Abstract. The adhesive and signaling functions of integrins are regulated through their cytoplasmic domains. We identified a novel 111 residue polypeptide, designated β3-endonexin, that interacted with the cytoplasmic tail of the β3 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 β3 cytoplasmic tail (S^{752}→P) that disrupts integrin signaling. Moreover, this interaction is integrin subunit specific since it was not observed with the cytoplasmic tails of the αIIb, β1, or β2 subunits. β3-Endonexin fusion proteins bound selectively to detergent-solubilized β3 from platelets and human umbilical vein endothelial cells, and β3-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 β3-endonexin for the β3 integrin subunit suggests potential mechanisms for selective modulation of integrin functions.

A dhesion 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 β3 integrins include α₀β₃, which is specific for the megakaryocytic lineage, and α₀β₃, which is also expressed on endothelial cells, vascular smooth muscle cells, monocytes, macrophages, osteoclasts, and certain subpopulations of lymphocytes (7, 17, 20, 57, 66). α₁β₃ and α₀β₃ 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, α₁β₃ 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 pp125FAK, 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 α₁β₃ tail (Shattil, S., L. Leong, C. Abrams, M. Cunningham, T. Parsons, T. O'Toole, and M. Ginsberg. 1994. Circulation. 90:1-86) or overexpression of a chimeric β3 tail (2, 49) results in anchorage-independent phosphorylation of pp125FAK 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 pp125FAK 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 (β3) and intracel-
ular proteins. We describe here a novel polypeptide named β₃-endonexin, initially identified using a yeast two-hybrid approach (18, 31), that interacts with the β₂ 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 (13, 33) 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(32-88) 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 × 10⁶ independent clones, had been ligated into the vector at a XhoI site, resulting in fusion to the 3′ end of the GAL4(32-88) 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 β₂ cytoplasmic tail, the yeast two-hybrid system was employed as described by Fields and co-workers (15, 53) using sense primers containing BamHI or EcoRI restriction sites at either end of a 285-bp putative coding region in clone 28. PCR was used to introduce 5′ BamHI and 3′ XhoI restriction sites into each of the primers (sense: 5′-GTAGTATACAGTGACAAAAGTG-3′; antisense: 5′-TAGACATGCACCTGCACAATGCAGTAG-3′). 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 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.

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

| Integrin tail | Residues* | Sequence† |
|---------------|-----------|-----------|
| β₃ | 716-762 | KLLETHDRKEFKFEEERARAKWFTANPNPYLEKTSFTNTYTGK |
| β₃ (S752→P) | 716-762 | KLLETHDRKEFKFEEERARAKWFTANPNPYLEKTSFTNTYTGK |
| β₂/β₃, Chimera | β₁: 740-775 β₂: 704-749 | CALIIHLSDLREYRIRFEEKELKQSWNNDNLPLKSATTTVWNPKFAEK |
| λ₀β | 999-1008 | KVFIFKNRRPILLEDEEDEEGE |

*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 β₁ (S752→P), the mutated residue is underlined. In the β₂/β₃, the β₂ 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 antisemur (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 β3. Platelets were washed (64) and resuspended to 2 × 10^6 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 α_5β_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 ~1 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 β3 (SSA6, 10 μg/ml) (1), a goat antiserum against human β3 (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 β3 and β3 integrin subunits and β3-endonexin, the latter polypeptide was bacteriologically-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 verified by mass spectrometry. An affinity matrix was prepared by binding 2 mg of his-tagged β3-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 Xhol digestion, labeled with (α-32P)dATP using a commercial kit (Prime-It II Random Primer Labeling Kit; Stratagene, La Jolla, CA), and used as a probe.

**Results**

**Detection of Proteins**

Expression of GAL4 DNA-binding domain fusion proteins containing either the α_5, β3, or β3 tails was confirmed by immunoblotting with rabbit antiserum (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 μl 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.

