A Conserved Sequence Motif in the Integrin β3 Cytoplasmic Domain Is Required for Its Specific Interaction with β3-Endonexin

(Received for publication, September 13, 1996, and in revised form, January 9, 1997)

Martin Eigenthaler‡§, Liane Höfferer‡%, Sanford J. Shattil‡, and Mark H. Ginsberg‡**

From the Departments of ‡Vascular Biology and §Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037

Integrin signaling is mediated by interaction of integrin cytoplasmic domains with intracellular signaling molecules. Recently, we identified a novel 111-amino acid polypeptide, termed β3-endonexin, which interacts selectively with the integrin β3 cytoplasmic domain. In the present study we conducted a systematic mutational analysis of both the integrin β3 cytoplasmic domain and β3-endonexin to map sites required for interaction. The interaction of the full-length β3 integrin subunit with β3-endonexin in vitro required the β3 cytoplasmic domain. In a yeast two-hybrid system, both membrane-proximal and membrane-distal residues of the β3 cytoplasmic domain were necessary for interaction with β3-endonexin. In particular, the membrane-distal NITY motif at β3 756–759 was critical for the interaction. Exchange of β3 residues 756–759 (NITY) for the corresponding residues in β1 (NPKY) endowed the β1 cytoplasmic domain with the ability to interact with β3-endonexin. Conversely, exchange of the NPKY motif at β3 772–775 for the NITY motif in β3 abolished interaction of this chimeric cytoplasmic domain with β3-endonexin. Because the NITY motif is present in the β3 but not the β1 cytoplasmic domain, these results explain the selective interaction of this cytoplasmic domain with β3-endonexin. In addition, deletional analysis suggested that a core 91-residue sequence of β3-endonexin is sufficient for specific binding to the β3 cytoplasmic domain. These studies have identified a cytoplasmic domain sequence motif that specifies an integrin-specific protein-protein interaction.

Integrins are heterodimeric adhesion receptors composed of α and β transmembrane subunits (1). The β3 integrin subfamily includes αInbβ3 and αbβ3. αInbβ3 is largely specific for cells of the megakaryocytic lineage and is required for platelet aggregation (2). αbβ3 is found in a number of cell types, including endothelial cells, vascular smooth muscle cells, and monocytes, where it is involved in the regulation of cell adhesion, migration, proliferation, and cell survival (2–4). The adhesive function of the integrin can be regulated by the cell (inside-out signaling), and the ligand-bound and clustered form of the integrin triggers cellular responses (outside-in signaling) (5).

Integrin signaling and the interaction of integrin cytoplasmic domains with intracellular signaling molecules are still poorly understood. For example, inside-out signaling is believed to involve interactions of integrin cytoplasmic domains with specific cytoplasmic elements. Studies of patients with rare defects in platelet aggregation or of recombinant human integrins expressed in various mammalian cells are consistent with a role for the β3 cytoplasmic domain in inside-out and outside-in signaling (6–9). In addition, the over-expression of isolated β cytoplasmic domains can disrupt or promote integrin signaling, conceivably by binding to factors that interact with the β cytoplasmic domain (10–12).

A few proteins have been found to interact directly with integrin cytoplasmic domains, and most of these studies have been performed in vitro (13). The αInb cytoplasmic domain has been reported to interact with calreticulin through a membrane-proximal GFFKR sequence that is highly conserved among all integrin α subunits (14). Cytohesin-1 has recently been identified as a specific integrin β3 cytoplasmic domain binding protein (15), and direct interaction of filamin with this tail has been described (16). Other β cytoplasmic domains have been found to interact with α-actinin, talin, pp125FAK, and integrin-linked kinase (17–21). However, these latter interactions may not be specific for one particular β cytoplasmic domain. Because most cells contain many different integrins, it is possible that cytoplasmic domain-specific binding proteins may exist that play a role in determining the specificity of integrin responses.

Recently, we identified a novel 111-amino acid polypeptide called β3-endonexin, which is present in platelets, mononuclear lymphocytes, and several tissues, which interacts selectively with the β3 cytoplasmic domain in a yeast two-hybrid system (22). As a first step in assessing the potential biological functions of β3-endonexin, we have conducted a systematic mutational analysis of both the β3 cytoplasmic domain and β3-endonexin to identify amino acid residues required for this unique interaction.

