this context, β_3^{S752} appears critical for this process in both integrins. Platelets from individuals homozygous for the $S^{752}\rightarrow P$ mutation do not bind soluble fibrinogen due to defective agonist-induced conversion of $\alpha_{IIb}\beta_3$ to a high affinity state (14). This same mutation abolishes $\alpha_{IIb}\beta_3$-mediated spreading of CHO cells on fibrinogen (16) and $\alpha_v\beta_3$-mediated clot retraction by melanoma cells (Chen, Y., and M. Ginsberg, unpublished observations). It is intriguing, therefore, that β_3-endonexin binds at or near a region of the β_3 tail that regu-

lates both the adhesive and signaling functions of these integrins. Thus, this novel interaction or others like it could provide a structural context to explain how a cell might be able to regulate the function of one integrin despite the presence of multiple integrins. Furthermore, the β_3 integrins have been implicated in several pathological processes, including thrombosis, coronary restenosis after angioplasty, osteoporosis and tumor angiogenesis (11, 32, 50, 67). The present studies suggest that it might be possible to develop therapeutic strategies that target a specific integrin tail or proteins that bind differentially to that tail.

We thank Joseph Loftus for many helpful suggestions and Jack Deng and Jim Gao for expert technical assistance.

These studies were supported by grants from the National Institutes of Health (HL 40387, HL 48728, AI 24227), the University of California TRDRP Program (3RT-0320), ThromboSys, Inc., and the Stein Endowment Fund. M. Eigenthaler is supported by a fellowship from the Deutsche Forschungsgemeinschaft.

Received for publication 30 May 1995 and in revised form 20 July 1995.

