Integrins play an important role in regulating cell adhesion, motility, and activation. In an effort to identify intracellular proteins expressed by activated T cells that interact with the cytoplasmic domain of β₁-integrin (CD29), we used the β₁-integrin cytoplasmic domain as bait in the yeast two-hybrid system. Here we report that the cytoplasmic domain of β₁-integrin specifically interacts with the cytoskeletal protein filamin. This interaction required all but the most carboxyl-terminal three residues of the cytoplasmic domain of β₁, and the carboxyl-terminal 477 residues of filamin containing the terminal 45–96-residue tandem repeats of filamin. To verify this interaction in vivo, we showed that filamin specifically coprecipitated with β₁ in mammalian cells. We also showed that recombinant filamin chimeric proteins were able to bind to the β₁ cytoplasmic domain in vitro. We observed that a subset of single point mutations in the cytoplasmic domain of β₁, which had been previously reported to impair its function, disrupt the interaction between β₁ and filamin. Taken together, these findings suggest that the interaction between β₁ and filamin, which in turn can bind actin, provides a mechanism for the interaction of this cell surface receptor with cytoskeletal proteins and that this interaction plays a role in normal receptor function.

The integrins are a family of closely related heterodimeric cell surface receptors, which play an important role in mediating cell-extracellular matrix and cell-cell interactions (1). The integrins are composed of an α chain, which is associated with a β chain. To date, 16 α chains and 8 β chains have been characterized at the molecular level. Each α and β chain combination results in a cell surface receptor with a unique ligand specificity. Although both chains are required for ligand binding, it appears that the interaction of integrins with cytoplasmic proteins is predominantly mediated by cytoplasmic residues of the β chain. Typically, integrins are expressed in a low affinity conformation on the surface of resting cells. Cell activation can result in increased numbers of receptors; however, more importantly, cellular activation results in a transient change in receptor affinity that is critical for receptor-ligand interaction.

The β₁-integrin (CD29) family of receptors is composed of at least 10 members by virtue of the association of β₁ with at least 10 different α chains, α₁–α₁₀, and αυ. This family of receptors is involved in mediating interactions between cells and extracellular matrix proteins including laminin, fibronectin, collagen, and vitronectin. We have been particularly interested in studying the α₁β₁ (VLA-4, or very late antigen-4; CD49d/CD29) receptor. This protein has been shown to have at least two ligands, the extracellular matrix protein fibronectin (2–5) and VCAM-1 (INCAM-110, CD106) (6), a member of the immunoglobulin superfamily expressed on the surface of activated endothelial cells.

Both β₁ integrins and VCAM-1 have been shown to be critical for normal development. Mice lacking β₁ are not viable (7), and studies with chimeric mice lacking expression of β₁ in blood cells and hematopoietic cells indicate that hematopoietic stem cells lacking β₁ can differentiate normally but are unable to populate the fetal liver (8). On the other hand, the majority of mice lacking VCAM-1 have abnormal placental development and die at the embryo stage within 3 days (9, 10); however, a small number of mice lacking VCAM-1 survive and become fertile, and their only observed defect is an elevation in the number of peripheral blood mononuclear leukocytes. In mature individuals the interaction between VLA-4 and its two ligands plays an important role in regulating the recruitment and migration of leukocytes to sites of inflammation. Interactions between VLA-4 and VCAM-1 are in part responsible for mediating the adhesion of leukocytes to activated vascular endothelial cells (11, 12), while interactions between VLA-4 and fibronectin play a role in allowing leukocytes to efficiently migrate to the sites of inflammation following leukocyte diapedesis (13–15).

The contribution of α₄ to the recruitment of leukocytes to sites of inflammation in mature animals has been demonstrated by examining the effects of anti-α₄ mAbs1 in various models of immune disease. Antibodies directed against α₄, VLA-4, and VCAM-1 have been shown to block antigen-induced eosinophil and T cell infiltration into bronchial tissue (16, 17). Using an experimental autoimmune encephalomyelitis model, anti-α₄ antibodies have been shown to prevent the accumulation of lymphocytes in the central nervous system and the development of experimental autoimmune encephalomyelitis (18). Antibodies to α₄ and VCAM-1 have also been shown to delay the onset of diabetes in an adoptive transfer model of insulin-dependent diabetes mellitus and block lymphocyte infiltration of the islets of Langerhans (19). Anti-α₄ antibodies also lower leukocyte infiltration at sites of inflammation in an in vivo contact hypersensitivity model (20, 21). These effects may reflect blocking of the interaction between VLA-4 and/or α₄/β₂-integrins and their respective ligands. In a similar experiment, a soluble VCAM-1-Ig fusion protein was shown to inhibit the onset of insulin-dependent diabetes in an adoptive transfer model and delay the appearance of islet-specific leukocyte infiltration (22).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: BMS-PRI, P. O. Box 4000, Princeton, NJ 08543. Tel.: 609-252-5780; Fax: 609-252-6058; E-mail: lood@bms.com.

1 The abbreviations used are: mAb, monoclonal antibody; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; SD, Specimen Diluent.
We have been interested in the interaction between VLA-4 and VCAM-1 and have examined the role of this interaction in cell adhesion and signaling. Several groups, including ours, have found that VCAM-1 and fibronectin binding to VLA-4 can modulate lymphocyte activation, cell adhesion and motility. Both the cell activation-driven inside-out signaling events that modulate VLA-4-mediated cell adhesion (23–27) and the outside-in signaling events that modulate lymphocyte activation, adhesion, and migration (28–30) are for the most part mediated by interactions between the cytoplasmic domain of β1 and a number of intracellular proteins (1, 31, 32). In this report, we describe the use of the yeast two-hybrid system to identify an interaction between the cytoskeletal protein filamin and the cytoplasmic domain of β1. This interaction was verified by examining the ability of filamin to coimmunoprecipitate with β1 in the Jurkat T cell line. Additionally, we show that recombinant filamin chimeric proteins were able to bind to the β1 cytoplasmic domain in vitro. We also show results of experiments that define the minimal regions of β1 and filamin required for their interaction. Finally, we show that mutations in the cytoplasmic domain of β1, which had previously been shown to disrupt β1 function, prevent filamin binding. Taken together, this work suggests that the molecular interaction between β1 and filamin plays a critical role in the function of the β1-integrin in vivo.

