Mapping In Vivo Associations of Cytoplasmic Proteins with Integrin $\beta 1$ Cytoplasmic Domain Mutants

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Integrins promote formation of focal adhesions and trigger intracellular signaling pathways through cytoplasmic proteins such as talin, α -actinin, and focal adhesion kinase (FAK). The $\beta 1$ integrin subunit has been shown to bind talin and α -actinin in in vitro assays, and these proteins may link integrin to the actin cytoskeleton either directly or through linkages to other proteins such as vinculin. However, it is unknown which of these associations are necessary in vivo for formation of focal contacts, or which regions of $\beta 1$ integrin bind to specific cytoskeletal proteins in vivo. We have developed an in vivo assay to address these questions. Microbeads were coated with anti-chicken $\beta 1$ antibodies to selectively cluster chicken $\beta 1$ integrins expressed in cultured mouse fibroblasts. The ability of cytoplasmic domain mutant $\beta 1$ integrins to induce co-localization of proteins was assessed by immunofluorescence and compared with that of wild-type integrin. As expected, mutant $\beta 1$ lacking the entire cytoplasmic domain had a reduced ability to induce co-localization of talin, α -actinin, F-actin, vinculin, and FAK. The ability of $\beta 1$ integrin to co-localize talin and FAK was found to require a sequence near the C-terminus of $\beta 1$. The region of $\beta 1$ required to co-localize α -actinin was found to reside in a different sequence, several amino acids further from the C-terminus of $\beta 1$. Deletion of 13 residues from the C-terminus blocked co-localization of talin, FAK, and actin, but not α -actinin. Association of α -actinin with clustered integrin is therefore not sufficient to induce the co-localization of F-actin.

INTRODUCTION

Integrins comprise a family of transmembrane proteins that attach the cell to the extracellular matrix (ECM) and anchor the cytoskeleton to the plasma membrane (Burridge *et al.*, 1988; Hynes, 1992). Integrins are heterodimers consisting of α and β subunits; the particular combination of α with β confers specificity for matrix protein ligands. Eight β subunits and 14 α subunits have been identified to date (Hynes, 1992).

In cultured cells, integrins are clustered in discrete sites known as focal adhesions. Within these sites are high concentrations of various cytoskeletal proteins, which form a dynamic link with actin microfilaments. The binding of integrins to the ECM initiates the reorganization of cytoskeletal proteins into focal contacts and the local assembly of actin filaments. The $\beta 1$ integrin cytoplasmic domain is required for cytoskeletal interactions, and in fact

contains all of the information necessary for localization to focal adhesions (Geiger *et al.*, 1992; LaFlamme *et al.*, 1992). In vitro, the $\beta 1$ cytoplasmic domain has been shown to bind directly to the cytoskeletal proteins α -actinin and talin (Horwitz *et al.*, 1986; Otey *et al.*, 1990). The general spreading of cells and the formation of focal contacts also appear to involve signaling events that possibly include activation of small G proteins, protein tyrosine phosphorylation (Dans *et al.*, 1991; Guan *et al.*, 1991; Kornberg *et al.*, 1991; Burridge *et al.*, 1992; Ridley and Hall, 1992), activation of protein kinase C (Woods and Couchman, 1992; Vuori and Ruoslahti, 1993), and production of arachidonate metabolites (Chun and Jacobson, 1992). Some evidence suggests that these signaling events may be necessary for focal contact formation (Burridge *et al.*, 1992; Chun and Jacobson, 1992; Ridley and Hall, 1992; Woods and Couchman, 1992; Vuori and Ruoslahti, 1993).

Because integrins are also capable of initiating activation of intracellular signaling pathways such as protein tyrosine phosphorylation, intracellular pH and calcium, and inositol lipid metabolism (reviewed in Schwartz, 1992; Damsky and Werb, 1992), current information suggests that contact of cells with extracellular matrices initiates a cascade of signaling events and cytoskeletal rearrangements that ultimately lead to cell spreading and focal adhesion formation. However, the exact structure of focal contacts *in vivo* and the sequence of events leading to their formation is not yet known.

