Integrin β Cytoplasmic Domains Differentially Bind to Cytoskeletal Proteins

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Integrin cytoplasmic domains connect these receptors to the cytoskeleton. Furthermore, integrin-cytoskeletal interactions involve ligand binding (occupancy) to the integrin extracellular domain and clustering of the integrin. To construct mimics of the cytoplasmic face of an occupied and clustered integrin, we fused the cytoplasmic domains of integrin β subunits to an N-terminal sequence containing four heptad repeat sequences. The heptad repeats form coiled coil dimers in which the cytoplasmic domains are parallel dimerized and held in an appropriate vertical stagger. In these mimics we found 1) that both conformation and protein binding properties are altered by insertion of Gly spacers C-terminal to the heptad repeat sequences; 2) that the cytoskeletal proteins talin and filamin are among the polypeptides that bind to the integrin β1A tail. Filamin, but not talin binding, is enhanced by the insertion of Gly spacers; 3) binding of both cytoskeletal proteins to β1A is direct and specific, since it occurs with purified talin and filamin and is inhibited in a point mutant (β1A(Y788A)) or in splice variants (β1B, β1C) known to disrupt cytoskeletal associations of β1 integrins; 4) that the muscle-specific splice variant, β1D, binds talin more tightly than β1A and is therefore predicted to form more stable cytoskeletal associations; and 5) that the β7 cytoplasmic domain binds filamin better than β1A. The structural specificity of these associations suggests that these mimics offer a useful approach for the analysis of the interactions and structure of the integrin cytoplasmic face.

Integrins, a large family of heterodimeric adhesion receptors, physically link the extracellular matrix to cytoskeletal elements within a cell (1–3). The structure of integrin cytoplasmic domains plays a key role in these linkages (4–6). Thus, deletion or mutations in the cytoplasmic domain block the co-localization of integrins with cytoskeletal elements such as vinculin and talin (7, 8). Furthermore, isolated β cytoplasmic domains, joined to other transmembrane proteins, target them to focal adhesions, sites of integrin-cytoskeletal linkages (9, 10). Thus, integrin β cytoplasmic domains link these receptors to the cytoskeleton.

Integrin α cytoplasmic domains limit certain cytoskeletal interactions of their β partners. In intact integrins, focal adhesion targeting usually requires binding of a ligand to the extracellular domain (10), a conformational change in the extracellular domain of the integrin (11) and an intact β tail (8). Ligand-independent focal adhesion targeting is induced by deletion of the α cytoplasmic domain (8, 12), suggesting that the α tail blocks cytoskeletal interaction with the β tail. Ligand binding appears to remove this block, permitting the β tail to target to focal adhesions. Consequently, isolated β cytoplasmic domains resemble ligand-occupied integrins in their interactions with the cytoskeleton.

The majority of integrin ligands are multivalent and cause receptor clustering after binding (13). Simple antibody-mediated clustering can initiate biochemical signals (14) and local accumulation of tensin and pp125Fβ2 near the clustered integrins (15). Nevertheless, accumulation of the full complement of cytoskeletal proteins (15) and functional cytoskeletal interaction (16, 17) requires ligand occupancy. Consequently, cytoskeletal interactions of integrins involve both receptor clustering and ligand occupancy.

We previously proposed a strategy for the chemical synthesis of structural models of the cytoplasmic domain of multisubunit transmembrane receptors (18). The cytoplasmic domains of integrin αβ2 were covalently linked via a helical coiled coil. In the present study we have extended and tested this approach by preparation of recombinant mimics of the cytoplasmic face of occupied and clustered integrins. By using recombinant proteins, we avoided limitations of polypeptide length and modest yield encountered in the initial synthetic approaches. Occupancy was mimicked by use of isolated β cytoplasmic domains; clustering was mimicked by use of covalent homodimers of these domains. Helical coiled coil architecture provided the desired parallel topology and vertical stagger of the tails. We now report that the binding of cytoplasmic proteins to mimics of the β1A tail is sensitive to conformational changes induced by the insertion of Gly residues C-terminal to the helical coiled coil moieties. The cytoskeletal proteins talin and filamin are among the polypeptides that bind to the β1A tail. Filamin, but not talin binding, is enhanced by the insertion of a Gly spacer. Binding of both proteins is direct and specific, since it occurs with the purified proteins and is inhibited in a point mutant (β1A(Y788A)) or in splice variants (β1B and β1C) known to disrupt cytoskeletal associations of β1 integrins (21–23). The muscle-specific splice variant β1D (24–26) binds talin better than β1A, and the β7 cytoplasmic domain binds filamin more tightly than β1A. Thus, these constructs can be used to analyze integrin class and splice variant-specific interactions with cytoskeletal proteins.

