Identification of Amino Acid Sequences in the Integrin $\beta_1$ Cytoplasmic Domain Implicated in Cytoskeletal Association

Alfred A. Reszka, Yokichi Hayashi, and Alan E. Horwitz
Department of Cell and Structural Biology, University of Illinois, Urbana, Illinois 61801

Abstract. Wild-type and mutant chicken integrin $\beta_1$ subunit ($\beta_{1o}$) cDNAs were expressed in NIH 3T3 cells and assayed for localization in focal adhesions of cells plated on fibronectin substrates. Focal adhesion localization in stable transfected cells was assayed by indirect immunofluorescent staining with chicken-specific anti-$\beta_{1o}$ antibodies. Mutant $\beta_{1c}$ integrins containing internal deletions of 13 amino acids adjacent to the membrane, $\Delta$759-771, and 20 centrally located amino acids, $\Delta$771-790, localized in focal adhesions demonstrating that sequences required for direction to focal adhesion structures were not limited to one region of the cytoplasmic domain. Point mutations revealed three clusters of amino acids which contribute to localization in focal adhesions. These three clusters or signals are: cyto-1 (764-774), cyto-2 (785-788), and cyto-3 (797-800). The 11-residue cyto-1 signal is only found on integrin $\beta$ subunit sequences, except $\beta_4$. Four residues within this region, D764, F768, F771, and E774, could not be altered without reducing focal adhesion staining intensities, and likely form a signal that occupies one side of an $\alpha$ helix. Mutations involving two cyto-1 residues, K770 and F771, also appeared to affect heterodimer affinity and specificity. Cyto-2 (785-788), NPIY, is an NPXY signal that forms a tight turn motif. Cyto-2 provides a structural conformation, which when perturbed by proline removal or addition, inhibits integrin localization in focal adhesions. Cyto-3 (797-800), NPKY, resembles cyto-2, however, the nonconserved proline residue can be replaced without alteration of the localization phenotype. Cyto-3, therefore, constitutes a unique integrin signal, NXXY. Both serine and tyrosine residues at positions 790 and 788, respectively, which have been implicated in integrin phosphorylation/regulation, were conservatively replaced without detectable effect on focal adhesion localization. However, acidic replacements for these amino acids reduced focal adhesion staining intensities, suggesting that phosphorylation at these sites may negatively regulate integrin function.

The integrin superfamily of heterodimeric cell surface receptors associates with extracellular matrix (ECM) proteins and with cytoskeletal-associated proteins such as talin and $\alpha$-actinin (Burrage et al., 1988; Albeda and Buck, 1990). There are at least four integrin families, each defined by a different common $\beta$ subunit, and at least four other $\beta$ subunits that associate with only one $\alpha$. At least 12 $\alpha$ subunits have been identified which associate with one or more of the eight $\beta$ subunits. The $\alpha$ subunits generally confer ECM substrate specificity (Hepler, 1990; Albeda and Buck, 1990; Springer, 1990), although $\alpha_\alpha$, which associates with several different $\beta$ subunits, does not follow this rule (Smith et al., 1990; Bodary and McLean, 1990; Vogel et al., 1990).

Both $\alpha$ and $\beta$ integrin subunits have large extracellular, single membrane-spanning, and small cytoplasmic domains. The avian $\beta_1$ subunit ($\beta_{1o}$) is 803 amino acids long (Tamkun et al., 1986). The extracellular domain consists of the 735 NH$_2$-terminal residues and the cytoplasmic domain, the 40-46 COOH-terminal residues. The $\beta_1$ cytoplasmic domain shows complete sequence conservation between the chicken, human, and Xenopus cDNA clones (Tamkun et al., 1986; Argraves et al., 1987; DeSimone et al., 1988). The $\beta_1$ cytoplasmic domain encodes two NPXY signals, which have been associated with internalization of the LDL receptor (Chen et al., 1990). A tyrosine phosphorylation signal similar to ones found in the EGF receptor, the neu oncogene, and the insulin receptor are also located in the $\beta_1$ cytoplasmic domain (Tamkun et al., 1988). Other studies suggest that signals for talin and $\alpha$-actinin binding are present in the $\beta_1$ subunit cytoplasmic domain (Tapley et al., 1989; Otey et al., 1990).