MATERIALS AND METHODS

Yeast Strains and Library Screen—The cytoplasmic domain of the β1-integrin subunit (CD29) containing residues 757–805 was obtained by PCR and cloned in-frame into the GAL4 DNA binding domain (GAL4bd; bait) vector pGBT9 (CLONTECH). The GAL4bd-β1 vector was cotransformed with an activated human leukocyte cDNA expression library fused to the activation domain of GAL4 (GAL4ad; prey) in the pGAD10 vector (CLONTECH) into the yeast strain HF7c using the method of Schiestl and Gietz (33). Prey plasmids were recovered from transformants that exhibited β-galactosidase activity in the yeast strain and were screened for their interaction. Finally, we show that mutations in the cytoplasmic domain of β1, which had previously been shown to disrupt β1 function, prevent filamin binding. Taken together, this work suggests that the molecular interaction between β1 and filamin plays a critical role in the function of the β1-integrin in vivo.

| Filamin residues | PCR primer set |
|-----------------|----------------|
| 2225–2647       | CGC GGA TCC GTC CTT TCC AGT TCA CCG TG |
| 2323–2647       | CAC GTG GAC TCA GCC CAC AAC GCG |
| 2418–2647       | CGG TCA GAC TCA GAG ATA TGA CAG CCC AGG TG |
| 2514–2647       | CAG GTC GAC TCA GGG CAC CAC AAG GCG |
| 2550–2647       | CAG GTC GAC TCA GAG ATA TGA CAG CCC AGG TG |
| 2171–2550       | CAG GTC GAC TCA GCC GGC CCC ATG GTC GGG GGC ACA GGT GGC |
| 2171–2516       | CAG GTC GAC TCA GAC TTT GCA CCT GAA GGG GCT CCC AAT |
| 2171–2420       | CAG GTC GAC TCA CCC AGC CCC GTG TTG GGT GGA GGT GCC CCC AAT |
| 2171–2325       | CAG GTC GAC TCA CCC AGC CCC GTG TTG GGT GGA GGT GCC CCC AAT |
| 2171–2230       | CAG GTC GAC TCA CCC AGC CCC GTG TTG GGT GGA GGT GCC CCC AAT |

We have been interested in the interaction between VLA-4 and VCAM-1 and have examined the role of this interaction in cell adhesion and signaling. Several groups, including ours, have found that VCAM-1 and fibronectin binding to VLA-4 can modulate lymphocyte activation, cell adhesion and motility. Both the cell activation-driven inside-out signaling events that modulate VLA-4-mediated cell adhesion (23–27) and the outside-in signaling events that modulate lymphocyte activation, adhesion, and migration (28–30) are for the most part mediated by interactions between the cytoplasmic domain of β1 and a number of intracellular proteins (1, 31, 32). In this report, we describe the use of the yeast two-hybrid system to identify an interaction between the cytoskeletal protein filamin and the cytoplasmic domain of β1. This interaction was verified by examining the ability of filamin to coimmunoprecipitate with β1 in the Jurkat T cell line. Additionally, we show that recombinant filamin chimeric proteins were able to bind to the β1 cytoplasmic domain in vitro. We also show results of experiments that define the minimal regions of β1 and filamin required for their interaction. Finally, we show that mutations in the cytoplasmic domain of β1, which had previously been shown to disrupt β1 function, prevent filamin binding. Taken together, this work suggests that the molecular interaction between β1 and filamin plays a critical role in the function of the β1-integrin in vivo.
plexes were recovered by addition of 50 cells and prepared in the pGAD10 prey vector to screen for in conjunction with a cDNA library generated from activated T

reader at dual wavelengths of 450 and 630 nm. The density was measured on an enzyme-linked immunosorbent assay plate probed with mAbs against CD29 or filamin followed by 125I-labeled (Amersham Pharmacia Biotech), electrophoresed on SDS-polyacrylamide gels containing 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, and 50 mM Tris-HCl, were prepared by lysis of matched mAb 2B12 (35) served as a negative control. Jurkat cell lysates precipitated from the Jurkat T cell line with mAbs directed against CD29 bodies against actin and tropomyosin (Sigma).

**Table II**

| CD29 residues | PCR primer set |
|---------------|----------------|
| 757–803       | CCG GAA TTC AAG CTT TTA ATG ATA ATT CAT GAC |
| 776–803       | CCG CTG CAG TGG CTA CTT TGG TCG ATC GAG GTC TGG |
| 757–780       | CCG GAA TTC ATG GTA GAC AAA TGG GAC ACG GGT GAA |
| 757–790       | CCG CTG CAG TAA ATG CAG GAA TAT CAA GAG ACT TTA |
| 757–796       | CCG CTG CAG TGA ACA ATG ATC AAC TTG CAA GTC TGG |
| 757–800       | CCG GAA TTC AAG CTT TTA ATG ATA ATT CAT GAC |