References

1. Abrams, C. S., Z. M. Ruggeri, R. Taub, J. A. Hoxie, C. Nagaswami, J. W. Weisel, and S. J. Shattil. 1992. Anti-idiotypic antibodies against an antibody to the platelet glycoprotein (GP) IIb-IIIa complex mimic GP IIb-IIIa by recognizing fibrinogen. *J. Biol. Chem.* 267:2775–2785.
2. Akiyama, S. K., S. S. Yamada, K. M. Yamada, and S. E. LaFlamme. 1994. Transmembrane signal transduction by integrin cytoplasmic domains expressed in single-subunit chimeras. *J. Biol. Chem.* 269:15961–15964.
3. Altschul, S. F., W. Gish, W. Miller, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403–410.
4. Argraves, W. S., S. Suzuki, H. Arai, K. Thompson, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the human fibronectin receptor. *J. Cell Biol.* 105:1183–1190.
5. Arroyo, A. G., P. Sanchez-Mateos, M. R. Campanero, I. Martin-Padura, E. Dejana, and F. Sanchez-Madrid. 1992. Regulation of the VLA integrin-ligand interactions through the β_1 subunit. *J. Cell Biol.* 117:659–670.
6. Bartel, P.L., C.-T. Chien, R. Sternglanz, and S. Fields. 1993. Using the two-hybrid system to detect protein-protein interactions. *In* Cellular Interactions in Development: A Practical Approach. D. A. Hartley, editor. Oxford University Press, Oxford, UK. 153–179.
7. Basson, C. T., O. Kocher, M. D. Basson, A. Asis, and J. A. Madri. 1992. Differential modulation of vascular cell integrin and extracellular matrix expression in vitro by TGF-beta-1 correlates with reciprocal effects on cell migration. *J. Cell. Biochem.* 153:118–128.
8. Boudreau, N., C. J. Sympson, Z. Werb, and M. J. Bissell. 1995. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science (Wash. DC).* 267:891–893.
9. Brass, L. F., S. J. Shattil, T. J. Kunicki, and J. S. Bennett. 1985. Effect of calcium on the stability of the platelet membrane glycoprotein IIb-IIIa complex. *J. Biol. Chem.* 260:7875–7881.
10. Breeden, L., and K. Nasmyth. 1985. Regulation of the yeast *HO* gene. *Cold Spring Harbor Symp. Quant. Biol.* 50:643–650.
11. Brooks, P. C., A. M. P. Montgomery, M. Rosenfeld, R. A. Reisfeld, T. Hu, G. Klier, and D. A. Cheresh. 1994. Integrin $\alpha_v\beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell.* 79:1157–1164.
12. Burridge, K., L. A. Petch, and L. H. Romer. 1992. Signals from focal adhesions. *Curr. Biol.* 2:537–539.
13. Chen, H.-C., and J.-L. Guan. 1994. Stimulation of phosphatidylinositol 3'-kinase association with focal adhesion kinase by platelet-derived growth factor. *J. Biol. Chem.* 269:31229–31233.
14. Chen, Y.-P., I. Djaffar, D. Pidard, B. Steiner, A.-M. Cieutat, J. P. Caen, and J.-P. Rosa. 1992. Ser-752 → Pro mutation in the cytoplasmic domain of integrin β_3 subunit and defective activation of platelet integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) in a variant of Glanzmann thrombasthenia. *Proc. Natl. Acad. Sci. USA.* 89:10169–10173.
15. Chen, Y.-P., T. E. O'Toole, T. Shipley, J. Forsyth, S. E. La Flamme, K. M. Yamada, S. J. Shattil, and M. H Ginsberg. 1994. "Inside-out" signal transduction inhibited by isolated integrin cytoplasmic domains. *J. Biol. Chem.* 269:18307–18310.
16. Chen, Y.-P., T. E. O'Toole, J. Ylänne, J.-P. Rosa, and M. H. Ginsberg. 1994. A point mutation in the integrin β_3 cytoplasmic domain (S^{752}→P) impairs bidirectional signaling through $\alpha_{IIb}\beta_3$ (platelet glycoprotein IIb-IIIa). *Blood.* 84:1857–1865.
17. Cheresh, D. A. 1987. Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor. *Proc. Natl. Acad. Sci. USA.* 84: 6471–6475.
18. Chien, C.-T., P. L. Bartel, R. Sternglanz, and S. Fields. 1991. A method to identify and clone for proteins that interact with a protein of interest. *Proc. Natl. Acad. Sci. USA.* 88:9578–9582.
19. Cobb, B. S., M. D. Schaller, T-H. Leu, and J. T. Parsons. 1994. Stable association of $pp60^{src}$ and $pp59^{fyn}$ with the focal adhesion-associated protein tyrosine kinase, $pp125^{FAK}$. *Mol. Cell. Biol.* 14:147–155.
20. Coller, B. S., D. A. Cheresh, E. Asch, and U. Seligsohn. 1991. Platelet vitronectin receptor expression differentiates Iraqui-Jewish from Arab patients with Glanzmann thrombasthenia in Israel. *Blood.* 77:75–83.