β3-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 TAPS, pH 11. Blots were probed with two different rabbit antisera (1:1,000) reactive with β3-endonexin. The first (antisemur 0834) was obtained using a tyrothryoglobin-conjugated synthetic peptide consisting of the predicted carboxy-terminal 17 residues of β3-endonexin. The second (antisemur 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 β3-endonexin; neither antibody reacted with GST.

**Detection of a Polypeptide That Binds to the Cytoplasmic Tail of the Integrin β3 Subunit**

A yeast two-hybrid system was used to screen for proteins that interact with the β3 cytoplasmic tail. Nucleotides encoding the entire 47-amino acid cytoplasmic tail of β3 (Ta-
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 β3 (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 CaCl2. This experiment is representative of three so performed.

Figure 3. Specific interaction in vitro between the polypeptide expressed by clone 28 as a GST fusion protein and the β3 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 β3 (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 CaCl2. This experiment is representative of three so performed.

Alternate Forms of Clone 28-related mRNA Encode Polypeptides That Bind Differentially to the β3 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 2. The fusion protein expressed by clone 28 binds to the cytoplasmic tail of β3 in yeast. Yeast were co-transformed as indicated with two of the following plasmids: pGBT9/β3, 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 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 β3-endonexin cDNA from various human tissues. As shown schematically on the top, a primer pair was selected that would prime β3-endonexin but not the longer mRNA species (clone 28 Long). The sense and antisense primers were 5'-GCAAAATTAAAGTAGTATAACAGTGAC-3' and 5'-CTCGTAGCAGTTGGCAGGTGCATGTCTA-3', respectively. As shown on the bottom, the predicted 265-bp β3-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 β3-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 in Fig. 5A. 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 carboxyl terminus (Fig. 5B).

The capacity of these two different clone 28-related mRNAs to express polypeptides that bind to the β3 cytoplasmic tail was tested in the two-hybrid system. The shorter 111-amino acid polypeptide bound to the β3 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 β3-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 β3 tail (Fig. 6). Not
shown is the fact that the longer polypeptide also failed to bind to the cytoplasmic tails of the β1 or β2 integrin subunits.

β3-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 sequence tag derives from mouse testis (MUSBO48A) 71). Despite this, the β2 cytoplasmic tail did not interact with β3-endonexin in the yeast two-hybrid system. Moreover, a chimeric β2/β1 cytoplasmic tail containing the membrane-proximal 11 residues of β1 and the distal 36 residues of β1 bound minimally to β3-endonexin. Also, no interaction was observed between β3-endonexin and the cytoplasmic tail of α1β1 (Fig. 6). This lack of interaction with integrin tails other than β3 was not likely to be due to insufficient expression of these tails in yeast. Expression of the α1β1 and β2 fusion proteins was confirmed by immunoblotting with tail-specific antibodies (Fig. 1, B and C). Altogether, these results indicate that β3-endonexin binds selectively to the β3 cytoplasmic tail, probably due to recognition of membrane-distal sequences unique to β3.

Further support for this conclusion was obtained by studying a point mutant of the β3 cytoplasmic tail, S752→P (Table I). In human platelets, this mutation is associated with a bleeding disorder due to defective agonist-induced activation of fibrinogen binding to α1β3 (14). Moreover, CHO cells expressing this mutant exhibit markedly reduced spreading and focal adhesion formation following adhesion to fibrinogen (16). When the S752→P cytoplasmic tail was tested in the two-hybrid system, it showed a 64% reduction in binding to β3-endonexin compared to the wild-type β3 tail (P < 0.001) (Fig. 6).

To determine whether β3-endonexin exhibited a selective interaction with the β3 integrin subunit in vitro, a histidine-tagged form of β3-endonexin was expressed in bacteria and attached non-covalently to a metal chelation affinity resin. The β3 integrin subunit from a detergent extract of platelets was retained and eluted from this β3-endonexin affinity matrix, while the β1 integrin subunit from the same cells was not (Fig. 7). Thus, studies in yeast and with recombinant β3-endonexin in vitro indicate that the binding of this polypeptide to the β3 integrin tail is structurally specific.