Integrins appear to be regulated through phosphorylation of serine, threonine, and tyrosine residues in the $\beta$ subunit cytoplasmic domains. Previous studies have shown that the $\beta_{1c}$ subunit can be phosphorylated by pp60$^{c-src}$ on Y788 (Hirst et al., 1986; Tapley et al., 1989; Horvath et al., 1990), and that integrins from RSV-transformed cells show reduced focal adhesion localization. Studies involving the murine F9 teratocarcinoma $\beta_1$ integrin suggest serine phosphorylation...
results in reduced localization and organization (Dahl and Grabel, 1989). Other studies with CD11/CD18 associate integrin activation with serine phosphorylation (Chatila et al., 1989) and threonine phosphorylation on β2 (Hillery et al., 1991).

The β2 subunit appears to connect with the cytoskeleton through interactions with cytoskeletal proteins such as talin (Horwitz et al., 1985) and α-actinin (Otey et al., 1990). The talin interaction has been demonstrated further by competitive inhibition assays using a peptide, derived from the integrin β2 cytoplasmic domain. Furthermore, integrin phosphorylated by pp60src did not bind talin (Talipay et al., 1989).

Recently it has been shown that the β2c cDNA can be expressed in mouse fibroblasts and form interspecies heterodimers that localize in adhesion plaques and function in cell adhesion (Solowska et al., 1989; Hayashi et al., 1990). Human αc and β2, when coexpressed on COS cells, mediate adhesion to ICAM-1 (Hibbs et al., 1991). β2c subunit cytoplasmic domain termination deletions resulted in reduced or null localization in focal adhesions (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990). The largest of the β2c deletions and most of the β2 deletions were unable to mediate adhesion in vivo (Hayashi et al., 1990; Hibbs et al., 1991). Taken together, these studies suggest that β2 subunit sequences near residues 766 and 799 contribute to integrin function.

In the present study, we examine the effects of internal deletions and point mutations in the β2c cytoplasmic domain on integrin focal adhesion localization. We find that three regions within the β2c cytoplasmic domain contribute to integrin localization. Single amino acid residue substitutions in each of the three regions reduce integrin localization in focal adhesions. Serine, threonine, and tyrosine residues were replaced conservatively, with nonhydroxyl-bearing residues, and localization was unaffected. Acidic substitutions for Y788 and S790, on the other hand, resulted in a reduced focal adhesion localization.

Materials and Methods

Vector Construction

The integrin β2c cDNA was removed from the pGEMI-integrin plasmid (Takun et al., 1986) by digestion with EcoRI and purified by agarose gel electrophoresis (Seakem GTG, FMC BioProducts, Rockland, ME). The cDNA was ligated into EcoRI-cleaved pTZ18R vector (United States Biochemical, Cleveland, OH) using T4 DNA ligase (Gibco BRL, Gaithersburg, MD). This construct was subsequently digested with HindIII and PvuII and a 2.8-kbp pTZ-cDNA fragment was isolated. This fragment was ligated into pMAMneo (Clontech Laboratories, Inc., Palo Alto, CA), which was partially cleaved with HindIII (sites: 91, 2688) at position 91, and SmaI (sites: 1550 and 4011) at position 1550 to remove the MMTV promoter. The resulting vector, designated pRneo-β2c, expressed the β2c subunit under the RSV promoter only and also expressed neomycin (pRneo) as well.

Multiple restriction sites and mutants were generated as described (Hayashi et al., 1990). Amino acid residues predicted not to act as structure breakers were used for these substitutions, unless otherwise indicated in the Results (Chou and Fasman, 1978). Aspartate, glutamate, and threonine codons were replaced with valine, arginine, histidine, and lysine codons were replaced with isoleucine. Phenylalanine, threonine, tyrosine, and proline codons were replaced with alanine (F771L was generated as part of a novel HindIII site.) Y788 and S790 were also replaced with aspartic and glutamic acid, respectively, in an attempt to simulate phosphorylation.

Cell Culture

NIH 3T3 (Swiss mouse embryo) cells were grown in DME (Sigma Chemical Co., St. Louis, MO) with 10% iron-supplemented bovine calf serum (HyClone Laboratories, Inc.; Logan, UT). Transfected cells were initially grown in the above medium supplemented with geneticin (G418 sulfate, Gibco BRL) at 1 mg/ml (selection medium). Stable transfecants were grown in media supplemented with geneticin at 200 μg/ml (maintenance medium).