Studies *in vitro* have shown that residues 780–789 of $\beta 1$ integrin can bind to talin (Tapley *et al.*, 1989), which could then bind to actin either directly (Muguruma *et al.*, 1990; Goldman and Isenberg, 1991) or indirectly through a vinculin- α -actinin link (Belkin and Kotelian-sky, 1987; Wachsstock *et al.*, 1987) or a vinculin-tensin link (Wilkins *et al.*, 1987). The $\beta 1$ cytoplasmic domain has also been shown to bind directly to α -actinin, which in turn could bind to actin (Otey *et al.*, 1990). More detailed studies have recently shown that α -actinin can bind to synthetic peptides representing two distinct regions within $\beta 1$, residues 768–777 and 785–794 (Otey *et al.*, 1993).

Studies *in vivo* have investigated the cellular distribution of chicken $\beta 1$ integrin cytoplasmic domain mutants expressed in mouse fibroblasts (Hayashi *et al.*, 1990; Marcantonio *et al.*, 1990). Deletion of the four or five C-terminal amino acids of $\beta 1$ only slightly reduced co-localization of chicken $\beta 1$ with the native mouse integrin in focal contacts, but longer deletions inhibited co-localization to focal contacts. As expected, a $\beta 1$ mutant lacking almost the entire cytoplasmic region ($\Delta 762$) did not localize to focal contacts, but one mutant only four amino acid residues longer ($\Delta 766$) did show some localization (Marcantonio *et al.*, 1990).

Reszka *et al.* (1992) have investigated in further detail which amino acid residues of the $\beta 1$ cytoplasmic region are required for localization of integrins to focal contacts. In mouse fibroblasts, they expressed the chicken $\beta 1$ subunit with point mutations at nearly every position in the cytoplasmic domain and assessed the ability of the chicken integrin to co-localize with endogenous mouse integrins in focal adhesions. Three clusters of amino acids were identified that, when altered, resulted in reduced localization of the $\beta 1$ in focal contacts. This reduced localization might reflect altered interactions of cytoskeletal proteins with specific amino acids that are necessary to stabilize $\beta 1$ in focal contacts. However, this approach did not permit study of which cytoskeletal interactions map to specific regions of $\beta 1$. Nor did it provide information concerning the ability of the mutants to participate in the initial formation of focal contacts, as opposed to co-localization to contacts initiated by endogenous, wild-type mouse $\beta 1$.

To address these questions, we have developed a system using antibody-coated microbeads to specifically cluster the transfected integrin. Previous work has shown that fibronectin-coated beads induce localization of integrins and focal adhesion proteins, but not other membrane or cytoskeletal molecules (Mueller *et al.*, 1989; Plopper and Ingber, 1993). We have used this system to assay the ability of cytoplasmic domain mutants of the $\beta 1$ integrin subunit to induce co-localization of talin, α -actinin, actin, and focal adhesion kinase (FAK) (Schaller *et al.*, 1992) in focal adhesion-like structures.

MATERIALS AND METHODS

Cell Culture

Mouse NIH 3T3 fibroblasts that expressed wild type or mutant chicken $\beta 1$ integrins ($\Delta 764$) were a generous gift from Dr. A. Horwitz (University of Illinois, Urbana, IL; Hayashi *et al.*, 1990; Reszka *et al.*, 1992). Mouse NIH 3T3 fibroblasts expressing other mutant chicken $\beta 1$ integrins ($\Delta 762$, $\Delta 766$, $\Delta 775$, $\Delta 790$, and $\Delta 799$) were a generous gift from Dr. Richard Hynes (Massachusetts Institute of Technology, Cambridge, MA) (Marcantonio *et al.*, 1990). These included two clonal cell lines for each mutant used. Cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) with 10% calf serum and 0.5 mg/ml G418 sulfate (GIBCO Laboratories, Grand Island, NY).

The notation for the mutants has been changed to make the data easier to comprehend. Although previous papers refer to each mutant by the position of the termination codon, we have defined each mutant by the number of amino acids still present. For example, the mutant previously described as 765t is described here as $\Delta 764$.