**Table III**

| CD29 point mutant | PCR primer set |
|-------------------|----------------|
| D764V             | GCT TTT AAT GAT GAT AAT TAA TG AAG GGA GCT TGC |
| N785I             | GAA CAC GGG TGA AAT TCC TAT TTA TAA GAG |
| N785D             | CTC TTA TAA AAT GGA AGG TCT TCA CCC GTG TCC |
| P786A             | GAC ACA GGG TGA AAA TGC TAT TTA TAA GAG |
| Y788E             | CAC GGG TAA CTC TCT TCT CAG TAT GAG TTT CAC CGG TGG |
| Y788A             | CAC GGG TAA CTC TCT TCT CAG TAT GAG TTT CAC CGG TGG |
| Y788F             | CAC GGG TAA CTC TCT TCT CAG TAT GAG TTT CAC CGG TGG |
| N797I             | CAG TGG TTA CAG CAC TCT TCT CAG TAT GAG TTT CAC CGG TGG |
| N797D             | CTG AAG TGG GAA TTA GGG TCA TAC TGG TGG TGG TGG TGG TGG |
| Y800E             | CAA CTG TGG TCA ATC TAA GGA GAA GAA AAT GAG TAC TGC |
| Y800A             | GAA CTG TGG TCA ATC TAA GGA GAA GAA AAT GAG TAC TGC |
| Y800F             | GAA CTG TGG TCA ATC TAA GGA GAA GAA AAT GAG TAC TGC |

Burlingame, CA) diluted 1:5000 in PBS/1× SD for 1 h at 20 °C, and then washed 10 times with PBS/Tween and treated with the EIA chromogen reagent (Genetics Systems, Seattle, WA) for 15 min at 20 °C. The colorimetric reaction was quenched with 1 × H2SO4, and the optical density was measured on an enzyme-linked immunosorbent assay plate reader at dual wavelengths of 450 and 630 nm.

Coommunoprecipitation—β3-Integrins and filamin were immunoprecipitated from the Jurkat T cell line with mAbs directed against CD29 (Upstate Biotechnology, Inc., Lake Placid, NY) and filamin (Chemicon, Temecula, CA) respectively, as described previously (34). The isotype matched mAb 2B12 (35) served as a negative control. Jurkat cell lysates were prepared by lysis of ∼2 × 107 cells in 0.5 ml of modified radioimmunoprecipitation assay buffer containing 1% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5, supplemented with proteinase inhibitors. Cell lysates were incubated with 5 μg of the indicated mAb for 2 h at 4°C. Immunocomplexes were recovered by addition of 50 μl of protein A-Sepharose beads (Amersham Pharmacia Biotech), electrophoresed on SDS-polyacrylamide gels, and blotted onto nitrocellulose membranes. Blots were probed with mAbs against CD29 or filamin followed by 123I-labeled anti-mouse IgG (36). Duplicate blots were similarly probed with antibodies against actin and tropomyosin (Sigma).

**RESULTS**

**Use of the Yeast Two-hybrid System to Identify Intracellular Proteins That Bind β1**—In an effort to identify cytoplasmic proteins that bind to the cytoplasmic domain of β1, we subcloned a cDNA fragment encoding the complete cytoplasmic domain of β1 into the pG&B89 bait vector. This vector was used in conjunction with a cDNA library generated from activated T cells and prepared in the pGAD10 prey vector to screen for β1 interactive proteins using the yeast two-hybrid system as described previously. A screen of approximately 2 × 108 transformants resulted in the isolation of two cDNA clones that encoded a polypeptide which could specifically bind to the cytoplasmic domain of β1. The recovered prey plasmids were tested in a cotransformation assay with GAL4bd-β1 vector but not the control heterologous baits, as determined using the β-galactosidase activation assay (data not shown). Characterization of these clones revealed that they were identical and encoded the carboxyl-terminal 4.5 tandem repeats including the hinge domain. The filamin fragment found to determined using the filamin subunit (CD61), which is highly homologous to the cytoskeletal protein filamin (residues 2171–2647). We also observed that the cytoplasmic domain of the β3-integrin subunit (CD61), which is highly homologous to the cytoplasmic domain of β3, could weakly interact with the isolated filamin clones in the yeast two-hybrid system (data not shown).

Electron microscopy studies have shown that filamin is an elongated homodimeric, wishbone-shaped structure (37). Each monomer consists of an amino-terminal actin binding domain followed by 24 tandem repeats, each ~96 amino acids in length, which provide for the elongated structure of filamin. The carboxy-terminal 65 amino acids of the terminal tandem repeat contains a self-assembly sequence that allows for dimerization. This repeat is separated from the previous 23 repeats by an ~34-amino acid “hinge” domain. The filamin fragment found to be capable of interacting with β1 contained the carboxy-terminal 4.5 tandem repeats including the hinge domain.
Independent Verification of the \(\beta_1\)-Filamin Interaction—We took two approaches to verify the \(\beta_1\)-filamin interaction. First, we examined the ability of a recombinant 6×His-filamin chimeric protein, containing the carboxyl-terminal 478 amino acids of filamin, to bind to a 47-amino acid synthetic polypeptide corresponding to the \(\beta_1\) cytoplasmic domain. As shown in Fig. 1A, the interaction between 6×His-filamin and the polypeptide corresponding to the \(\beta_1\) cytoplasmic domain was specific and saturable, and was not blocked by the addition of 0.5% bovine serum albumin (data not shown). Neither 6×His-IL-6 nor 6×His-CD40 control proteins bound to the \(\beta_1\) cytoplasmic domain peptide. Binding of purified \(\alpha\)-actinin to the \(\beta_1\) cytoplasmic domain is shown in Fig. 1B. Similar results were obtained using a recombinant flag-epitope-tagged filamin chimeric protein (data not shown).

Second, we performed coimmunoprecipitation assays to test whether \(\beta_1\) interacted with filamin in mammalian cells. Cell lysates from the T cell line Jurkat were incubated with a control mAb or a mAb against \(\beta_1\), and coprecipitating filamin was detected by Western blotting with a mAb against filamin. As shown in Fig. 2, filamin specifically coprecipitated with \(\beta_1\). Neither \(\beta_1\) nor filamin was detected in the control immunoprecipitation. We were unable to detect \(\beta_1\) in anti-filamin mAb immunoprecipitates. This is not unlike the results reported by Sharma et al. (38), who were able to coimmunoprecipitate filamin with an anti-\(\beta_2\) mAb, but were unable to coimmunoprecipitate \(\beta_2\) with anti-filamin mAbs. Neither actin nor the actin-binding protein tropomyosin was detected in anti-\(\beta_1\) mAb immunoprecipitates (data not shown).