MATERIALS AND METHODS

Antibodies—Monoclonal antibodies against the extracellular domain of the human β3 integrin subunit (monoclonal antibody 15) or the extracellular domain of the hamster β1 integrin subunit (monoclonal antibody 7E2) have been described (23, 24). A monoclonal antibody against the extracellular domain of human β3 (antibody B-D15) was purchased from BioSource International (Camarillo, CA). A monoclonal antibody against the GALA DNA binding domain was purchased from Clontech Laboratories (Palo Alto, CA).

This paper is available on line at http://www-jbc.stanford.edu/jbc/
The cytoplasmic domain of the integrin β1 subunit is required for interaction with β3-endonexin. As described under “Materials and Methods,” an affinity matrix was prepared with histidine-tagged β3-endonexin bound covalently to a metal chelation resin. CHO cell lysates (0.7 ml) containing the full-length β3 integrin subunit (A), the β3Δ717 subunit (B), or the full-length β1 subunit (C) were incubated with the affinity resin for 12 h at 4 °C. After washing, proteins were eluted from the resin in 0.7 ml of lysis buffer containing 1 M imidazole. Lysate (lanes 1), last column wash (lanes 2), and resin eluate (lanes 3) were then analyzed by SDS-PAGE under nonreducing conditions, transferred to nitrocellulose, and immunoblotted with monoclonal antibodies specific for the extracellular domain of β3 (A and B) or β1 (C). β3 integrins appear as a double band representing β3 and a precursor form of this integrin subunit. The autoradiographs shown are representative for three separate experiments. Integrin bands on the Western blots were analyzed by densitometry. Integrins in the elution fraction were expressed as percentages of integrins in the lysate (which was defined as 100%). The data represent the means ± S.E. of three separate experiments.

Fig. 1. The cytoplasmic domain of the integrin β1 subunit is required for interaction with β3-endonexin. 

Cell Culture and Transfection—CHO cells stably expressing α1β3 or α1α1β3Δ717 (a β3 truncation mutant lacking the cytoplasmic domain) have been described (25). CHO cells stably expressing human integrin β1 paired with endogenous hamster α subunits were obtained by transfecting β1 cDNA in pCDM8 using neomycin resistance as a selectable marker. Expression of human β3 or β1 integrins was quantified by Western blot technique using monoclonal antibodies 15 (5 μg/ml) or B-D15 (1:400), respectively (22). The intensity of the bands of the β subunits on scanned images of these Western blots was quantified by densitometry on a Macintosh computer using NIH Image software (version 1.55). All labeled bands were analyzed within the linear range for the chemiluminescence reaction.

Binding of Integrin Cytoplasmic Domains to a β3-Endonexin Affinity Matrix—Bacterial expression of β3-endonexin as an amino-terminal histidine-tagged protein and preparation of a Nickel-agarose β3-endonexin affinity resin were performed as described (22). Stably transfected CHO cell lines expressing approximately equivalent amounts of the indicated human integrins were lysed in 0.4 ml of lysis buffer containing 50 mM Tris, pH 7.2, 0.9% NaCl, 1 mM CaCl2, 1% Triton X-100, and protease inhibitors (100 units/ml aprogin, 0.5 mM leupeptin, 4 mM Pefabloc, 0.1 mM E64) at 4 °C for 30 min while shaking. Cell lysates were spun in a microfuge at 14,000 rpm for 20 min at 4 °C. Then 0.35 ml of each supernatant were added to 0.35 ml of the lysis buffer containing no Triton, such that the final concentration of Triton was 0.5%. Each diluted lysate was batch-incubated with 1 ml of packed volume of β3-endonexin affinity resin for 12 h at 4 °C while shaking. Resins were then packed in columns and washed with 15 ml of lysis buffer, and bound proteins were eluted into 0.7-ml fractions of lysis buffer after the addition of 1 M imidazole. Fractions were collected and run on SDS-PAGE gels under nonreducing conditions. After electrophoresis and Western blotting using the antibodies specific for the extracellular portion of the integrins, proteins were visualized by fluorography. The autoradiographs shown are representative for three separate experiments. The data represent the means ± S.E. of three separate experiments.