Antibodies

A hybridoma producing the monoclonal antibody CSAT, directed against chicken $\beta 1$ integrin (Neff *et al.*, 1982), was a gift from Dr. A. Horwitz. Ascites was raised in BALB/c mice. The polyclonal antibody Ab814 and the monoclonal antibodies W1B10 and V9C3 were a generous gift from Dr. A. Horwitz. Fluorescein-conjugated affinity-purified goat anti-mouse IgG and Fc-specific sheep anti-mouse IgG were purchased from Cappel Laboratories (Organon Teknika, West Chester, PA). A polyclonal antibody directed against the conserved cytoplasmic domain of $\beta 1$ was a gift from Dr. E. Marcantonio (Marcantonio and Hynes, 1988). The rat monoclonal antibody 5D2-27, directed against mouse CD-44, was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA) (Hughes and August, 1981; Aruffo *et al.*, 1990; Carter and Wayner, 1988), and IgG was purified on protein G Sepharose (Pharmacia, Piscataway, NJ).

Reagents

Human plasma fibronectin (FN) was prepared by affinity chromatography on gelatin-Sepharose (Mieka *et al.*, 1982). The 40-kDa C-terminal fragment of FN was purchased from GIBCO Laboratories. Tosyl-activated 4.5- μ m polystyrene beads were obtained from Dynal, Inc. (Great Neck, NY). Rhodamine phalloidin was obtained from Molecular Probes (Eugene, OR). All other chemicals, unless otherwise noted, were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Sorting

For each experiment, all cells expressing chicken $\beta 1$ integrin were sorted and processed in parallel. For each cell type, approximately 2.5×10^7 cells were rinsed once in phosphate-buffered saline (PBS)

and detached in PBS containing 2 mM EDTA for 2–3 min. Cells were collected and washed in 20 mM HEPES, pH 7.4, containing 140 mM NaCl, 5 mM KCl, and 5 mM glucose (HNKG) and resuspended in 2 ml HNKG containing 2% BSA and 20 $\mu\text{g}/\text{ml}$ W1B10. Cells were incubated for 30 min on ice, washed once with cold HNKG, and resuspended in 2 ml HNKG containing 2% BSA and 4 $\mu\text{g}/\text{ml}$ affinity-purified fluorescein isothiocyanate-sheep anti-mouse IgG. After a 30-min incubation on ice, cells were washed once and resuspended in 4 ml HNKG. Sorting was performed on a FACStar (Becton Dickinson, Mountain View, CA) with a window set to select for similar fluorescence intensities. Cells were replated and allowed to recover in DME containing 10% CS and G418 (250 $\mu\text{g}/\text{ml}$) for 38 h before use.

Preparation of CSAT Fab Fragments

CSAT-IgG was purified on a protein A-agarose column (BRL, Gaithersburg, MD). Papain (Sigma Chemical Co.; 25 $\mu\text{g}/\text{ml}$) was added to 3 mg/ml CSAT-IgG in PBS containing 1 mM EDTA and 5 mM DTT, and the mixture was incubated for 15 min at 37°C. To stop the reaction, 2 $\times$ vol of 200 μM iodoacetic acid in 1 M Tris-Cl, pH 8.0, was added. The Fab fragments were separated from the Fc portion of the antibodies by incubation with protein A agarose. Purity was assessed by SDS-PAGE, which resulted in a single band as visualized by Coomassie staining.

Conjugation of Proteins to Beads

Fibronectin or BSA in pH 9.5 borate (50 μM), at 500 $\mu\text{g}/\text{ml}$ final concentrations, were conjugated to 2×10^8 tosyl-activated 4.5- μm polystyrene beads/ml (Dyna, Inc.). CSAT Fab fragments or other antibodies were conjugated at 0.75 mg/ml final concentration, in pH 9.5 borate (500 mM). Samples were incubated for 24 h at room temperature. Conjugated beads were washed four times with 50 mM Tris-HCl, pH 9.6, and free tosyl groups were blocked in the same buffer by incubation for 16 h at room temperature. Beads were washed twice and stored in sterile PBS containing 1% protease-free BSA.