Transfections

Transfections were performed by calcium phosphate precipitation (Graham and van der Eb, 1973) as modified by Frost and Williams (1978). NIH 3T3 cells were grown in DME containing 10% calf serum (HyClone Laboratories). 0.25 μg pRneo-β2c DNA and 15 μg NIH 3T3 carrier DNA were cotransfected for 48 h into 2–4 × 10⁵ NIH 3T3 cells in 60-mm tissue culture dishes and then were transferred (1:4–1:16) to 2–100-mm dishes containing selection media. After 7–9 d, 12–24 individual clones were isolated using cloning rings and then expanded. β2c-expressing clones were identified by immunofluorescence staining using anti-β2c antibodies (see below). Focal adhesion staining phenotypes were verified by analyzing 12 or more clones from different transfection experiments. Populations with a high percentage of cells expressing these mutants were subcloned, by limiting dilution, to homogeneity before further biological and biochemical analyses.

Antibodies

Polyclonal antibodies (Ab 814) and mAbs (WIB10) directed against the integrin β2c subunit have been described previously (Hayashi et al., 1990). FITC- or rhodamine-conjugated goat anti-mouse or anti-rabbit IgG (Fab')2 were purchased from Cappel Laboratories (Organon Teknika, West Chester, PA).

Immunofluorescent Staining

NIH 3T3 cell cultures were rinsed 2× with PBS-EDTA and then treated with 0.0025% trypsin (Gibco BRL) in calcium and magnesium-free PBS (CMF-PBS) for 5–10 min at 37°C. Cells were suspended in 5 vol DME with 0.5% calf serum and then seeded on fibronectin-coated (20 μg/ml in CMF-PBS, overnight at 4°C) glass slides, which were divided into grids of 12–15 squares using a PAP pen (Research Products, International; Mount Prospect, IL). After 5 h incubation at 37°C, the cells were stained either live with Ab 814 or WIB10 (Boycezio et al., 1989) or live with Ab814 followed by permeabilization and a second staining with WIB10. All immunofluorescent staining procedures were carried out at room temperature. Ab814 and WIB10 antibodies were diluted to 20 μg/ml in PBS containing 5% goat serum. Incubations with primary and secondary antibodies lasted 20–30 min at room temperature. Live cell staining was followed by fixation with 3% formaldehyde (Polysciences, Washington, PA) and then quenched with 0.15 M glycine in PBS. For internal staining, cells were permeabilized with Triton X-100 after formaldehyde fixation and glycine quenching. FITC- and rhodamine-conjugated goat antimouse and rabbit antibodies were diluted (10 μg/ml in PBS containing 5% goat serum and incubated on the cells for 20–30 min. Cells were mounted and observed under an axiptop fluorescein microscope (Carl Zeiss, Inc., Thornwood, NY).

Flow Cytometry and Cell Sorting

Cell sorting was performed as described (George-Weinstein et al., 1988). Cells were released from culture dishes using calcium and magnesium-free Hepes-Hanks buffer (CMF-HH) containing 0.02% EDTA, collected by cen-
trifugation, and suspended in 1 ml CMF-HH buffer containing 2% BSA and 0.02% Na3H (HHA). Cell numbers were adjusted to 10⁶ cells/ml. 20 µg WIB10 in HHA was added to 1 ml cell suspension and incubated for 30 min. Cells collected and rinsed 2× with HHA, were then resuspended in 1 ml HHA containing FITC-conjugated goat antimouse antibody (Cappel), at a ratio of 1:200 dilution, for 30 min. Cells were finally washed 2× with HHA, then 2× with CMF-HH containing 0.02% NaN₃.

Cell populations were examined using an EPICS™ cell sorter employing MDADS™ analysis software for analyzing fluorescence intensities (Coulter Electronics, Inc., Miami Lakes, FL). Fluorescence excitation at 488 nm was generated using an argon ion laser. Window limits for cell sort populations were set at 30–50 fluorescence units (fu) which was 10–13-fold higher than mean background fluorescence (3.5 fu). Cell populations were allowed to recover in DME containing 10% calf serum and G418 (250 µg/ml) for 15–24 h before immunofluorescent staining.