RESULTS AND DISCUSSION

Interaction of the β3 Integrin Subunit with β3-Endonexin Requires the Integrin Cytoplasmic Domain—Previous studies have shown that β3-endonexin binds to the cytoplasmic domain of the β3 integrin subunit when the isolated cytoplasmic domain is expressed in a yeast two-hybrid system (22). This interaction is structurally specific, because it was not observed with the cytoplasmic domains of the integrin α1β1 or β3 subunits. Therefore, we conducted a mutational analysis using the yeast two-hybrid system to identify sites within these two proteins that are necessary for this binary interaction. Prior to undertaking such an analysis, experiments were performed to determine the specificity with which β3-endonexin binds to the β3 cytoplasmic domain in the context of an intact integrin. CHO cell lines were prepared that stably expressed the human α1β1 subunit paired with either human β3 or β3Δ717, a truncated form of β3 missing the entire cytoplasmic domain except for a putative membrane-proximal lysine residue (β3 Lys716) (33). As an additional control, a CHO cell line expressing human β1 paired with endogenous hamster α subunits was prepared. Analyzing these cell lines by SDS-PAGE and Western blotting using antibodies specific for the extracellular portion of human β3 or β1 showed that they expressed similar levels of their respective β subunits (data not shown). As a source of β3-endonexin for in vitro binding studies, a histidine-tagged form of β3-endonexin was expressed in bacteria and coupled noncovalently to a metal chelation affinity resin. Then the yeast vector, pACT, was used to construct fusions of wild-type or truncated forms of β3-endonexin cDNAs with the GAL4 activation domain (22–27). Amino-terminal truncations of β3-endonexin were cloned into pACT by PCR using a 5′ primer containing a BamHI restriction site and a 3′ primer encoding an appropriate stop codon. PCR products were cut with BamHI and PstI and ligated into pACT. Carboxy-terminal truncated forms of β3-endonexin were constructed by splice overlap PCR, and the PCR products were cut with BamHI and Xhol and ligated into BamHI- and Xhol-cut pACT.

Yeast Two-Hybrid System—Yeast strain maintenance and transformation were described (22). The yeast two-hybrid system was used to quantify the extent of binary interactions between β3-endonexin and integrin β cytoplasmic domains. Protein expression in transformed yeast was analyzed by SDS-PAGE and Western blotting using a specific monoclonal antibody for the GAL4 DNA binding domain (28). The extent of expression of the reporter gene, lacZ, was determined by quantitative liquid β-galactosidase assay (29) and taken as an indicator of the strength of interaction between the two fusion proteins (29–32). A one-tailed Student’s t test for unpaired samples was used for statistical calculations.
equal aliquots of affinity resin were incubated with equal volumes of detergent extracts from each of the three CHO cell lines, and the amount of human integrin β subunit retained on the β3-endonexin resin was determined by Western blotting (Fig. 1). Approximately 53% of the full-length β3 integrin subunit that was applied to the β3-endonexin affinity matrix was retained, compared with only 14% of β3Δ717 and 5% of the β3 integrin subunit. The differences between β3 and β3Δ717 and between β3 and β3Δ717 and between β3 and β1 were significant (p < 0.006). In contrast, the difference between β3Δ717 and β1 was not (p > 0.05) (Fig. 1). Furthermore, using a monoclonal antibody specific for hamster β3 (monoclonal antibody 7E2) to detect β3 in CHO cell lysates, no significant binding of endogenous hamster β3 integrin to β3-endonexin could be detected by Western blotting (data not shown). These results demonstrate that interaction of the full-length β3 integrin subunit with β3-endonexin is mediated by the integrin cytoplasmic domain.