MATERIALS AND METHODS

Antibodies and cDNAs—Antibodies for immunoblot analysis were either obtained commercially (goat serum against filamin (Sigma), rabbit serum against α-actinin (Sigma), monoclonal antibodies against

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The abbreviations used are: PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazineethanesulfonic acid.
additional Gly residues inserted between the coiled coil and the cytoplasmic domain. Insertion of glycines sharply reduced the minima at 208 and 222 nm. Consequently, predicted α-helical content in the protein model was reduced from 65 to 36% (Fig. 3). 29% of the amino acids in the construct are in the four heptad repeats; therefore, 36% helical content is consistent with most of the helical structure being limited to these repeats. Thus, the Gly insertion appears to eliminate α-helical structure induced in the cytoplasmic domain by the direct linkage to the coiled coil sequence.

Cytoplasmic Domain Conformation Affects Cytoskeletal Protein Binding—To assess functional consequences of the structural changes induced by the Gly insertions, we produced protein models of the β1A cytoplasmic domain with one, two, and three additional Gly residues inserted after the heptad repeat motif (G2-, G3-, G4-β1A) and compared these with the G1-β1A construct. As an additional control, we produced a variant of the G4-β1A peptide, with a Tyr to Ala substitution in the membrane-proximal NPXY-motif (G4-β1A(Y788A)) (Fig. 1). This mutation interferes with focal adhesion targeting (22) and activation (29) of integrins. The purified proteins were bound via their N-terminal His tag to a Ni²⁺ resin and used in affinity chromatography experiments with lysates of human platelets. Marked changes in protein binding properties were observed as a consequence of the Gly insertions (Fig. 4): In Coomassie Blue-stained gels, polypeptides migrating at 45, 56, 58, 140, and 240 kDa bound only to the mimics with Gly insertions. Moreover, comparison to the cell lysate (ly in Fig. 4A) showed that this binding was selective. Immunoblotting identified the 240-kDa and 45-kDa proteins as filamin and actin, respectively (Fig. 5). The enriched 56-, 58-, and 140-kDa polypeptides have not been identified. They failed to react with antibodies specific for pp60⁵⁺⁺ (Fig. 5), paxillin, pp125⁵⁺⁺, α-actinin, and vinculin (data not shown) in immunoblotting experiments. Talin bound to both the G1- and G4-β1A protein. In addition, we used biotinylated extracts to enhance sensitivity of protein detection. In such extracts, we confirmed talin binding to the G1-β1A protein and enhanced filamin binding with the Gly insertions (Fig. 4B). Thus, the structural changes (Fig. 3) induced by the insertion of Gly between the coiled coil motif and the integrin cytoplasmic domain sequence alter interactions of these proteins with cellular components.

To establish specificity of the binding of cellular proteins to G4-β1A, we examined the effect of structural changes known to
Peroxidase and chemiluminescence. Note equal loading with the model proteins; panel B depicts biotinylated proteins as detected by streptavidin.

Thus, alterations of the G4-β1 tail, which block cytoskeletal interactions, also reduce binding to talin and filamin.