Binding of Beads to Cells

FACS-sorted cells were detached with trypsin and collected in DMEM containing 0.1% protease- and nuclease-free BSA, insulin, selenium, and transferrin (1:100 dilution of GIBCO BRL GMS-G) ("DBI"). Soybean trypsin inhibitor was added to a concentration of 250 $\mu\text{g}/\text{ml}$. Cells were centrifuged and resuspended in DBI at $1\text{--}5 \times 10^5$ cells/ml. For each sample, 100 μl of cells were transferred to a well of a 96-well suspension culture plate; suspension culture plates were prepared by coating tissue culture plastic with heat-denatured 10 mg/ml BSA in PBS for 5 min. Care was taken to avoid shear stress on cells by using only wide-bore (cut) pipet tips. Either fibronectin-, BSA-, or CSAT Fab-conjugated beads were added (approximately 2×10^5 beads per 100 μl cells), and each sample was mixed by pipetting two times. Cells and beads were incubated for 1 h at 37°C, after which each cell had from zero to four beads bound.

Immunofluorescence

Glass coverslips were washed in 5% KOH in methanol and stored in ethanol. Poly-L-lysine at 10 mg/ml was dried onto the surface and washed with PBS just before use. Suspended cells with beads bound were transferred to the treated coverslips using a wide-bore pipet tip, and cells were allowed to adhere for 5 min. Cells were extracted in ice-cold 100 mM piperazine-*N,N*-bis(2-ethanesulfonic acid) (PIPES), pH 6.8, containing 2 mM MgCl_2 , 1 mM EGTA, 0.5% NP-40, and 10% vol/vol glycerol for 1 min to remove noncytoskeletal-associated cell material (Ben Ze'ev *et al.*, 1979). Extracted cells were gently washed once with PBS, then fixed in 3.7% methanol-free formaldehyde (Polysciences, Warrington, PA) in 100 mM PIPES

with 2 mM MgCl_2 and 1 mM EGTA, for 20 min at room temperature.

Fixed cells were permeabilized in 0.5% NP-40 and blocked in 10% normal goat serum (GIBCO BRL) in PBS. Primary and secondary antibodies or rhodamine-phalloidin were diluted into the blocking solution and were incubated at 37°C for 90 min and 45 min, respectively. To visualize mouse primary antibodies without labeling of the CSAT-Fab-coated beads, secondary antibodies specific for the Fc portion of the antibodies were employed. Coverslips were mounted in Fluoromount G (Fisher Scientific, Pittsburgh, PA) to which 2.5% wt/vol 1,4-diazabicyclo-octane (DABCO) had been added as a fluorescence stabilizer. Fluorescent samples were viewed on a Nikon inverted fluorescence microscope equipped with an Olympus 60X oil objective, and photographed using Kodak Ektar 1000 or Tmax 400 film.

Quantitation

For each sample, 200 beads were scored for the presence or absence of patches of fluorescent label around the beads. Conditions were adjusted so that the ratio of beads bound per cell was approximately 1:1. For the few cells with multiple beads bound, usually only one of the beads was positive. Therefore, any positive bead on a cell was scored as a positive interaction. For every mutant, two clones from separate transfections were analyzed. Values are means $\pm$ SD from four to five such experiments.

RESULTS

Specificity of Cytoskeletal Associations

Beads (4.5 μm) coated with Fab fragments of the anti-chicken $\beta 1$ monoclonal antibody CSAT were used to specifically cluster the chicken $\beta 1$ subunit in transfected cells. Although the anti-chicken integrin beads bound with high efficiency to the transfected NIH 3T3 cells, they showed virtually no binding to untransfected cells (Figure 1, A and B). To test the ability of these beads to induce co-localization of cytoskeletal proteins, suspended mouse 3T3 cells expressing chicken $\beta 1$ were incubated with the beads, then processed for immunofluorescence microscopy (Figure 1, C-G). As a control, cells were also incubated with BSA-coated beads (Figure 1H). Although the anti-integrin beads bound to cells with high efficiency, few of the BSA beads bound. The few that did bind to the cells failed to induce clustering of either $\beta 1$ or cytoskeletal proteins. By contrast, CSAT antibody-coated beads clustered chicken $\beta 1$ and induced co-localization of actin, talin, α -actinin, vinculin, and FAK, which are characteristic of focal contacts. Although the presence or absence of a patch was all-or-none, differences in the size and intensity of patches between the different cytoskeletal proteins were noticeable, which could be caused by differences in distribution or by the use of different antibodies or reagents to detect each protein.