21. D'Souza, S. E., T. A. Haas, R. S. Piotrowicz, V. Byers-Ward, D. E. McGrath, H. R. Soule, C. Cierniewski, E. F. Plow, and J. W. Smith. 1994. Ligand and cation binding are dual functions of a discrete segment of the integrin β_3 subunit: cation displacement is involved in ligand binding. *Cell.* 79:659–667.
22. Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science (Wash. DC).* 264:1415–1421.
23. Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12:387–395.
24. Diamond, M. S., and T. A. Springer. 1994. The dynamic regulation of integrin adhesiveness. *Curr. Biol.* 4:506–517.
25. Durfee, T., K. Becherer, P. L. Chen, S. H. Yeh, Y. Yang, A. E. Kilburn, W. H. Lee, and S. J. Elledge. 1993. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes & Dev.* 7: 555–569.
26. Edwards, J. B., J. Delort, and J. Mallet. 1991. Oligodeoxyribonucleotide ligation to single-stranded cDNAs: a new tool for cloning 5' ends of mRNAs and for constructing cDNA libraries by in vitro amplification. *Nucleic Acids Res.* 19:5227–5232.
27. Elledge, S. J., J. T. Mulligan, S. W. Ramer, M. Spottswood, and R. W. Davis. 1991. λYES: A multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia Coli* mutations. *Proc. Natl. Acad. Sci. USA.* 88:1731–1735.
28. Felding-Habermann, B., and D. A. Cheresh. 1993. Vitronectin and its receptors. *Curr. Opin. Cell Biol.* 5:864–868.
29. Feramisco, J. R., D. B. Glass, and E. G. Krebs. 1980. Optimal spatial requirements for the location of basic residues in peptide substrates for the cyclic AMP-dependent protein kinase. *J. Biol. Chem.* 255:4240–4245.
30. Fields, S. 1993. The two-hybrid system to detect protein-protein interactions. *METHODS: A Comp. Methods Enzymol.* 5:116–124.
31. Fields, S., and O. Song. 1989. A novel genetic system to detect protein-protein interactions. *Nature (Lond.).* 340:245–247.
32. Fisher, J. E., M. P. Caulfield, M. Sato, H. A. Quartuccio, R. J. Gould, V. M. Garsky, G. A. Rodan, and M. Rosenblatt. 1993. Inhibition of osteoclast bone resorbtion in vivo by echistatin, an arginyl-glycyl-aspartyl (RGD)-containing protein. *Endocrinology.* 132:1411–1413.
33. Fitzgerald, L. A., B. Steiner, S. C., Rall, Jr., S. S. Lo, and D. R. Phillips. 1987. Protein sequence of endothelial glycoprotein IIIa derived from a cDNA clone. Identity with platelet glycoprotein IIIa and similarity to "integrin". *J. Biol. Chem.* 262:3936–3939.
34. Frangione, J. V., and B. G. Neel. 1993. Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. *Anal. Biochem.* 210:179–187.
35. Glass, D. B., M. R. el-Maghrabi, and S. J. Pilkis. 1986. Synthetic peptides corresponding to the site phosphorylated in 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase as substrates of cyclic nucleotide-dependent protein kinases. *J. Biol. Chem.* 261:2987–2993.
36. Haimovich, B., L. Lipfert, J. S. Brugge, and S. J. Shattil. 1993. Tyrosine phosphorylation and cytoskeletal reorganization in platelets are triggered by interaction of integrin receptors with their immobilized ligands. *J. Biol. Chem.* 268:15868–15877.
37. Hibbs, M. L., S. Jakes, S. A. Stacker, R. W. Wallace, and T. A. Springer. 1991. The cytoplasmic domain of the integrin lymphocyte function-associated antigen 1 β subunit: Sites required for binding to intercellular adhesion molecule 1 and the phorbol ester-stimulated phosphorylation site. *J. Exp. Med.* 174:1227–1238.
38. Hoffman, C. S., and F. Winston. 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene.* 57:262–272.
39. Horwitz, A., K. Duggan, C. Buck, M. C. Beckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin-a transmembrane linkage. *Nature (Lond.).* 320:531–533.
40. Hynes, R. O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell.* 69:11–25.
41. Juliano, R. L., and S. Haskill. 1993. Signal transduction from the extracellular matrix. *J. Cell Biol.* 120:577–585.
42. Kawaguchi, S., J. M. Bergelson, R. W. Finberg, and M. E. Hemler. 1994. Integrin α^2 cytoplasmic domain deletion effects: loss of adhesive activity parallels ligand-independent recruitment into focal adhesions. *Mol. Biol. Cell.* 5:977–988.
43. Kishimoto, T. K., K. O'Connor, A. Lee, T. M. Roberts, and T. A. Springer. 1987. Cloning of the β subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family.