Both Membrane-proximal and Membrane-distal Residues of the β3 Cytoplasmic Domain Are Necessary for Interaction with β3-Endonexin—To study the structural basis for the interaction of the β3 cytoplasmic domain and β3-endonexin in more detail, a series of truncation mutants of the β3 cytoplasmic domain (Table I) were fused in-frame to the carboxyl terminus of the GAL4 DNA binding domain and co-expressed in yeast with β3-endonexin fused to the GAL4 DNA activation domain. In this system, the extent of expression of the reporter gene, lacZ, can be taken as an indication of the strength of interaction between the two fusion proteins (29–32). Deletion of the carboxyl-terminal 1–3 amino acids from the cytoplasmic domain of β3 (β3Δ762, β3Δ761, or β3Δ760) caused no significant reduction in its interaction with β3-endonexin (Fig. 2, C). In fact, deletion of the carboxyl-terminal threonine residue actually increased apparent binding (p < 0.0002) (Fig. 2). However, deletion of the carboxyl-terminal 4 residues from the β3 cytoplasmic domain (β3Δ759) reduced binding to β3-endonexin, whereas deletion of 8 residues from the carboxyl terminus of the β3 cytoplasmic domain (β3Δ755) virtually abolished binding (Fig. 2). This result was not due to lack of expression of any of the GAL4 DNA binding domain fusion proteins (Fig. 3).

To further elucidate the role of the carboxyl terminus of the β3 cytoplasmic domain for interaction with β3-endonexin, we attached the carboxyl-terminal 7 residues of β3 to the α1b cytoplasmic domain (α1bΔ589–1008/β3 756–762) (Table I). Although expressed in yeast, this construct did not interact with β3-endonexin (Figs. 2 and 3), indicating that the carboxyl-terminal region of the integrin β3 cytoplasmic domain is necessary but not sufficient for the interaction with β3-endonexin.

To determine whether residues in the membrane-proximal region of the β3 cytoplasmic domain are necessary for the interaction with β3-endonexin, the effects of amino-terminal truncations of the β3 cytoplasmic domain were assessed. Deletion of even a single residue from the amino terminus (Lysβ716) caused a more than 92% reduction in binding to β3-endonexin (p < 0.0001). Additional constructs containing deletions of 3, 6, or 11 residues from the amino terminus of the β3 cytoplasmic domain also failed to interact (data not shown).

Taken together with the results of the carboxyl-terminal cytoplasmic domain truncations, these data indicate that both the amino and carboxyl termini of the β3 cytoplasmic domain are required for the interaction with β3-endonexin. This could mean that both membrane-proximal and membrane-distal regions of the β3 cytoplasmic domain are directly involved in binding and/or that overall folding of the cytoplasmic domain is a critical determinant of its interaction with β3-endonexin.

The NITY Motif at β3 756–759 Is Critical for Interaction of the β3 Cytoplasmic Domain with β3-Endonexin—Despite the fact that the β3 and β1 cytoplasmic domains exhibit high overall similarity (60% identical; 68% identical plus conservative substitutions) only the β3 cytoplasmic domain interacts with β3-endonexin (Fig. 1) (22). It is therefore of particular interest to identify the residues within the β3 cytoplasmic domain that account for the specific binding to β3-endonexin. Because the region of greatest dissimilarity between these two cytoplasmic domains is at the extreme carboxy terminus (Table I), we wondered if exchange of certain residues in this region would

| β3, 716 | β3, 762 |
|---------|---------|
| β3 wild type | KLLITI HDRKE FAKFEEARAKWDTAN NPLY KEATSTFT NITY RGT |
| β3Δ759 | KLLITI HDRKE FAKFEEARAKWDTAN NPLY KEATSTFT NITY RGT |
| β3Δ751 | KLLITI HDRKE FAKFEEARAKWDTAN NPLY KEATSTFT NITY RGT |
| β3 717–755/β3 772–778 | KLLITI HDRKE FAKFEEARAKWDTAN NPLY KEATSTFT NITY RGT |
| β3Δ757P | KLLITI HDRKE FAKFEEARAKWDTAN NPLY KEATSTFT NITY RGT |
| β3Δ732 | KLLITI HDRKE FAKFEEARAKWDTAN NPLY KEATSTFT NITY RGT |
| β3 wild type | KLLMII HDRRE FAKFEEKMNAWDTGE NPIY KSATTVV NPKY EGK |
| β3 755–762 | KLLMII HDRRE FAKFEEKMNAWDTGE NPIY KSATTVV NPKY EGK |
| β3P7731 | KLLMII HDRRE FAKFEEKMNAWDTGE NPIY KSATTVV NPKY EGK |
| α1b, 989–1008/β3 756–762 | KVGFFKRRPPLEEDDEEGQ NITY RGT |