Tissue and Cellular Expression of β3-Endonexin

In order to begin to assess the significance of β3-endonexin expression in cells, PCR of several cDNA libraries was carried out using oligonucleotides specific for β3-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 β3-endonexin was confirmed.

Next, platelets and a mononuclear fraction of peripheral blood leukocytes were examined by immunoblotting to
characterize β₃-endonexin expression at the protein level. Using a rabbit anti-peptide antiserum specific for the putative carboxy terminus of β₃-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 β₃ integrin subunit. Designated β₃-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 β₃-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 β₃-endonexin and detergent-solubilized β₃ from platelets or human umbilical vein endothelial cells. (b) mRNA specific for β₃-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 β₃ integrins. (c) Binding of β₃-endonexin to the β₃ cytoplasmic tail was structurally specific: A Ser -> Pro mutation at position 752 of the β₃ tail caused markedly reduced binding to β₃-endonexin, and the cytoplasmic tails of the β₁ and β₂ integrin subunits failed to bind. Moreover, a cDNA related to β₃-endonexin was cloned from B lymphocytes that encoded a larger polypeptide containing an extra 59 amino acids at the carboxy terminus of β₃-endonexin. When expressed in the yeast system, it failed to bind to the cytoplasmic tails of β₁, β₂, or β₃.

Expression of β₃-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 β₃-endonexin found evidence for this mRNA in B lymphocytes, brain and placenta, again suggesting a wide tissue distribution. In addition, β₃-endonexin mRNA

Figure 7. Specific interaction between recombinant β₃-endonexin and the β₃ integrin subunit. As described in Materials and Methods, an affinity matrix was prepared containing histidine-tagged β₃-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 immuno-

Figure 8. Detection of β₃-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 β₃-endonexin. Note that both platelets (100 μg protein, or 4 × 10⁷ 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 × 10⁹ platelets/lane) and a bacterially expressed recombinant form of β₃-endonexin (3 ng/lane) were also analyzed using antiserum specific for the GST/β₃-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 β3 integrin expression appears relatively restricted (e.g., endothelial cells, platelets, monocytes/macrophages, osteoclasts and certain lymphocyte subsets), the apparent wide tissue distribution of β3-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 β3-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. β3-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 antibodies. Based on the immunoreactivity of recombinant β3-endonexin and β3-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 β3 integrin molecules per platelet (63). Preliminary attempts to examine whether β3-endonexin co-immunoprecipitates with β3 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 β3-endonexin interacts with and modulates the functions of β3 integrins within cells.

**Structural Specificity of the Interaction between β3-Endonexin and the β3 Cytoplasmic Tail**

Divalent cations are essential for adhesive ligand binding to β3 integrins and for αβ subunit association (9, 21). In the present study, a GST/β3-endonexin–derived fusion protein bound specifically to detergent-solubilized β3 from platelets, whether or not CaCl2 was present to maintain the αtββ3 complex or EDTA was present to dissociate it. Thus, unlike extracellular integrin ligands, the binding of β3-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 β3-endonexin and the β3 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 α3β2 and insulin receptor substrate-1, a protein that mediates insulin signaling by specifically binding to several intracellular targets. This interaction required the β3 subunit but not α3.