Use of \(\beta_1\) and Filamin Truncations to Identify the Minimal Requirements for the \(\beta_1\)-Filamin Interaction—To define the minimal \(\beta_1\) and filamin protein fragments required for the productive interaction of these two proteins, we prepared a series of cDNA constructs containing different fragments of each of these two proteins and examined their ability to interact using the yeast two-hybrid system. A total of 10 filamin constructs were prepared (Fig. 3). Five constructs encoded filamin fragments progressively lacking tandem repeat domains from the amino terminus. The other five encoded filamin fragments progressively lacking tandem repeat domains from the carboxyl terminus. None of the truncated filamin fragments interacted with \(\beta_1\) (Figs. 3 and 4). We occasionally observed a weak interaction of \(\beta_1\) with the construct containing the amino-terminal 3.5 tandem repeat (residues 2171–2518) but lacked the hinge and carboxyl-terminal tandem repeat that contains the dimerization domain (data not shown).

Likewise, we prepared a series of 5 constructs containing different fragments of the cytoplasmic domain of \(\beta_1\) (Fig. 5) and examined their ability to bind filamin using the yeast two-hybrid system. We observed that, with one exception, truncations in the cytoplasmic domain of \(\beta_1\) prevented the \(\beta_1\)-filamin interaction. The only truncation that was tolerated was the...
removal of the carboxyl-terminal three residues (EGK) of β1 (Figs. 5 and 6).

Identification of β1 Residues Required for the β1-Filamin Interaction—A number of individual residues in the cytoplasmic domain of β1 have been shown to be critical for the function of β1-containing integrins (39, 40). However, the molecular basis for these effects is not known. We wished to examine if residues that had been previously shown to be important for β1 function were involved in the β1-filamin interaction. A series of 12 single point mutations in the cytoplasmic domain of β1 were prepared (Fig. 7), and their ability to bind to filamin was examined using the yeast two-hybrid system. Our mutagenesis studies predominantly targeted the two NPXY motifs found in the cytoplasmic domain of β1. The Asn residue in both motifs

| Filamin Constructs | Residues | Interaction |
|--------------------|----------|------------|
| NH2 | 2171-2647 | +++ |
| NH2 | 2225-2647 | - |
| NH2 | 2323-2647 | - |
| NH2 | 2418-2647 | - |
| NH2 | 2514-2647 | - |
| NH2 | 2550-2647 | - |
| NH2 | 2171-2550 | - |
| NH2 | 2171-2516 | +/- |
| NH2 | 2171-2420 | - |
| NH2 | 2171-2325 | - |
| NH2 | 2171-2230 | - |

Fig. 3. Summary of the interaction of the β1 cytoplasmic domain with the filamin amino-terminal and carboxyl-terminal truncation constructs. Y190 yeast strain cotransformants containing the Gal4bd-β1 vector and the indicated Gal4ad-filamin constructs (numbers denote amino acid residues of the filamin primary sequence) were selected on medium lacking leucine and tryptophan, and individual colonies were patched onto filters and assayed for β-galactosidase activity as described under “Materials and Methods.” The relative blue color development of individual patches was scored visually, with +++ indicating dark blue patches and – indicating white patches; +/- indicates the appearance of faint blue patches in some of the colonies examined. The filled black ovals represent tandem repeat domains 20–24; the line between tandem repeat domains 23 and 24 represents the hinge region.

Fig. 4. Interaction of β1 cytoplasmic domain with amino-terminal and carboxyl-terminal truncated filamin proteins. Saccharomyces cerevisiae strain Y190 was cotransformed with Gal4bd-β1 vector and the indicated Gal4ad-filamin constructs (numbers denote amino acid residues of the filamin primary sequence) and transformants were selected on medium lacking leucine and tryptophan. Four independent colonies from each transformation were patched onto filters and assayed for β-galactosidase activity as described under “Materials and Methods.”
interaction between $\beta_1$ and filamin could be independently demonstrated by coimmunoprecipitation studies that showed that filamin specifically coprecipitated with $\beta_1$ in the T cell line Jurkat. Neither actin nor the actin-binding protein tropomyosin was detected in parallel $\beta_1$ immunoprecipitates, supporting our evidence of a direct interaction between $\beta_1$ and filamin. Additionally, fusion proteins containing the carboxy-terminal 478 amino acids of filamin specifically bound to a synthetic peptide containing the complete cytoplasmic domain of $\beta_1$ in vitro. Studies with a series of constructs encoding truncated $\beta_1$ and filamin polypeptides showed that the interaction between these two proteins in the yeast two-hybrid system required all but the most carboxy-terminal three amino acids of $\beta_1$ and the carboxy-terminal tandem repeats 20–24 of filamin that includes the self-association region. It is presently unclear if these are the minimal fragments required for $\beta_1$-filamin binding in vivo or if these are the minimal fragments required to detect the interaction using the yeast two-hybrid system. The occasional detection of a weak interaction between $\beta_1$ and the carboxy-terminal filamin truncation lacking the hinge and 24th tandem repeat may reflect a lack of robustness of the yeast two-hybrid system to detect very weak interactions. It is of interest to note that the glycoprotein Ib has also been shown to bind to filamin (41, 42), and the binding domain has been mapped to the amino-terminal end of filamin near the actin-binding site (43). Thus, filamin may associate with multiple membrane proteins that interact at discreet sites on filamin. Finally, we demonstrate that residues in the NPXY motifs in the cytoplasmic domain of $\beta_1$, which had been shown previously to play a key role in $\beta_1$ function, are critical for filamin binding. Of these residues, the Asn-785, Asn-797, and Tyr-788 residues appear to be the most important for binding. If the Asn-785 residue is replaced with Asp or Ile, the interaction between $\beta_1$ and filamin is reduced, while replacement of Asn-785 with Asp but not Ile reduced the interaction. Likewise replacing the Tyr residue in the first NPXY motif (Tyr-788) with Glu or Ala, but not Phe, prevented $\beta_1$-filamin interactions. On the other hand, similar changes at the second NPXY motif (Tyr-800) had no effect on the formation of $\beta_1$-filamin complexes. Replacement of the Pro residue in the first NPXY (Pro-786) with Ala reduced the $\beta_1$-filamin interaction (Figs. 7 and 8).