Cell. 48:681–690.
44. Kozak, M. 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.* 266:19867–19870.
45. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105–128.
46. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
47. Law, S. K. A., J. Gagnon, J. E. K. Hildreth, C. E. Wells, A. C. Willis, and A. J. Wong. 1987. The primary structure of the β-subunit of the cell surface adhesion glycoproteins LFA-1, CR3 and p150,95 and its relationship to the fibronectin receptor. *EMBO J.* 6:915–919.
48. Leung-Hagesteijn, C. Y., K. Milankov, M. Michalak, J. Wilkins, and S. Dedhar. 1994. Cell attachment to extracellular matrix substrates is inhibited upon downregulation of expression of calreticulin, an intracellular integrin α-subunit-binding protein. *J. Cell Sci.* 107:589–600.
49. Lukashev, M. E., D. Sheppard, and R. Pytela. 1994. Disruption of integrin function and induction of tyrosine phosphorylation by the autonomously expressed β_1 integrin cytoplasmic domain. *J. Biol. Chem.* 269:18311–18314.
50. Matsuno, H., J. M. Stassen, J. Vermylen, and H. Deckmyn. 1994. Inhibition of integrin function by a cyclic RGD-containing peptide prevents neointima formation. *Circulation.* 90:2203–2206.
51. McEver, R. P., and M. N. Martin. 1984. A monoclonal antibody to a membrane glycoprotein binds only to activated platelets. *J. Biol. Chem.* 259: 9799–9804.
52. Miyamoto, S., S. K. Akiyama, and K. M. Yamada. 1995. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science (Wash. DC).* 267:883–885.
53. O'Toole, T. E., Y. Katagiri, R. J. Faull, K. Peter, R. Tamura, V. Quaranta, J. C. Loftus, S. J. Shattil, and M. H. Ginsberg. 1994. Integrin cytoplasmic domains mediate inside-out signaling. *J. Cell Biol.* 124:1047–1059.
54. Otey, C. A., G. B. Vasquez, K. Burridge, and B. W. Erickson. 1993. Mapping of the α-actinin binding site within the β_1 integrin cytoplasmic domain. *J. Biol. Chem.* 268:21193–21197.
55. Pavalko, F. M., and C. A. Otey. 1994. Role of adhesion molecule cytoplasmic domains in mediating interactions with the cytoskeleton. *Proc. Soc. Exp. Biol. Med.* 205:282–293.
56. Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA.* 85:2444–2448.
57. Rabinowich, H., W. Lin, A. Amoscato, R. B. Herberman, and T. L. Whiteside. 1995. Expression of vitronectin receptor on human NK cells and its role in protein phosphorylation, cytokine production, and cell proliferation. *J. Immunol.* 154:1124–1135.
58. Reszka, A. A., Y. Hayashi, and A. F. Horwitz. 1992. Identification of amino acid sequences in the integrin β_1 cytoplasmic domain implicated in cytoskeletal association. *J. Cell Biol.* 117:1321–1330.
59. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1.42–1.45 and 7.79–7.83.
60. Sastry, S. K., and A. F. Horwitz. 1993. Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signaling. *Curr. Opin. Cell Biol.* 5:819–831.
61. Schiestl, R. H., P. Manivasakam, R. A. Woods, and R. D. Gietz. 1993. Introducing DNA into yeast by transformation. *METHODS: A Comp. Methods Enzymol.* 5:79–85.
62. Schlaepfer, D. D., S. K. Hanks, T. Hunter, and P. Van der Geer. 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature (Lond.).* 372:786–791.
63. Shattil, S. J. 1995. Function and regulation of the β_3 integrins in hemostasis and vascular biology. *Thromb. Haemost.* 74:149–155.
64. Shattil, S. J., J. A. Hoxie, M. Cunningham, and L. F. Brass. 1985. Changes in the platelet membrane glycoprotein IIb-IIIa complex during platelet activation. *J. Biol. Chem.* 260:11107–11114.
65. Shaw, L. M., and A. M. Mercurio. 1993. Regulation of $\alpha_6\beta_1$ integrin laminin receptor function by the cytoplasmic domain of the α_6 subunit. *J. Cell Biol.* 123:1017–1025.
66. Stupack, D. G., C. Shen, and J. A Wilkins. 1992. Induction of $\alpha_v\beta_3$ integrin-mediated attachment to extracellular matrix in β1 integrin (CD29)-negative B cell lines. *Exp. Cell Res.* 203:443–448.
67. The Epic Investigators. 1994. Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty. *New Engl. J. Med.* 330:956–961.
68. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
69. Ullrich, A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell.* 61:203–212.
70. Vuori, K., and E. Ruoslahti. 1994. Association of insulin receptor substrate-1 with integrins. *Science (Wash. DC).* 266:1576–1578.
71. Williams, M. J., P. E. Hughes, T. E. O'Toole, and M. H. Ginsberg. 1994. The inner world of cell adhesion: integrin cytoplasmic domains. *Trends Cell Biol.* 4:109–112.
72. Woodgett, J. R., K. L. Gould, and T. Hunter. 1986. Substrate specificity of protein kinase C. Use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. *Eur. J. Biochem.* 161:177–184.