Dependence on Integrin Cytoplasmic Domain

To demonstrate that the co-localization of cytoskeletal proteins with integrins depends on the $\beta 1$ cytoplasmic region, cells transfected with wild-type $\beta 1$ were com-

Figure 1. CSAT-antibody-coated beads bind specifically and induce clustering of $\beta 1$ and cytoskeletal proteins. Polystyrene beads coated with CSAT-IgG linked to goat anti-mouse IgG were incubated for 45 min with adherent NIH 3T3 cells expressing chicken $\beta 1$ (A) or untransfected 3T3 cells (B) and unbound beads were removed by washing. CSAT-coated beads (C-G) or BSA-coated beads (H) were incubated for 60 min at 37°C with suspended cells expressing chicken $\beta 1$ integrin. Cells were fixed onto poly-L-lysine-coated coverslips, and indirect immunofluorescence was performed by labeling with primary antibodies specific for either chicken $\beta 1$ (using the monoclonal antibody V9C3; C and H), talin (D), α -actinin (E), FAK (F), or actin (G). A and B, bar = 40 μ m; C-H, bar = 10 μ m.

pared with those transfected with $\beta 1$ lacking most of the cytoplasmic domain ($\Delta 764$). This truncated integrin subunit forms dimers with α subunits and is expressed on the plasma membrane (Hayashi *et al.*, 1990). FN-coated beads, which bind the endogenous mouse integrins, induced co-localization of talin in both cell lines (Figure 2, B and D). This result shows that talin is present and capable of associating with integrins in these cell lines. CSAT antibody-coated beads, however, which are specific for the chicken $\beta 1$, rarely induced detectable co-localization of talin within the cells expressing the $\Delta 764$ mutant (Figure 2C). These results suggest that the appearance of talin patches around the beads, as predicted from *in vitro*

studies, required the presence of the cytoplasmic domain of $\beta 1$ integrin.

We also attempted to determine whether the CSAT antibody-coated beads clustered the endogenous mouse integrins along with the transfected chicken integrin. To differentiate between the chicken and mouse $\beta 1$, cells transfected with the $\Delta 762$ chicken $\beta 1$ mutant lacking the cytoplasmic domain were employed. An antibody specific for the cytoplasmic domain of $\beta 1$ labeled the full-length mouse $\beta 1$, but not the truncated chicken $\beta 1$, in these cells. As expected, FN-coated beads induced clustering of both chicken and mouse integrins, detected by a polyclonal anti-chicken $\beta 1$ and the anti-cytoplasmic region antibody,

Figure 2. Talin co-localizes with wild-type chicken $\beta 1$, but not mutant $\beta 1$. Polystyrene beads coated with either CSAT Fab fragments (A and C) or FN (B and D) were incubated for 60 min at 37°C with mouse 3T3 fibroblasts expressing either wild-type chicken $\beta 1$ integrin (A and B) or the $\Delta 764$ mutant (C and D). Cells were fixed onto poly-L-lysine-coated coverslips and processed for indirect immunofluorescence. All cells were labeled with antibodies specific for talin. Arrows indicate position of beads; the cell in panel C has five beads attached. Bar, 10 μm .

respectively (Figure 3, A and B). When CSAT-coated beads were used (Figure 3, C and D), the chicken $\beta 1$ was found to be concentrated at the bead-cell interface, but no mouse $\beta 1$ could be detected. Quantitation of this experiment showed that the anti-cytoplasmic domain antibody stained 64% of the FN beads, whereas only 3% of the CSAT beads were stained. Thus, within the resolution of the technique, endogenous integrins do not appear to associate with the $\Delta 762$ mutant.