**DISCUSSION**

Elucidating the interactions between the cytoplasmic domain of the $\beta_1$ chain of the integrins with intracellular proteins is critical to our understanding of the molecular mechanisms that regulate integrin function. Here, we provide evidence that the cytoplasmic domain of $\beta_1$ can bind to the cytoskeletal protein filamin. The interaction was initially identified using the $\beta_1$ cytoplasmic tail as bait in a yeast two-hybrid screen. The interaction between $\beta_1$ and filamin could be independently demonstrated by coimmunoprecipitation studies that showed that filamin specifically coprecipitated with $\beta_1$ in the T cell line Jurkat. Neither actin nor the actin-binding protein tropomyosin was detected in parallel $\beta_1$ immunoprecipitates, supporting our evidence of a direct interaction between $\beta_1$ and filamin. Additionally, fusion proteins containing the carboxy-terminal 478 amino acids of filamin specifically bound to a synthetic peptide containing the complete cytoplasmic domain of $\beta_1$ in vitro. Studies with a series of constructs encoding truncated $\beta_1$ and filamin polypeptides showed that the interaction between these two proteins in the yeast two-hybrid system required all but the most carboxy-terminal three amino acids of $\beta_1$ and the carboxy-terminal tandem repeats 20–24 of filamin that includes the self-association region. It is presently unclear if these are the minimal fragments required for $\beta_1$-filamin binding in vivo or if these are the minimal fragments required to detect the interaction using the yeast two-hybrid system. The occasional detection of a weak interaction between $\beta_1$ and the carboxy-terminal filamin truncation lacking the hinge and 24th tandem repeat may reflect a lack of robustness of the yeast two-hybrid system to detect very weak interactions. It is of interest to note that the glycoprotein Ib has also been shown to bind to filamin (41, 42), and the binding domain has been mapped to the amino-terminal end of filamin near the actin-binding site (43). Thus, filamin may associate with multiple membrane proteins that interact at discreet sites on filamin. Finally, we demonstrate that residues in the NPXY motifs in the cytoplasmic domain of $\beta_1$, which had been shown previously to play a key role in $\beta_1$ function, are critical for filamin binding. Of these residues, the Asn-785, Asn-797, and Tyr-788 residues appear to be the most important for binding. If the Asn-785 residue is replaced with Asp or Ile, the interaction between $\beta_1$ and filamin is reduced, while replacement of Asn-785 with Asp but not Ile reduced the interaction. Likewise replacing the Tyr residue in the first NPXY motif (Tyr-788) with Glu or Ala, but not Phe, prevented $\beta_1$-filamin interactions. On the other hand, similar changes at the second NPXY motif (Tyr-800) had no effect on the formation of $\beta_1$-filamin complexes. Replacement of the Pro residue in the first NPXY (Pro-786) with Ala reduced the $\beta_1$-filamin interaction (Figs. 7 and 8).
either the $\alpha_5$ or $\beta_1$ subunit induced transmembrane aggregation of filamin in cultured fibroblasts. In a later study, Sharma et al. (38) showed by a communoprecipitation approach that filamin could associate with the cytoplasmic domain of $\beta_2$, a closely related integrin $\beta$ chain. These investigators generated three peptides corresponding to either the amino-terminal 24 amino acids of the cytoplasmic domain of $\beta_2$ (peptide N), the carboxyl-terminal 27 amino acids of the cytoplasmic domain of $\beta_2$ (peptide C), or a decapeptide (peptide N1), which is a subfragment of peptide N and contained residues 733–742 of $\beta_2$. These peptides were coupled to Sepharose and their ability to precipitate filamin examined. These investigators reported that only peptide N was capable of binding filamin. Our analysis of the $\beta_1$-filamin interaction using $\beta_1$ truncations is consistent with this finding. We also observed that removal of membrane proximal residues prevented the $\beta_1$-filamin interaction. Additionally, the finding that residue Asp-764, which is conserved in both $\beta_2$ and $\beta_1$ and is contained within the $\beta_2$, N peptide, contributes to the $\beta_2$-filamin interaction is consistent with our observation that residues in the amino-terminal region of the cytoplasmic domain of $\beta_2$ are required for filamin binding. However, unlike the $\beta_2$-filamin interaction, our data from the truncation and site-directed mutagenesis studies suggest that the interaction between $\beta_1$ and filamin requires residues located in the carboxyl-terminal region of the cytoplasmic domain of $\beta_1$. This difference may reflect intrinsic differences between the $\beta_1$- and $\beta_2$-integrin cytoplasmic tails. However, because of the high degree of homology between the two integrin tails, it seems more likely that the difference observed may result from differences in binding avidity of the yeast-based assay versus the solid-phase binding assay. It is interesting to note that the CD18 cytoplasmic domain has two NPXF motifs at the approximately same location as the NPXY motifs in $\beta_1$. Our mutagenesis studies indicate that the $\beta_1$-filamin interaction tolerates the replacement of Tyr with Phe, indicating that even though these motifs appear not to be as important to the CD18-filamin interaction as they are to the $\beta_1$-filamin interaction, they can support an interaction with filamin in the context of $\beta_1$. Furthermore, the ability of the $\beta_1$-filamin interaction to tolerate the replacement of Tyr with Phe in the NPXY motifs indicates that tyrosine phosphorylation is not required for the $\beta_1$-filamin interaction.