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 β1 tail that bind α-actinin (54) and another membrane-proximal linear sequence in β1 and β3 that binds pp125 FAK (Otey, A. C., 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 αtββ3. Cells expressing wild-type αtββ3 adhered to fibrinogen, exhibited tyrosine phosphorylation of pp125 FAK and underwent spreading. Cells expressing a truncated form of β3 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. 90:1–86). Other studies have identified an NPXY sequence in the β1 or β3 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 β3-endonexin bound only to the cytoplasmic tail of β3. The specificity of this interaction is underscored by the inability of the larger clone 28-related polypeptide to interact with the β3 tail (Fig. 6). Neither the β2 tail nor a chimeric tail made up of the membrane–proximal 11 residues of β3 and the distal 36 residues of β1 interacted with β3-endonexin. Since the sequences of the membrane-proximal portions of the three β subunits are very similar (Table 1), residues unique to the distal portion of the β3 tail must be responsible for binding. This notion is supported by two other observations. First, interaction of the β3 tail with β3-endonexin in the yeast system was markedly reduced when proline was substituted for serine at β3 residue 752 (Fig. 6). Second, recent studies have demonstrated that the human β3 integrin subunit expressed as αtββ3 complex in CHO cells binds to a β3-endonexin affinity column. In contrast, a β3 subunit lacking the COOH-terminal 39 residues does not bind (Eigenthaler, M., S. J. Shattil, and M. H. Ginsberg, unpublished observations).

Both αtββ3 and αβ3 are involved in integrin-mediated signaling (63). Of note in this context, β3572 mutant appears critical for this process in both integrins. Platelets from individuals homozygous for the S572→P mutation do not bind soluble fibrinogen due to defective agonist-induced conversion of αtββ3 to a high affinity state (14). This same mutation abolishes αtββ3-mediated spreading of CHO cells on fibrinogen (16) and αβ3-mediated clot retraction by melanoma cells (Chen, Y., and M. Ginsberg, unpublished observations). It is intriguing, therefore, that β3-endonexin binds at or near a region of the β3 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 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.

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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. Argov, W. S., S. Suzuki, H. Arau, 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. G. Klier, and D. A. Cheresh. 1994. Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibronectin and von Willebrand factor. Proc. Natl. Acad. Sci. USA. 84:6471–6475.

6. Chien, C.-T., P. L. Bartlet, R. Stern glanz, and S. Fields. 1991. A method to identify and clone proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA. 88:9578–9582.

7. Cobb, B. S., M. D. Schaller, T.-H. Lee, and J. T. Parsons. 1994. Stable association of pp60csrc and p90rsk with the focal adhesion-associated protein kinase, pp125FAD. Mol. Cell. Biol. 14:4765–4775.

8. Coller, B. S., D. A. Cheren, E. Asch, and U. Selkon. 1991. Platelet vitronectin receptor expression differentiates Iraqi-Jewish from Arab patients with Glanzmann thrombasthenia in Israel. Blood. 77:75–83.

9. D’Souza, S. E., T. A. Haas, R. S. Potrovica, 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 β3 subunit: cation placement is involved in ligand binding. Cell. 79:659–667.

10. Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other cytokines. Science (Wash. DC). 264:1415–1421.

11. Deveraux, J. P., H. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12:387–395.

12. Diamond, M. S., and T. A. Springer. 1994. The dynamic regulation of integrin adhesiveness. Curr. Biol. 4:506–517.

13. 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.

14. Edwards, J. B., J. D. Delori, and J. Mallet. 1991. Oligodexosaminouronucleotide-like signal 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.

15. Elledge, S. J., T. T. Mulligan, S. W. Ramer, M. Spotswood, and R. W. Davis. 1991. YES: A functional DNA binding vector for the isolation of genes by complementation of yeast and Escherichia coli mutations. Proc. Natl. Acad. Sci. USA. 88:1713–1725.

16. Felding-Habermann, B., and D. A. Cheresh. 1995. Vitronectin and its receptors. Curr. Opin. Cell Biol. 7:647–650.

17. Feramisco, J. R., D. B. Glass, and E. G. Krebs. 1980. Optimal spatial requirements for the localization of basic residues in peptide substrates for the cAMP-dependent protein kinase. J. Biol. Chem. 255:4340–4345.