**TABLE I**

| Protein | Expected average mass (Da) | Measured mass (Da) |
|---------|---------------------------|--------------------|
| G1-β1A | 10,843.4                  | 10,844 ± 1.5       |
| G2-β1A | 10,900.4                  | 10,900 ± 1.0       |
| G3-β1A | 10,957.5                  | 10,957 ± 2.6       |
| G4-β1A | 11,014.6                  | 11,016 ± 1.0       |
| G1-β1D | 11,311.9                  | 11,313 ± 3.1       |
| G4-β1D | 11,483.1                  | 11,484 ± 1.6       |
| G1-β7  | 11,551.9                  | 11,551 ± 2.5       |
| G4-β7  | 11,723.0                  | 11,723 ± 1.7       |
| G4-β1A (Y788A) | 10,922.5                 | 10,921 ± 1.2       |
| G4-β1B | 10,045.5                  | 10,044 ± 1.0       |
| G4-β1C | 14,219.4                  | 14,217 ± 1.7       |
| G4-αT  | 6,354                     | 6,354 ± 1.2        |
| G4-αTβ1 | 7,864                     | 7,864 ± 1.6        |
| G4-αT β1A | 17,367                  | 17,368 ± 2.0       |

**FIG. 3.** Circular dichroism spectroscopy of G1-β1A and G4-β1A model proteins. Polypeptides were dissolved at 110 μg/ml in 50 mM sodium borate, pH 7.0, and analyzed in an AVIV 60DS spectropolimeter. After CD spectroscopy, protein concentrations were determined by sodium borate, pH 7.0, and analyzed in an AVIV 60DS spectropolarimeter.

**FIG. 4.** Gly insertions change the protein binding properties of the model proteins. Ni²⁺ resin was loaded with G1-β1A (G1), G2-β1A (G2), G3-β1A (G3), or G4-β1A (G4). As a control, the resin was loaded with a construct containing a β1 mutation known to disrupt cytoskeletal associations of integrins (G4-β1A(Y788A) (YA)). Unloaded Ni²⁺ resin (Co) was also used. Depicted are reduced SDS-PAGE (4–20%) analyses of proteins bound from biotinylated platelet lysates and of the lysate itself (Ly). Panels A and C represent Coomassie Blue-stained gels; panel B depicts biotinylated proteins as detected by streptavidin peroxidase and chemiluminescence. Note equal loading with the model proteins shown in panel C.

**FIG. 5.** Western blot analysis of samples from the experiments shown in Fig. 4 with antibodies specific for filamin, talin, actin, and pp60 src. Lanes: Ly, lysate; Co, unloaded Ni²⁺ resin; YA, G4-β1A(Y788A).

The β1A cytoplasmic domain is dimerized in these model proteins. To assess the role of β1A dimerization in cytoskeletal protein binding, we constructed a heterodimer containing a single G4-β1A subunit. The other subunit (G4-αT) was identical to G4-β1A through the four glycines. C-terminal to the glycines, we placed a KLGFFKR sequence, representing the conserved membrane proximal region of integrin cytoplasmic domains. The structure of the heterodimer was confirmed by mass spectroscopy (Table I). The G4-αTβ1A heterodimer bound filamin and talin to the same extent as the G4β1A dimer (Fig. 6). Specificity of binding in this experiment was confirmed by the failure of an α1B cytoplasmic domain construct to bind either cytoskeletal protein. Thus, the dimerization of the β1A cytoplasmic domain is not essential for talin and filamin binding.

**FIG. 6.** Role of β1A dimerization in talin and filamin binding. Ni²⁺ resin was loaded with the heterodimer G4-αTβ1A or homodimers of G4-β1A or G4-αT. Bound proteins from platelet extracts were separated on 4–20% SDS-polyacrylamide gels under reducing conditions, transferred to nitrocellulose membranes, and stained with antibodies specific for talin or filamin. In addition, Coomassie Blue-stained gels were examined to assess loading of the resin. Note the migration position of the G4-αT construct (αT) and the similar quantities of G4-β1A or G4-αTβ1A subunit in each lane.

Differential Binding of Talin and Filamin to Integrin β1D and β7 Cytoplasmic Domains—To extend our affinity chromatography results, we prepared similar G1 and G4 model proteins of the splice variant, β1D, and the β7 integrin subunits (Fig. 1). When incubated with platelet lysates, the β1D constructs bound more talin, and β7 constructs bound more filamin compared with β1A (Fig. 7A). In addition, these differences in binding were consistently observed when lysates of a human T-cell leukemia cell line (Jurkat), a human fibrosarcoma cell line (HT 1080), or differentiated myotubes derived from a mouse myoblast cell line (C2C12) were used for affinity chromatography (Fig. 7B). Moreover, stronger binding of the β1D constructs to talin and of the β7 constructs to filamin was observed both with the G1 (data not shown) as well as the G4 variants of the model proteins, indicating that structural
Changes induced by Gly insertions do not alter the relative strengths of these interactions.