**Evaluation of Focal Adhesion Staining Intensities**

Focal adhesion staining intensities were initially determined on cells stained both on the surface with Ab 814 and, after Triton X-100 permeabilization, with WIB10. Cells expressing wild-type and mutant β₆ with equivalent internal WIB10 staining intensities were compared for focal adhesion intensities detected with Ab 814. From each transfection, a minimum of twelve clones were examined by immunofluorescent staining, and two to three clones containing a high percentage of cells expressing the β₆ subunit were analyzed for further analyses. Within each clonal population, localization phenotypes were determined by examination of hundreds of cells in multiple fields under the microscope. Cells showing various sizes and degrees of spreading were compared to equivalent wild-type β₆-expressing cells. Cells expressing mutant β₆ integrins with reduced focal adhesion staining intensities were then normalized for equivalent surface expression levels by flow cytometry using the WIB10 mAb. This insured that differing β₆ surface expression levels did not bias the estimation of focal adhesion staining intensities. Cells expressing the A771-790 deletion mutant were sorted at slightly lower intensity levels (5–7-fold over background) than wild-type, (10–13-fold greater). Focal adhesions were detected with Ab814. From each transfection, a minimum of twelve clones were examined by immunofluorescent staining, and two to three clones containing a high percentage of cells expressing the β₆ subunit were analyzed for further analyses. Within each clonal population, localization phenotypes were determined by examination of hundreds of cells in multiple fields under the microscope. Cells showing various sizes and degrees of spreading were compared to equivalent wild-type β₆-expressing cells. Cells expressing mutant β₆ integrins with reduced focal adhesion staining intensities were then normalized for equivalent surface expression levels by flow cytometry using the WIB10 mAb. This insured that differing β₆ surface expression levels did not bias the estimation of focal adhesion staining intensities. Cells expressing the A771-790 deletion mutant were sorted at slightly lower intensity levels (5–7-fold over background) than wild-type, (10–13-fold greater).

**Immunoprecipitations**

Immunoprecipitations were performed as described (Hayashi et al., 1990). Cells were labeled metabolically with [35S]methionine (1,000 Ci/ml; Dupont New England Nuclear, Boston, MA) for 24 h. After labeling, cell cultures were washed three times with CMF-PBS then collected with cell scrapers (GIBCO BRL). The cells were incubated with the WIB10 mAb or Ab 814 (50 µg/ml) for 1 h at 4°C, and then washed three times with CMF-PBS. The cells were then solubilized in TNC (0.01 M Tris, pH 8.0; 0.15 M NaCl; 0.5 mM CaCl₂, 0.5% NP-40) and a protease inhibitor cocktail (pepstatin, 20 µg/ml; leupeptin, 200 µg/ml; O-phenanthroline, 9 µg/ml; PMSF, 0.09 µg/ml). NP-40 extracts (5 x 10⁶ cpm) were combined with 100 µl of 50% (vol/vol) anti-rabbit IgG-conjugated polyclarimide beads (Bio Rad Laboratories). Beads were previously blocked by incubation in an unlabeled TNC extract of NIH 3T3 cells. After incubation, beads were washed repeatedly with TNC buffer. Proteins were released from the beads by heating to 60°C in 40 µl Laemmli sample buffer (Laemmli, 1970). Samples were centrifuged in a microfuge for 10 min at 4°C, and supernatant aliquots (3,500 cpm) were loaded on 7% SDS polyacrylamide gels. Gels were visualized after drying by fluorography (Laskey, 1980).

**Results**

**β₆ Deletion and Insertion Mutants**

**Deletions.** Previous studies of the integrin β₆ cytoplasmic domain suggest that more than one location is required for function (Tapley et al., 1989; Hayashi et al., 1990; Marcontonio et al., 1990). The A759-771 and A771-790 β₆ deletion mutants were created to narrow the search for amino acid sequences that direct integrin to focal adhesions (Fig. 1). The Δ759-771 deletion removed 10 amino acids adjacent to the membrane. This region is reported to mediate localization of β₆ in focal adhesions (Marcontonio et al., 1990) and cellular adhesion through α₅β₆ (Hibbs et al., 1991). The Δ771-790 deletion removed 20 central residues. A peptide included in this sequence (WDTGENPYK) inhibited integrin–talin interactions in vitro (Tapley et al., 1989). Both deletions were generated by molecular cloning of novel HindIII sites, introduced by oligonucleotide-directed mutagenesis as described in Materials and Methods.