Several groups had previously reported on a series of $\beta_1$ cytoplasmic domain truncations and point mutants that affected different aspects of $\beta_1$ function. Marcantonio et al. (45) generated a series of $\beta_1$ cytoplasmic domain truncations and showed that $\beta_1$ lacking only the carboxyl-terminal four residues retained the ability to localize to focal adhesions, while larger truncations did not localize efficiently to focal adhesions. Additionally, Hayashi et al. (46) reported that deletions of

---

**Fig. 7. Summary of the interaction of the $\beta_1$ cytoplasmic domain point mutants with filamin.** As shown in Fig. 5, Y190 yeast strain cotransformants containing Gal4ad-filamin (amino acid residues 2171–2647) and the indicated Gal4bd-$\beta_1$ point mutant constructs were selected on medium lacking leucine and tryptophan and individual colonies were patched onto filters and assayed for $\beta$-galactosidase activity as described under “Materials and Methods.” The relative blue color development of individual patches was scored visually, with + + + indicating dark blue patches, + indicating light blue patches, and − indicating white patches. The numbers above the amino acid sequence represent the primary amino acid positions in $\beta_1$. Single amino acid residues indicate the positions and nature of each substitution. Dots represent unchanged amino acids.

**Fig. 8. Interaction of $\beta_1$ cytoplasmic domain point mutants with filamin.** S. cerevisiae strain Y190 was cotransformed with Gal4ad-filamin (amino acid residues 2171–2647) and the indicated Gal4bd-\$\beta_1$ constructs (numbers denote the location of the amino acid residue change in the $\beta_1$ primary sequence) and transformants were selected on medium lacking leucine and tryptophan. Four independent colonies from each transformation were patched onto filters and assayed for $\beta$-galactosidase activity as described under “Materials and Methods.”

| CD29 Cytoplasmic Domain Sequence | Mutant | Interaction |
|---------------------------------|--------|-------------|
|                                 |        |             |
| KLMIHDFRPEAKFRKGMNTGENTGIYFVQTEHK | WT     | +++         |
|                                 | D764V  | +           |
|                                 | N785I  | +++         |
|                                 | N785D  | +           |
|                                 | Y786A  | −           |
|                                 | Y788A  | −           |
|                                 | Y788F  | +           |
|                                 | Y797I  | +           |
|                                 | Y800E  | +++         |
|                                 | Y800A  | +++         |
|                                 | Y800F  | +++         |

| 1    | 1    | 1    |
|-------------------|------|------|
| D                 | T    | F    |
|                  |     |      |
| T                 |     |      |
|                  |     |      |
| F                 |     |      |
|                  |     |      |
greater than 5–15 residues from the carboxyl terminus of β1 prevented its ability to localize to focal adhesions. We observed that removal of the carboxyl-terminal three residues of β1 did not alter its ability to interact with filamin, while truncations of seven or more carboxyl-terminal residues prevented its interaction with filamin. Reszka et al. (39) examined the role of individual residues in the cytoplasmic domain of β1 on localization to focal adhesions. These investigators systematically targeted all residues in the cytoplasmic domain of β1. Among the residues which they found impaired the localization of β1 to focal adhesions were Asp-764 and the Asn-785, Pro-786, and Tyr-788 residues in the first NPXY motif of β1 and the Asn-797 and Tyr-800 residues in the carboxyl-terminal NPXY motif of β1. We observed that with the exception of changes at the Tyr residues in the carboxyl-terminal NPXY motif (Tyr-800) mutations which blocked focal adhesion localization also inhibited the interaction between β1 and filamin. Interestingly, in a previous study these investigators showed that a more conservative substitution of Tyr-788 with Phe did not prevent β1 localization to focal adhesions (46). As noted above, we found that the β1-filamin interaction tolerated the replacement of Tyr-788 with Phe.

Nhieu et al. (40) extended the studies on these point mutations by examining the effects of single point mutations in the cytoplasmic domain of β1 on bacterial internalization. A number of bacteria have been shown to bind to integrins and be endocytosed. In particular, the interaction between Yersinia pseudotuberculosis and β1 has been well studied. These studies have revealed that this bacterium binds to a number of different β1 integrins via the protein invasin, leading to bacterial uptake and a local rearrangement of the actin network at the site of uptake. These investigators found that mutations at the Asn and Tyr residues in the amino-terminal NPXY motif (N785I, N785A, and Y788E) but not at the carboxyl-terminal NPXY motif (N797I and Y800A) affected bacterial uptake. The effect of mutations at Asp-764 were not examined in this study. The finding that β1 mutations which affect bacterial uptake also affect filamin binding, in conjunction with the finding that filamin binds actin, may provide a molecular explanation for the rearrangement in the local actin network observed following bacterial uptake.

At least four different β1 isoforms have been described which differ from one another in the amino acid sequence of their cytoplasmic domains (β1A = CD29, β1B = CD29b, β1C = CD29c, and β1D). Unlike β1A, β1B (47, 48) and β1C (49, 50) were unable to localize to focal adhesion. Analysis of the amino acid differences in the cytoplasmic domain of these different β1 isoforms shows that they are missing the NPXY motifs that are critical for filamin binding. β1D, which is most homologous to β1A and retains both NPXY motifs, localizes to focal adhesions (51). Taken together, these observations are consistent with a role for β1-filamin interactions in focal adhesion localization. It will be of interest to further dissect the possible role of β1-filamin interactions in localization of integrins to focal adhesions.