**Table I** Amino acid sequences of selected integrin cytoplasmic domains used in the yeast two-hybrid system.
The interaction between \(\beta_3\) integrin and \(\beta_3\)-endonexin

**Fig. 3.** Expression of integrin cytoplasmic domain constructs as GAL4 DNA binding domain fusion proteins in yeast. Yeast cells were transfected with cDNAs encoding integrin cytoplasmic domain constructs in pGAL9 vector as described under “Materials and Methods.” Expression of GAL4 DNA binding domain fusion proteins was assessed by SDS-PAGE and Western blot analysis using a monoclonal antibody against the GAL4 DNA binding domain. Each lane contains the protein derived from 2.0 A_{600} units of yeast cells. Ab, antibody.

**Fig. 4.** Binding of \(\beta_3\)-endonexin to chimeras of integrin \(\beta_3\) and \(\beta_3\) cytoplasmic domains. Interactions between \(\beta_3\)-endonexin and integrin \(\beta_3\) or \(\beta_3\) cytoplasmic domain chimeras were studied in yeast. Carboxyl-terminal amino acid sequences of the cytoplasmic domain constructs are shown. Residues from \(\beta_3\) are indicated by white boxes, and residues from \(\beta_3\) by filled boxes. The data represent the means ± S.E. of a minimum of six single colonies from two independent transformations.

Influence the ability of these domains to interact with \(\beta_3\)-endonexin. Indeed, exchange of carboxyl-terminal 7 residues of the \(\beta_3\) cytoplasmic domain amino acids 756–762 for the corresponding region of the \(\beta_3\) cytoplasmic domain resulted in a strong interaction of the new chimeric \(\beta_3/\beta_3\) cytoplasmic domain with \(\beta_3\)-endonexin (Fig. 4). In fact, exchange of only 4 \(\beta_3\) residues 756–759 (NITY) for the corresponding residues in \(\beta_3\) (NPKY) or exchange of even a single amino acid of \(\beta_3\) (Ile757) for Pro773 in \(\beta_3\) (\(\beta_3/P773I\)) now endowed the \(\beta_3\) cytoplasmic domain with the ability to interact with \(\beta_3\)-endonexin (Fig. 4). Conversely, swapping the carboxyl-terminal 7 residues of the \(\beta_3\) cytoplasmic domain into the corresponding region of \(\beta_3\) or exchange of the NPKY motif at \(\beta_3/\beta_3\) 772–775 for the NITY motif in \(\beta_3\) abolished interaction of these chimeric cytoplasmic domains with \(\beta_3\)-endonexin. Moreover, introduction of Pro773 of \(\beta_3\) into the \(\beta_3\) cytoplasmic domain, resulting in \(\beta_3/P773I\), decreased binding to \(\beta_3\)-endonexin by more than 70% (p < 0.004) (Fig. 4). These data could not be accounted for by differences in levels of expression of the \(\beta_3/I757P\) and \(\beta_3/P773I\) fusion proteins (Fig. 3). These data indicate that the \(\beta_3\) linear sequence, 756NITY, is critical for the interaction of the \(\beta_3\) cytoplasmic domain with \(\beta_3\)-endonexin.

To examine the importance of individual components of the NITY motif further, alanine was substituted individually for each amino acid in this motif. Alanine substitution at Ile757 (\(\beta_3/I757A\)) or Tyr759 (\(\beta_3/Y759A\)) in \(\beta_3\) resulted in a 75 or 92% reduction in interaction of the \(\beta_3\) cytoplasmic domain with \(\beta_3\)-endonexin, respectively (Fig. 5). On the other hand, more conservative substitutions of Ile757 (\(\beta_3/I757L\)) for Tyr759 (\(\beta_3/Y759F\)) or alanine substitutions of Asn756 (\(\beta_3/N756A\)) or Thr758 (\(\beta_3/T758A\)) had little or no effect on binding (Fig. 5). This demonstrates that Ile757 and Tyr759 in \(\beta_3\) are critical residues for interaction with \(\beta_3\)-endonexin. As shown in Fig. 6, despite its dissimilarity with the corresponding region in several other human \(\beta\) cytoplasmic domains, the NITY motif is highly conserved among \(\beta_3\) integrins of various species. Thus, we propose that this motif is responsible for the \(\beta\) subunit specificity of \(\beta_3\)-endonexin.