**Plasmids encoding wild-type and mutant β₆ subunit cDNAs were transfected into NIH 3T3 cells and then selected with G418. Cells from a number of individual colonies expressing wild-type and mutant β₆ subunits were screened for focal adhesion intensities both by surface and internal indirect immunofluorescent staining (see Materials and Methods). Determination of focal adhesion staining intensities of mutant β₆ subunits were based on consistency within and among clonal populations. When mutants were initially found to have lowered focal adhesion staining intensities, transfections were repeated two to four times, and clonal and nonclonal populations were analyzed. Cell populations expressing these β₆ mutants were passed through an EPICS™ cell sorter to collect cells with equivalent surface expression, i.e., 10–13-fold over background (Fig. 2). This insured accurate comparisons of focal adhesion staining intensities. After sorting, cells were plated on fibronectin and then surface stained by indirect immunofluorescence.

Transfected cells expressing the Δ759-771 (Fig. 3a) and Δ771-790 β₆ deletions demonstrated trace (+), but detectable, β₆ immunofluorescent staining in focal adhesions relative to that of the wild-type (Fig. 3a). Reduced focal adhesion staining intensities of the Δ759-771 and Δ771-790 β₆ deletion mutants suggests that these regions contain sequences that contribute to localization in focal adhesions. The presence of these mutant β₆ subunits in focal adhesions demonstrates that localization signals also exist outside of the deleted regions, presumably in the remaining β₆ sequences. Termination mutants extending from 799–803 and 800–803 (Hayashi et al., 1990; Marcontonio et al., 1990) have already been shown to localize in focal adhesions.
Surface immunofluorescence intensities of cells expressing wild-type and mutant \( \beta_{ic} \) integrins. Cells were labeled with the WIB10 mAb and then with FITC-conjugated goat antimouse polyclonal antibodies. Cells with immunofluorescence intensities 10-13-fold above background (indicated by parallel vertical lines) were sorted for subsequent analyses of focal adhesion localization. (a) Wild-type \( \beta_{ic} \), (b) D764V (\( \beta_{ic} \)), (c) F771L (\( \beta_{ic} \)), (d) N785I (\( \beta_{ic} \)) transfected, and (e) untransfected NIH 3T3 cells.

**Figure 2.** Surface immunofluorescence intensities of cells expressing wild-type and mutant \( \beta_{ic} \) integrins. Cells were labeled with the WIB10 mAb and then with FITC-conjugated goat antimouse polyclonal antibodies. Cells with immunofluorescence intensities 10-13-fold above background (indicated by parallel vertical lines) were sorted for subsequent analyses of focal adhesion localization. (a) Wild-type \( \beta_{ic} \), (b) D764V (\( \beta_{ic} \)), (c) F771L (\( \beta_{ic} \)), (d) N785I (\( \beta_{ic} \)) transfected, and (e) untransfected NIH 3T3 cells.

Taken together, these results suggest that multiple localization signals exist in the cytoplasmic domain of \( \beta_{ic} \) and that not all sequences need be present for localization to focal adhesions.

**Insertions.** The \( \Delta 759-771 \) \( \beta_{ic} \) deletion maintained, but shifted residues 772-803 closer to the membrane. The intact sequences contributed to localization in focal adhesions, suggesting that a precise phasing of the \( \alpha \) and \( \beta \) cytoplasmic domains is not necessary for function. Therefore, we investigated the effects of shifting the intact \( \beta_{ic} \) cytoplasmic domain away from the transmembrane domain by insertion mutations. Molecular cloning of novel HindIII sites was used to create \( \beta_{ic} \) insertions, between the cytoplasmic and transmembrane domains, of 6 and 32 residues. The 764::757 \( \beta_{ic} \) insertion mutant introduced the sequence, KLLMMM, between residues 756 and 757 (Fig. 1), resulting in only reduced focal adhesion staining intensities (+++, data not shown). The 788::757 \( \beta_{ic} \) insertion mutant duplicated residues 757-788 as a direct repeat (Fig. 1), also resulting in reduced focal adhesion staining (+++, data not shown). These findings suggest that precise alignment of \( \beta \) and \( \alpha \) subunit cytoplasmic sequences is not required for \( \beta_{ic} \) integrin sequences to contribute to localization.

**\( \beta_{ic} \) Missense Mutants**

Phenotypes of \( \beta_{ic} \) deletion mutants suggest that more than one region in the cytoplasmic domain is required for localization in focal adhesions. Therefore, we generated missense mutations to identify specific residues that contribute to localization in focal adhesions. Wild-type and 50 mutant \( \beta_{ic} \) cDNAs were transfected into NIH 3T3 cells and screened as described in Materials and Methods. Below we describe the localization phenotypes of 42 single and 8 double missense mutations.