In addition to binding filamin, it has been previously reported that β1 can bind focal adhesion kinase (52) and the cytoplasmic proteins talin (53), paxillin (52), and α-actinin (54). Detailed analysis of the residues in the cytoplasmic domain of β1 that might be involved in binding to these proteins has only been carried out for α-actinin. In these studies, the ability of an overlapping set of decapeptides that span the cytoplasmic domain of β1 to preferentially bind α-actinin was examined (55). These studies suggest that the two NPXY motifs, but not Asp-764, might play a role in α-actinin binding to β1. These studies suggest that the filamin and α-actinin binding sites on β1 might overlap considerably. In an independent study, Lewis et al. (56) examined the ability of various cytoplasmic domain truncation mutants of β1 to co-localize with talin, α-actinin, and focal adhesion kinase in live cells. They observed that β1 truncations which lacked the carboxyl-terminal NPXY motif still could co-localize with α-actinin but not with actin, while a truncation lacking the last four residues of β1, including the Tyr in the carboxyl-terminal NPXY motif of β1, was able to co-localize with both α-actinin and actin. Considering that our mutagenesis study demonstrates that the Asn residue, but not the Tyr residue in the carboxyl-terminal NPXY motif of β1, is important for filamin binding, and that both α-actinin and filamin are actin binding proteins, the results reported by Lewis et al. (56) suggest that in their experimental system the association between β1 and actin might be preferentially mediated by filamin.

Three additional proteins have recently been identified using yeast two-hybrid approaches that appear to regulate integrin function. Hannigan et al. (31) identified a serine/threonine kinase, termed p56Lck (integrin-linked kinase), which interacts with and phosphorylates the β1 cytoplasmic tail. Overexpression of this protein in IEC-18 intestinal epithelial cells reduced the ability of these cells to adhere to extracellular matrix proteins. Kolanus et al. (57) identified a cytoplasmic protein that contains a pleckstrin homologous domain and a domain homologous to the yeast SEC7 gene product and interacts with the β1-integrin (CD18) subunit cytoplasmic tail. Overexpression of this protein, termed cytohesin-1, results in constitutive activation of α5β1 integrin and constitutive binding of Jurkat cells to ICAM-1. Overexpression of the SEC7 homologous domain alone also induces constitutive activation, while overexpression of the pleckstrin homologous domain alone leads to inhibition of α5β1 integrin-mediated adhesion to ICAM-1. Finally, Shattil et al. (58) identified a 111 residue polypeptide, designated β5-endonexin, which specifically interacts with the β5-integrin subunit. The role of β5-endonexin in integrin signaling remains to be elucidated. Based on our observations, it will be of interest to examine whether the interaction of filamin with integrin β-subunits plays a role in the regulation of integrin function by these cytoplasmic proteins.

The ability of the cytoplasmic domain of β1 to bind filamin provides an additional molecular mechanism for the coupling of the cytoplasmic domain of this integrin to actin. Although no site-directed mutagenesis studies have been undertaken to investigate the residues in the cytoplasmic domain of β1 which are critical for α-actinin binding, the data available to date suggest that both α-actinin and filamin might bind to similar sites on β1 and provide a mechanism for bridging β1 to actin. Presently, there is no information on the relative importance of these two interactions on integrin function. The findings presented in this study provide the basis for future experiments that will address the role of filamin binding in β1 function.

Acknowledgments—We thank Bill Bear for preparation of oligonucleotides; Mike Neubauer, Trent Youngman, and Joe Cook for DNA sequencing; Gary Carlton and Dr. Robert Reife for help in preparation of the figures; Jim Blake for peptide synthesis; Debby Baxter for help in preparation of this manuscript; and Tori Amos.

REFERENCES
1. Hynes, R. O. (1992) Cell 69, 11–25
2. Wayner, E. A., Garcia-Pardo, A., Humphries, M. J., McDonald, J. A., and Carter, W. G. (1989) J. Cell Biol. 109, 1321–1330
3. Mould, A. P., Wheldon, L. A., Komoriya, A., Wayner, E. A., Yamada, K. M., and Humphries, M. J. (1990) J. Biol. Chem. 265, 4920–4924
4. Guan, J. L., and Hynes, R. O. (1990) Cell 60, 53–61
5. Mould, A. P., and Humphries, M. J. (1991) EMBO J. 10, 4089–4095
6. Elices, M. J., Osborn, L., Takada, Y., Crouse, C., Lubowsky, S., Hemler, M. E., and Lobb, R. R. (1990) Cell 60, 577–584
7. Fassler, R., and Meyer, M. (1995) Genes Dev. 9, 1986–1990
8. Hirsch, E., Igleias, A., Potencin, A. J., Hartmann, U., and Fassler, R. (1996) J. Cell Biol. 135, 1231–1241
β₁-Integrin Residues That Mediate Filamin Binding