Given the key role of the NITY motif in this interaction, it should be noted that this motif is also important for localization of \(\beta_3\) integrins to focal adhesions and for integrin signaling (34). For example, deletion of \(\beta_3\) residues 756YRGT (\(\beta_3/\Delta 756\)) significantly reduced cell spreading and recruitment of \(\beta_3\) integrins to focal adhesion sites, whereas deletion of \(\beta_3\) residues 757ITYRGT (\(\beta_3/\Delta 757\)) totally abolished cell spreading and formation of focal contacts (34). Retaining Ile757 partially pre-
served these functions. The point mutation β3 Y759A also significantly reduced cell spreading and β3 integrin recruitment to focal adhesions (34). Thus, the region of the β3 cytoplasmic domain that is necessary for interaction with β3-endonexin is also necessary for post-adhesive functions of β3 integrins. Whether β3-endonexin modulates the adhesive or post-adhesive functions of β3 integrins remains to be determined. In this context, preliminary studies show that overexpression of β3-endonexin in an αβ3/β3 CHO cell model system increases the affinity state of integrin αβ3/β3 (35).

Recent studies have demonstrated tyrosine phosphorylation of the integrin β3 cytoplasmic domain as well as calpain-induced cleavage at various sites within the β3 cytoplasmic domain during thrombin-induced activation of platelets (36, 37). Although the latter might be expected to cause release of β3-endonexin from the β3 cytoplasmic domain, we cannot predict the effects of tyrosine phosphorylation on the binding of β3-endonexin, and we have not observed tyrosine phosphorylation of the β3 cytoplasmic domain in the yeast system.2

Both Amino- and Carboxyl-terminal Residues of β3-Endonexin Are Required for Interaction with the β3 Cytoplasmic Domain—As a first approach to identify the residues within β3-endonexin that are critical for binding to the β3 cytoplasmic domain, we investigated the binding of carboxyl-terminal and amino-terminal truncation mutants of β3-endonexin to this cytoplasmic domain. The carboxyl terminus of β3-endonexin contains three heptad repeats that may form coiled-coil structures (residues 89–111) (38). Removal of part of the last of these repeats by deleting 2 amino acids from the carboxyl terminus of β3-endonexin (residues 110 and 111 of β3-endonexin, EN 82) decreased binding to the β3 cytoplasmic domain more than 80% (Fig. 7). Deletion of 7 or more amino acids (corresponding to at least one heptad repeat) from the carboxyl terminus of β3-endonexin abolished binding completely (Fig. 7). Furthermore, deletion of 9 or 20 residues from the amino term-

---

2 H. Kashiwagi and S. J. Shattil, unpublished data.
Interaction between $\beta_3$ Integrin and $\beta_3$-Endonexin

31. Yang, M., Wu, Z., and Fields, S. (1995) *Nucleic Acids Res.* **23**, 1152–1156
32. Yan, K., Kalyanaraman, V., and Gautam, N. (1996) *J. Biol. Chem.* **271**, 7141–7146
33. Williams, M. J., Hughes, P. E., O'Toole, T. E., and Ginsberg, M. H. (1994) *Trends Cell Biol.* **4**, 109–112
34. Ylanne, J., Huuskonen, J., O'Toole, T. E., Ginsberg, M. H., Virtanen, I., and Gahmberg, C. G. (1995) *J. Biol. Chem.* **270**, 9550–9557
35. Kashiwagi, H., Eigenthaler, M., Ginsberg, M. H., and Shattil, S. J. (1996) *Blood* **88**, Suppl. 1, 140 (abstr.)
36. Law, D. A., Nannizzi-Alaimo, L., and Phillips, D. R. (1996) *J. Biol. Chem.* **271**, 10811–10815
37. Du, X., Saito, T. C., Tsubuki, S., Indig, F. E., Williams, M. J., and Ginsberg, M. H. (1995) *J. Biol. Chem.* **270**, 26146–26151
38. Cohen, C., and Parry, D. A. (1990) *Trends Biochem. Sci.* **11**, 245–248
39. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) *Annu. Rev. Cell Biol. Dev. Biol.* **11**, 549–599