Except where noted, amino acid substitutions were designed to avoid the use of \( \alpha \)- or \( \beta \)-breakers (Chou and Fasman, 1978), and to maintain the general size and shape and/or the hydrophobicity of the R-group being replaced. Mutations produced by degenerate oligonucleotides, however, sometimes resulted in substitutions that were either too conservative or nonconservative. When overly conservative mutations were generated, other less conservative mutations were also generated at the same site. Nonconservative mutants were assayed for focal adhesion staining, and if reductions in focal adhesion staining intensities were not detected, no further mutations were generated.

To simplify descriptions of the missense mutants, we have divided the \( \beta_{ic} \) cytoplasmic domain into three regions: I (763-775), II (776-790), and III (791-803). A summary of all single mutations in the \( \beta_{ic} \) cytoplasmic domain and their localization phenotypes is shown in Fig. 4.

**Single Mutants**

Region I contains putative residues capable of directing \( \beta_{ic} \) localization to focal adhesions in NIH 3T3 cells and \( \beta_{ic} \)-mediated cellular adhesion (via \( \alpha_{ic}\beta_{ic} \)) in COS cells (Mar cantonio et al., 1990; Hibbs et al., 1991). 15 individual mutations were generated in this region, replacing 12 residues. Replacements of 8 of these 12 residues resulted in normal focal adhesion staining intensities (+++: histidine at position 763 was replaced with isoleucine (H763I, ++++) and with lysine (H763K, ++++). Arginines at positions 765 and 766 were replaced with isoleucine (R765I and R766I, ++++), or more conservatively with lysine (R765K, ++++), all resulting in normal focal adhesion staining. Glutamates at posi-
Figure 3. Localization of representative wild-type, deleted, and point mutated β_{c} integrins on the cell surfaces of NIH 3T3 cells. Cells were plated on fibronectin substrates 15 h after cell sorting, allowed to spread for 5 h, labeled with Ab814, fixed with formaldehyde, and then labeled with a FITC-conjugated goat antirabbit polyclonal antibody. (a) Wild-type (+++ [localization phenotype]); (b) untransfected (n.a.); (c) Δ759-771β_{c} (+); (d) D764V (++); (e) Y800A (++); (f) F771L/N797I (+). Bar, 30 μm.

Mutations 767 and 772 were replaced with valine (E767V and E772V, +++), both without effect. Lysines at three positions: 770, 773, and 775, were replaced with isoleucine (K770I, K773I, and K775I, ++++) and more conservatively with arginine (K770R, +++), all resulting in normal focal adhesion staining intensities.

Mutations of four residues in region I of β_{c} resulted in reduced focal adhesion staining intensities relative to wild-type: Aspartate at position 764 was replaced with valine (D764V), resulting in reduced focal adhesion staining (++; Fig. 3 d). Phenylalanine at positions 768 and 771 was replaced with either alanine (F768A) or leucine (F771L), each resulting in reduced focal adhesion staining (+). Leucine was used to replace F771 since it was a byproduct of forming a novel HindIII site. Since F771L reduced the focal adhesion staining intensity of β_{c}, an alanine substitution was not generated. Glutamate at position 774 was replaced with valine (E774V), also resulting in reduced focal adhesion staining intensities (++). Glutamate to valine substitutions did not alter the β_{c} staining of E767V or E772V, therefore, E to V substitutions in this region did not universally result in altered focal adhesion staining.

Region II contains sequences that have been reported to inhibit integrin–talin interactions in vitro, a tyrosine residue that is phosphorylated by pp60^src in vivo and in vitro, and a serine which is phosphorylated in vivo (Tapley et al., 1990; Hirst et al., 1986; Horvath et al., 1990; Dahl and Grabel, 1989). Sixteen substitutions at 11 sites in region II were assayed for focal adhesion localization. Alterations of seven of these residues resulted in normal focal adhesion staining intensities (+++). Asparagine at position 777 was replaced with isoleucine (N777I, +++). Lysine at positions 779 and 789 was replaced with isoleucine (K779I, +++) or leucine and valine (K789L, +++ and K789V, +++). Replacement of lysine at position 789 with proline (K789P), which is both an α-breaker and a β-breaker, resulted in reduced localization (++). Since both leucine and valine substitutions at this site resulted in normal focal adhesion localization, this suggests that the K789P phenotype is due to the presence of proline rather than the absence of lysine. Tyrosine at position 786, which itself is an α-breaker and an β-breaker, was replaced with alanine (T786A, +++) which is neither an α-breaker nor a β-breaker. Tyrosine at position 788 was replaced previously with phenylalanine.
Marcantonio et al., 1990). 11 mutations of 8 residues in this region were examined for focal adhesion staining intensities. Six of eight substitutions did not alter focal adhesion staining intensities: threonines at positions 793 and 794 were simultaneously replaced with valines (T793IV, ++ +). Proline at position 798 was replaced with alanine (P798A), which surprisingly resulted in normal focal adhesion staining intensities (++++). Lysine at positions 799 and 803 were replaced with both isoleucine (K799I, ++++ and K803I, ++++) and more conservatively with arginine (K799R, ++++) and K803R, ++++). Finally, glutamate was conservatively replaced with aspartate (E801D, ++++) and with valine (E801V, ++++).