18. Fields, S. 1993. The two-hybrid system to detect protein-protein interactions. METHODS: A Comp. Methods Enzymol. 5:116–124.

19. Fields, S., and O. Song. 1989. A novel genetic system to detect protein-protein interactions. Nature (Lond.). 340:245–246.

20. Coller, B. S., D. A. Cheresh, E. Asch, and U. Selkon. 1991. Platelet vitronectin receptor expression differentiates Iraqi-Jewish from Arab patients with Glanzmann thrombasthenia in Israel. Blood. 77:75–83.

21. Haimovich, B., L. Lipfert, J. S. Brugge, and S. J. Shattil. 1993. Tyrosine phosphorylation and cytoskeletal reorganization in platelets are trig-

22. Damell, J. E., Jr., I. M. Kerr, and G. R. Stark. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other cytokines. Science (Wash. DC). 264:1415–1421.
54. Otey, C. A., G. B. Vasquez, K. Burridge, and B. W. Erickson. 1993. Map-
49. Lukashev, M. E., D. Sheppard, and R. Pytela. 1994. Disruption of integrin
47. Law, S. K. A., J. Gagnon, J. E. K. Hildreth, C. E. Wells, A. C. Willis, and
55. Pavalko, F. M., and C. A. Otey. 1994. Role of adhesion molecule cytoplas-
57. Rabinowieh, H., W. Lin, A. Amoscato, R. B. Herberman, and T. L. White-
56. Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological se-
53. O'Toole, T. E, Y. Katagiri, R. J. Faull, K. Peter, R. Tamura, V. Quaranta,
52. Miyamoto, S., S. K. Akiyama, and K. M. Yamada. 1995. Synergistic roles
50. Matsuno, H., J. M. Stassen, J. Vermylen, and H. Deckmyn. 1994. Inhibition
46. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly
45. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hy-
44. Kozak, M. 1991. Structural features in eukaryotic mRNAs that modulate
43. Jung, J. U., H. Lee, H. R. Sheng, and J. A. Wilkins. 1992. Interaction of the
42. Jänne, P. A., J. Dedhar. 1994. Cell attachment to extracellular matrix substrates is inhib-
41. Dedhar, S. M., and S. M. Banerjee. 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Jr.
40. Shattil, S. J. 1995. Function and regulation of the β3 integrins in hemostasis and vascular biology. Thromb. Haemost. 74:149-155.
39. 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. 260:1107-11114.
38. Shaw, L. M., and A. M. Mercurio. 1993. Regulation of αβ₃ integrin laminin receptor function by the cytoplasmic domain of the α₃ subunit. J. Cell Biol. 123:1017-1025.
37. Stupack, D. G., C. Shen, and J. A. Wilkins. 1992. Induction of α₅β₃ integrin-mediated attachment to extracellular matrix in β1 integrin (CD29)-negative B cell lines. Exp. Cell Res. 203:443-448.
36. 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.
35. Ullrich, A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. Cell. 61:203-212.
34. Vuori, K., and E. Ruoslahti. 1994. Association of insulin receptor sub-
33. Schlesinger, J. H., and S. A. Flanagan. 1990. Identification of αα₃ integrin laminin receptor function by the cytoplasmic domain of the α₃ subunit. J. Cell Biol. 123:1017-1025.
32. Stupack, D. G., C. Shen, and J. A. Wilkins. 1992. Induction of α₅β₃ integrin-mediated attachment to extracellular matrix in β1 integrin (CD29)-negative B cell lines. Exp. Cell Res. 203:443-448.
31. 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.
30. Ullrich, A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. Cell. 61:203-212.
29. Vuori, K., and E. Ruoslahti. 1994. Association of insulin receptor sub-
28. Schlesinger, J. H., and S. A. Flanagan. 1990. Identification of αα₃ integrin laminin receptor function by the cytoplasmic domain of the α₃ subunit. J. Cell Biol. 123:1017-1025.