To determine whether the recruitment of cytoplasmic proteins is specific to integrins and is not a general result of clustering membrane proteins by beads, FN-coated beads were compared with those coated with either a 40-kDa C-terminal heparin-binding fragment of FN (Woods *et al.*, 1993) or an antibody specific for CD44. FN beads were far more effective at inducing localization of $\beta 1$ integrin to the bead-cell interface than were 40kDa or anti-CD44 beads (Figure 4). However, the 40kDa and CD44 beads did co-localize with a

Figure 3. CSAT-antibody-coated beads induce clustering of chicken $\beta 1$, but not endogenous mouse $\beta 1$. Polystyrene beads coated with either FN (A and B) or CSAT Fab fragments (C and D) were incubated for 60 min at 37°C with 3T3 mouse fibroblasts expressing the $\Delta 762$ chicken $\beta 1$ mutant. Cells were fixed onto poly-L-lysine-coated coverslips and processed for indirect immunofluorescence. Cells were labeled with antibodies specific for either chicken $\beta 1$ (Ab814; A and C), or the $\beta 1$ cytoplasmic region, which detects only mouse $\beta 1$ in these cells (B and D). Bar, 10 μm .

Figure 4. Beads coated with either antibody specific for CD44 or a 40-kDa fragment of FN adhere specifically to cells, but induce little clustering of focal adhesion proteins. Polystyrene beads coated with either FN (solid bars, A and B), anti-CD44 IgG (hatched bars, A), or 40-kDa C-terminal heparin-binding fragment of FN (hatched bars, B) were incubated for 60 min at 37°C with mouse 3T3 fibroblasts expressing wild-type chicken $\beta 1$ integrin. The ability of each type of bead to induce clustering of integrin $\beta 1$ or co-localization of cytoskeletal proteins was assessed by immunofluorescence. For each protein, 200 beads were scored as four groups of 50. Data are expressed as the mean $\pm$ SD.

distinct patch of $\beta 1$ integrin staining in a small proportion of samples. These patches are likely to be caused by some combination of nonspecific staining, nonspecific binding of membrane proteins to the

beads, or weak interactions of the $\beta 1$ integrin with the bound CD44 or heparan sulfate proteoglycan. In these instances, the patch was indistinguishable from the co-localization induced by FN beads. These results could therefore readily be quantitated by scoring interactions on an all-or-none basis. Quantification revealed that FN beads induced patches of $\beta 1$ integrin at an approximately eightfold higher frequency than did 40kDa or anti-CD44 beads (Figure 4).

The beads coated with intact FN also induced the formation of patches containing F-actin, talin, α -actinin, and FAK (Figure 4). Beads coated with either the anti-CD44 antibody or the 40-kDa fragment sometimes induced distinct patches of α -actinin and talin, but much less frequently. Anti-CD44 induced a somewhat higher level of actin localization, which is likely to represent a real association of CD44 with actin (Camp *et al.*, 1991). Both anti-CD44 and FN 40kDa fragment showed some co-localization with anti-FAK staining. These controls suggest that baseline levels of co-localization are approximately 5–15% for actin, talin, α -actinin, and $\beta 1$, and 20–30% for FAK. FN-coated beads induced co-localization of these proteins 3- to 10-fold more effectively than 40kDa or anti-CD44 beads. These results show that integrin clustering induces specific co-localization of cytoskeletal proteins, which can readily be detected above baseline levels.

Analysis of Deletion Mutants

To map which regions of the $\beta 1$ cytoplasmic domain bind to specific elements of the cytoskeleton, a series of deletion mutants was examined (Figure 5). Cells transfected with wild-type or mutant $\beta 1$ variants were first sorted by FACS to select populations expressing equivalent levels of chicken integrins (Figure 6). This step ensured that any differences observed in co-localization of cytoplasmic proteins did not merely reflect differences in $\beta 1$ expression. Cells were then replated and allowed to recover for 38 h. A second FACS analysis at this time showed that levels of chicken $\beta 1$ expression had not changed. Cells were then detached from the substratum, incubated with beads, processed for immunofluorescence, and scored for the presence

Figure 5. Schematic representation of deletion mutants of the cytoplasmic domain of chicken $\beta 1$ integrin. The ability to localize to focal contacts is denoted by + or – (Marcantonio *et al.*, 1990).