Replacement of two residues in region III resulted in reduced focal adhesion staining intensities: asparagine at position 797 was replaced with isoleucine (N797I), and like N785I, resulted in reduced focal adhesion staining intensities (++++). Tyrosine was replaced with alanine (Y800A), resulting in reduced focal adhesion staining (++ +, Fig. 3e), while a phenylalanine substitution at this site (Y800F) resulted in normal focal adhesion intensities (++++). Both Y800 and Y788 (Hayashi et al., 1990) phenylalanine substitutions did not alter focal adhesion intensities, while alanine substitutions did.

Double Amino Acid Substitutions

None of the aforementioned single missense mutations resulted in trace or null focal adhesions localization phenotypes. We generated double mutations in regions I/II, II, and II/III to investigate whether the inhibitions were additive.

**Figure 5.** Focal adhesion staining intensities for wild-type βc and cytoplasmic domain double point mutants. Locations for amino acid changes and relative focal adhesion staining intensities are as described in Fig. 4.

**Figure 6.** Immunoprecipitation of surface chicken βc/mouse α chimeric integrins from NIH 3T3 cells. Cells were labeled with [35S]methionine, and the βc integrins were immunoprecipitated from cell extracts made after preincubation with the polyclonal antibody, Ab 814. (Lane 1) Untransfected NIH 3T3 cells; (lane 2) wild-type βc; (lane 3) Y788E (βc); (lane 4) F771L/N797I (βc); (lane 5) N785I/N797I (βc); (lane 6) F771L (βc); (lane 7) K770R (βc); (lane 8) N797I (βc). Arrows indicate the locations of the four integrin bands: mature α's (double arrows); mature β; and pre-βc (from lysed cells).
or trace focal adhesion localization phenotypes: Y788E individual components that comprise F771L/N797I, namely were present in all other immunoprecipitates including the controls. These data were similar to those seen for all other It appears unique to this mutation as both α subunit bands was seen in analyses of four individual F771L/N797I clones. phenotype mutation in combination with a localization-altered mutants (data not shown) except the (lane 8), and normal localization phenotypes: K770R (lane 6). The combination of two normal-phenotype mutants: D764V (+ +) with N797I (+ +) [D764V/N797I], F771L (+ +) with N797I (+ +) [F771L/N797I, Fig. 3 J], N7851 (+ +) with N797I (+ +) [N7851/N797I], and Y788A (+ +) with N797I (+ +) [Y788A/N797I], resulted in trace focal adhesions staining intensities (+). Therefore, these loci in regions I, II, and III appear to work together to direct localization.

Dimerization and Surface Expression of Mutant Integrins

Previous studies have shown that αβ dimerization is required for surface expression of integrin β subunits (Heino et al., 1989). All previously described βEC termination deletion mutants that failed to localize in focal adhesions formed αβ homodimers (Hayashi et al., 1990; Marcantonio et al., 1990; Hayashi, data not shown). Thus, it is unlikely that the deduced focal adhesion staining intensities of βEC mutants in this study were due to deficient dimerization, especially since EPICS™ flow cytometry demonstrated that these mutant βEC subunits were expressed on the cell surface. To demonstrate directly that dimerization occurred between mutant βEC subunits and host α subunits, we immunoprecipitated the wild-type and mutant βEC integrins with chicken-specific antibodies and assayed for the coprecipitation of the murine α subunits. Because deletion and double mutants tended to show lower mean surface expression levels by EPICS™ flow cytometry, we sorted these populations, selecting for expression levels at least threefold over background, before immunoprecipitation. We immunoprecipitated 35S-labeled wild-type and mutant βEC from the surface of transfected NIH 3T3 cells using the βEC-specific polyclonal antibody Ab 814. Immunoprecipitates of wild-type βEC (Fig. 6, lane 2) yielded four bands in the 95-140-kD range, which were not present in immunoprecipitates from untransfected cells (lane 1). These bands represent a doublet of two 140-kD α subunits, the 120-kD mature βEC subunit, and the 95-kD βEC subunit precursor (pre-β), whose presence in these assays is due to a small amount of cell lysis during preparation (Hayashi et al., 1990).