9. Gurtner, G. C., Davis, V., Li, H., McCoy, M. J., Sharpe, A., and Cybulsky, M. I. (1995) *Gene Dev.* 9, 1–14
10. Kwee, L., Baldwin, H. S., Shen, H. M., Stewart, C. L., Buck, C., Buck, C. A., and Labow, M. A. (1995) *Development* 121, 489–503
11. Carlos, T. M., Schwartz, B. R., Kovach, N. L., Yee, E., Rosso, M., Osborn, L., Chi-Rosso, C., Newman, B., Lobd, R., and Harlan, J. M. (1996) *Blood* 80, 965–970
12. Schwartz, B. R., Wayner, E. A., Carlos, T. M., Ochs, H. D., and Harlan, J. M. (1990) *J. Clin. Invest.* 85, 2019–2022
13. Hauenberger, D., Kliminek, J., and Sundquist, K. G. (1995) *J. Immunol.* 154, 960–971
14. Molossi, S., Elices, M., Arrhenius, T., and Rabenitch, M. (1995) *J. Cell. Physiol.* 164, 620–633
15. Ratner, S. (1992) *Invasion Metastasis* 12, 82–100
16. Pretolani, M., Ruffie, C., Lapa e Silva, J.-R., Joseph, D., Lobb, R. R., and Vargas, B. B. (1994) *J. Exp. Med.* 180, 795–805
17. Nakajima, H., Sano, H., Nishimura, T., Yoshida, S., and Iwamoto, I. (1994) *J. Exp. Med.* 179, 1145–1154
18. Yednock, T. A., Cannon, C., Fritz, L. C., Sanchez, M. F., Steinman, L., and Karin, N. (1992) *Nature* 356, 63–66
19. Baron, J. L., Reich, E.-P., Visintin, I., and Janeway, C. A. J. (1994) *J. Clin. Invest.* 93, 1706–1708
20. Ferguson, T. A., and Kupper, T. S. (1993) *J. Immunol.* 150, 1172–1182
21. Chisholm, P. L., Williams, C. A., and Lobb, R. R. (1993) *Eur. J. Immunol.* 23, 682–688
22. Jakubowski, A., Ehrenfeld, B. N., Pepinsky, R. B., and Burkly, L. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 2671–2675
23. Shimizu, Y., Van Seventer, G. A., Horgan, K. J., and Shaw, S. (1990) *Nature* 345, 250–253
24. Barr, M. W., Alon, R., and Springer, T. A. (1997) *Immunity* 4, 179–187
25. Maselli-Smith, A., and Shaw, A. R. E. (1996) *Science* 225, 768–777
26. Shimizu, Y., Van Seventer, G. A., Eainis, E., Newman, W., Horgan, K. J., and Shaw, S. (1992) *J. Exp. Med.* 175, 577–582
27. Weber, C., Alon, R., Moser, B., and Springer, T. A. (1996) *J. Cell Biol.* 134, 1063–1073
28. Chambon, P., and Aruffo, A. (1993) *J. Biol. Chem.* 268, 24655–24664
29. Chaloupka, H. E., and Issekutz, A. C. (1993) *J. Clin. Invest.* 92, 2768–2777
30. Massia, S. P., and Hubbard, J. A. (1992) *J. Biol. Chem.* 267, 14019–14026
31. Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Filmus, J., Bell, J. C., and Dedhar, S. (1996) *Nature* 379, 81–96
32. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) *Annu. Rev. Cell Dev. Biol.* 11, 549–599
33. Schiestl, R. H., and Gietz, R. D. (1989) *Curr. Genet.* 16, 339–346
34. Kanner, S. B., Grosmaire, L. S., Ledbetter, J. A., and Damle, N. K. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 7099–7103
35. Kanner, S. B., Reynolds, A. B., Vines, R. R., and Parsons, T. J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 3328–3332
36. Kanner, S. B., Damle, N. K., Blake, J., Aruffo, A., and Ledbetter, J. (1992) *J. Immunol.* 149, 2023–2029
37. Gorlin, B. J., Yamin, R., Egan, S., Stewart, M., Stossel, T. P., Kwiatkowski, D. J., and Hartwig, J. H. (1996) *J. Cell Biol.* 111, 1089–1105
38. Sharma, C. P., Ezzell, R. M., and Arnaout, M. A. (1995) *J. Immunol.* 154, 3461–3470
39. Reszka, A. A., Hayashi, Y., and Horwitz, A. F. (1992) *J. Cell Biol.* 117, 1321–1330
40. Van Nhieu, G. T., Krukonis, E. S., Reszka, A. A., Horwitz, A. F., and Isberg, R. R. (1996) *J. Biol. Chem.* 271, 7665–7672
41. Okiro, J. A., Pidard, D., Newman, P. J., Montgomery, R. R., and Kunicki, T. J. (1985) *J. Cell Biol.* 100, 317–321
42. Fox, J. E. B. (1985) *J. Biol. Chem.* 260, 11970–11977
43. Ezzell, R. M., Kenney, D. M., Egan, S., Stossel, T. P., and Hartwig, J. H. (1988) *J. Biol. Chem.* 263, 13303–13309
44. Miyamoto, S., Akiyama, S. K., and Yamada, K. M. (1995) *Science* 267, 883–885
45. Marcantonio, E. E., Guan, J. L., Trevithick, J. E., and Hynes, R. O. (1996) *Cell Regul.* 7, 597–604
46. Hayashi, Y., Haimovich, B., Reszka, A., Boettiger, D., and Horwitz, A. (1990) *J. Cell Biol.* 116, 175–184
47. Balzac, F., Altruda, F., Koteliansky, V. E., Balabanov, Y. V., Altudra, F., Silengo, L., and Tarone, G. (1993) *J. Cell Biol.* 121, 171–178
48. Altruda, F., Cervella, P., Tarone, G., Botta, C., Balzac, F., Stefanuto, G., and Silengo, L. (1990) *Gene* (Amst.) 95, 261–266
49. Languno, L. R., and Russo, C. (1992) *J. Biol. Chem.* 267, 7116–7120
50. Meredith, J. J., Tukada, Y., Fornaro, M., Languno, L. R., and Schwartz, M. A. (1995) *Science* 269, 1730–1732
51. Belkin, A. M., Zhidkova, N. I., Balzac, F., Altudra, F., Tomatis, D., Moda, A., Tarone, G., Koteliansky, V. E., and Burridge, K. (1996) *J. Cell Biol.* 132, 211–226
52. Schaller, M. D., Otey, C. A., Hildebrand, J. D., and Parsons, T. J. (1995) *J. Cell Biol.* 130, 1181–1187
53. Horwitz, A., Duggan, K., Buck, C., Beckerle, M. C., and Burridge, K. (1990) *J. Cell Biol.* 111, 721–729
54. Otey, C. A., Pavalko, F. M., and Burridge, K. (1990) *J. Cell Biol.* 111, 721–729
55. Otey, C. A., Vasquez, G. B., Burridge, K., and Erickson, B. W. (1993) *J. Biol. Chem.* 268, 21183–21187
56. Lewis, J. M., and Schwartz, M. A. (1995) *Mol. Biol. Cell* 6, 151–160
57. Kolarus, W., Nagel, W., Schiller, B., Zeilmann, L., Goda, S., Stockinger, H., and Seed, B. (1996) *Cell* 86, 233–242
58. Shattil, S. J., O'Toole, T., Eigestaer, M., Thon, V., Williams, M., Babiak, B. M., and Ginsberg, M. H. (1995) *J. Cell Biol.* 131, 807–816