Figure 6. Indirect immunofluorescence of cells selected for resistance to BFA. Wild-type (CHO-WT) or BFA-resistant (CHO-BR) cells were plated on coverslips, treated with BFA for 30 min, fixed, and stained with polyclonal antibodies to mannosidase II. The BFA concentration for CHO-WT was 1.5 μ g/ml, and for the two mutants the concentration was 20 μ g/ml.

of chicken $\beta 1$ or cytoskeletal protein staining at the site of contact between the bead and the cell.

The ability of CSAT antibody-coated beads to cluster wild-type or mutant $\beta 1$ for each cell line is shown in Figure 7A. Approximately 80% of the beads induced detectable clustering of chicken $\beta 1$ for all of the mutants examined. When co-localization of F-actin was assayed (Figure 7C), wild-type $\beta 1$ induced efficient co-localization. The $\Delta 799$ mutant, missing four amino acids, induced actin localization almost as well, consistent with its ability to localize to focal adhesions almost as well as wild type (Marcantonio *et al.*, 1990). However, truncation of an additional nine amino acids ($\Delta 790$) resulted in a substantial decrease in the co-localization of F-actin, consistent with loss of focal adhesion localization. This decrease is statistically significant ($p < 0.025$) when analyzed using Student's paired t test. Longer truncations ($\Delta 762$, $\Delta 766$, $\Delta 775$)

Figure 7. Co-localization of cytoplasmic proteins with integrin $\beta 1$ mutants. Suspended mouse 3T3 cells expressing either wild-type chicken $\beta 1$ or cytoplasmic domain mutants were incubated with CSAT-antibody-coated beads, processed for immunofluorescence, and scored for co-localization as described in MATERIALS AND METHODS. Panels show the following: localization of the transfected integrin (A), co-localization of talin (B), actin (C), α -actinin (D), and FAK (E). For each mutant, two clones from separate transfections were analyzed. In each experiment, 200 beads were scored for the presence of each protein for each mutant. Data are expressed as the mean $\pm$ SD of four to five separate experiments. Y axis: percent of beads scored as positive.

also localized actin poorly and were statistically different from wild type or $\Delta 799$ ($p < .025$).

The co-localization of talin is shown in Figure 7B. The ability of $\beta 1$ mutants to induce talin localization paralleled that of F-actin. Clustering of wild type or the $\Delta 799$ deletion mutant induced efficient co-localization of talin, but larger deletion mutants ($\Delta 790$, $\Delta 775$, $\Delta 766$, $\Delta 762$) induced co-localization of talin very poorly. A similar pattern was observed for FAK (Figure 7E). In all of these cases, the differences between the wild-type or $\Delta 799$ $\beta 1$ and the longer deletions were statistically significant ($p < 0.025$). These results

show that interactions of the $\beta 1$ integrin subunit with talin, F-actin, and FAK depend upon residues 791–799.

Staining for α -actinin (Figure 7D) showed that wild-type $\beta 1$ and the $\Delta 799$ mutant induced co-localization at bead contact sites with similar efficiency. Unlike actin, talin, or FAK, however, the $\Delta 790$ mutant still induced efficient localization of α -actinin. This localization of α -actinin was nearly abolished in the $\Delta 775$, $\Delta 766$, and $\Delta 762$ mutants, and the differences between active and inactive mutants were statistically significant ($p < 0.025$) in all cases. These results demonstrate that the interaction of $\beta 1$ integrin with α -actinin requires the region 776–790, but is independent of residues C-terminal to 790.