Interactions with Purified Talin and Filamin—To learn whether the observed interactions with talin and filamin in the cell extracts are direct, we used purified preparations of these proteins. The relative binding of purified filamin and talin to the model proteins was similar to that observed with cell lysates (Fig. 8). Specifically, the model proteins bound more talin, and β7 constructs bound more filamin than β1A (Fig. 8). In addition, binding of both cytoskeletal proteins to the G4-β1A(Y788A) construct and to the G4-β1B and G4-β1C variants was markedly reduced compared with G4-β1A (Fig. 8). Moreover, G4 constructs of β1A, β1D, and β7 integrin cytoplasmic domains bound more purified filamin than the corresponding G1 constructs. However, the G1-β7 model protein still bound more filamin than G4-β1A or G4-β1D (data not shown). A densitometric analysis of the Coomassie Blue-stained gels indicated that the β1D construct bound 9 times more talin, and the β7 construct bound 8.4 times more filamin than the β1A model protein (Fig. 8). Thus, filamin and talin bind differentially to the different integrin β subunit cytoplasmic domain constructs.

Discussion

To study integrin-cytoskeletal interactions, we designed structural mimics of the cytoplasmic face of occupied and clustered integrins. We joined integrin cytoplasmic domains to an N-terminal parallel coiled coil dimer, and we found that the introduction of Gly spacers between the coiled coil and the β1A integrin tail leads to a change in the conformation of the protein. Concomitantly, the polypeptide binding properties are altered, resulting in an increase in the strength of the interaction with the cytoskeletal protein, filamin. Talin and filamin binding to these structural mimics are direct, specific, and biologically relevant. These interactions are observed with purified talin and filamin and are abrogated in splice variants and a mutant that disrupts cytoskeletal interactions of the intact integrin. The β1D integrin tail binds talin, and the β7 integrin tail binds filamin much more tightly than the β1A cytoplasmic domain. Thus, we provide insight into integrin class and splice variant-specific interactions with cytoskeletal proteins and their dependence on the conformation of integrin β cytoplasmic domains.

The model proteins described here have the topology of clustered integrin cytoplasmic domains. The tropomyosin-derived heptad repeats employed (30) are known to form parallel dimers (31, 32). Moreover, they were dimeric and disulfide-bonded (Fig. 2C) via their N termini. Thus, they must have been parallel, and the integrin tails must have been in the desired vertical stagger. The conformation of these constructs was sensitive to Gly insertions between the coiled coil and the integrin tails. The coiled coil structure formed by the heptad repeats should not be disrupted by these Gly insertions, and the estimated 36% residual helix in the G4-β1A construct is consistent with maintenance of the coiled coil. Consequently, the Gly insertions may have disrupted induced helical structure in the β1A tail in the G1-β1A construct.

These model proteins permitted direct detection of talin and filamin binding to integrin β1A subunit cytoplasmic domains. Binding of both talin and filamin was specific since it was sensitive to alterations in the β tail sequence known to block cytoskeletal associations in vivo. Previously, equilibrium gel filtration was required to demonstrate low affinity β1A integrin-talin interactions (33). Furthermore, affinity chromatography or yeast two-hybrid screens with linear β1A cytoplasmic domains.
Taken together, these data suggest that the folded structure of variant, yet the major differences in amino acid sequence be-
creased binding of the muscle-specific splice variant, clustered, and actin filaments insert end-on into submembra-
In contrast, talin concentrates at sites in which integrins are 
grins and contribute their role in lymphocyte homing (40, 41).

We found that talin binding was increased in the 
1A sequences. Their marked differences in cy-
1A, it was reported to bind to the 

The differential binding of β cytoplasmic domains to talin and filamin suggests functionally different linkages of integ-
rins with the cytoskeleton. Talin and non-muscle filamin are 

The model proteins described here provide new approaches 
provide powerful tools for the elucidation of such structures.

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