DISCUSSION

In this study, we have found that clustering of integrins with an antibody induces localization of focal adhesion proteins. Previous work has shown that immobilized anti-integrin antibodies induce cell spreading and trigger the same signaling pathways as ECM proteins (Werb *et al.*, 1989; Guan *et al.*, 1991; Schwartz *et al.*, 1991; Schwartz, 1993) providing further evidence that adhesion to antibodies provides a suitable model for adhesion to the ECM. When the effects of mutations in the $\beta 1$ integrin subunit were examined, we found that the co-localization of actin, talin, and FAK with $\beta 1$ depends upon amino acid residues 791–799. These residues lie adjacent to a sequence that Tapley showed to bind talin *in vitro* (residues 780–789; Tapley *et al.*, 1989). Taken together, the data suggest that the information required for binding talin involves both regions. This notion is consistent with the work of Reszka *et al.* (1992) in which residues 780–789 and 791–799 both contain NPXY motifs. Because this motif forms tight turns, these regions are likely to be closely opposed. Thus, amino acids from both these sectors could form one binding site for talin *in vivo*, so that removal of residues 791–799 weakens the affinity sufficiently to prevent association *in vivo*.

An alternative interpretation is that deletion of the 791–799 region inhibits production of a signal that is required for association of talin. The correlation between talin and FAK association is consistent with this interpretation. Other work demonstrating that co-localization of talin with clustered integrins in lymphoid cells requires activation of protein kinase C (Burn *et al.*, 1988) further supports this view.

Our data also show that residues 776–790 of $\beta 1$ integrin are necessary for interaction with α -actinin *in vivo*. Recent studies by Otey *et al.* (1993) using overlapping sequential decapeptides from the $\beta 1$ cytoplasmic region localized the *in vitro* binding of α -actinin to two different regions, residues 768–778 and 785–794. Our results are therefore generally consistent with the *in vitro* binding studies.

The most surprising and interesting conclusion from these studies is that the association of α -actinin in the absence of talin and FAK is not sufficient to promote localization of actin. This conclusion is based on the data indicating that the $\Delta 790$ mutant induces low levels of actin localization but high levels of α -actinin, and that further truncation to $\Delta 775$ brings α -actinin down to very low levels but has no further effect on actin. These results are surprising in light of the well-characterized ability of α -actinin to bind actin. The likeliest explanation in our view is that the binding of α -actinin to actin is also regulated and depends upon signals that require residues not present in the $\Delta 790$ mutant. One signaling molecule that has been shown to regulate the interaction between α -actinin and actin is the lipid phosphatidylinositol bisphosphate (Lassing and Lindberg, 1985), whose levels have indeed been found to depend upon integrin-mediated cell adhesion (McNamee *et al.*, 1993).

It is also highly interesting that localization of talin, FAK, and actin correlate, as all three depend on a stretch of nine amino acids from position 791 to 799. This result suggests that the three proteins may be functionally linked. It has been found that disruption of actin filaments with cytochalasin D blocks FAK phosphorylation (Huang *et al.*, 1993), consistent with this view. However, the existence of other proteins or signaling events that require this region of the $\beta 1$ cytoplasmic domain cannot be excluded.

Our data provide support for certain models of how focal contacts form. Microinjection studies have suggested that talin participates in the initial formation of focal adhesions, whereas α -actinin is important for their maintenance (Nuckolls *et al.*, 1992; Pavalko *et al.*, 1991). The effects we have observed probably correspond more closely to the initial formation of focal adhesions, and we have found that the presence of talin parallels that of F-actin. Our data also show that the presence of α -actinin alone is not sufficient to induce localization of actin filaments. However, our data do not rule out an important role for α -actinin in focal adhesions. Possibly, after the initial formation of focal contacts, a second set of signals is required to shift the F-actin link in focal adhesions from talin to α -actinin, similar to the interaction between α -actinin and $\beta 2$ after activation of neutrophils (Pavalko and La Roche, 1993). Alternatively, it may be that both α -actinin and talin are required for normal spreading and cytoskeletal organization.

Finally, we suggest that the method we have used in these studies may be generally applicable to the analysis of integrin mutants. Although a good deal of work has been carried out analyzing the effects of integrin cytoplasmic domain mutations on biological function, it has been difficult to interpret these data in terms of specific interactions. Our methods may pro-

vide a needed link between in vivo studies of function and in vitro measurements of binding interactions.

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