Immunoprecipitates from mutant integrins with reduced or trace focal adhesion localization phenotypes: Y788E (lane 3), N7851/N797I (lane 5), F771L (lane 6), and N797I (lane 8), and normal localization phenotypes: K770R (lane 7), showed the same four integrin bands found in wild-type controls. These data were similar to those seen for all other localization-altered mutants (data not shown) except the F771L/N797I double mutant (lane 4), which failed to copurify the larger of the two α subunit bands. This phenotype was seen in analyses of four individual F771L/N797I clones. It appears unique to this mutation as both α subunit bands were present in all other immunoprecipitates including the individual components that comprise F771L/N797I, namely F771L and N797I (compare lane 4 to lanes 6 and 8) and the other double mutation involving cyto-1 and -3 (D764V/N797I, data not shown).

Discussion

The observations presented here confirm previous conclusions that the integrin βEC cytoplasmic domain contains sequences essential for integrin function. With missense mutagenesis we have identified active regions in the βEC cytoplasmic domain that contribute to localization in focal adhesions. Although the contribution of the α subunit cytoplasmic domain to integrin localization in focal adhesions has not been investigated, the present evidence suggests that the β subunit cytoplasmic domain contains most if not all of the sequences required for this function. The βEC cytoplasmic domain binds to both talin (Tapley et al., 1989) and α-actinin (Otey et al., 1990) in vitro. Previous avian integrin deletion studies have shown that βEC cytoplasmic sequences are required for integrin localization and function in adhesion (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990). Analogous observations on the LFA-1 integrin (CD11a/CD18) also point to the importance of the βEC cytoplasmic domain in adhesion to ICAM-1. Deletions of the αE cytoplasmic domain did not inhibit adhesion to ICAM-1, while deletions in βEC did (Hibbs et al., 1991). Electron microscopic images show that the integrin αE and β cytoplasmic domains are separate (Carrell et al., 1985; Kelly et al., 1987; Nermut et al., 1988), suggesting that each may function independently. In this study, insertion mutants, 764:757:764 and 788:757:788 localized in focal adhesions, despite imprecise phasing of the αE and βEC cytoplasmic sequences. This also suggests that the β cytoplasmic domain sequences can function independently from the sequences of the α subunit. Taken together, these observations focus attention on β subunit sequences in integrin–cytoskeletal interactions.

These experiments were designed to identify specific sequences in the βEC cytoplasmic domain involved in the localization of integrin to focal adhesions. Our internal deletion studies and other previous COOH-terminal deletion studies (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990) show that the localization signals do not reside in any single region of the cytoplasmic domain. Through missense mutagenesis, three clusters of amino acids were identified: cyto-1 (residues 763–771), cyto-2 (residues 785–788), and cyto-3 (residues 797–800), which are implicated in the localization of integrin to focal adhesions. Mutations in these three clusters, while causing reductions in βEC localization to focal adhesions, did not cause changes in the focal adhesions themselves. Through immunofluorescent staining for both talin and vinculin, we found that focal adhesions in cells expressing mutant βEC subunits were comparable to those in untransfected or wild-type βEC-expressing cells (data not shown). Hayashi et al. (1990) reached similar conclusions with respect to the integrity of vinculin and the murine β subunit in cells expressing wild-type or null mutant βEC subunits. Taken together, these findings suggest that the amino acid substitutions in the three cyto-clusters affected primarily the localization of the βEC subunit to focal adhesions.

The amino acid residues that comprise these three clusters are highly conserved among integrin β subunits (Fig. 7),
suggested that their importance extends beyond the \( \beta \) family. Excluding \( \beta_4 \), whose cytoplasmic domain is completely dissimilar (Suzuki and Naitoh, 1990; Hogervorst et al., 1991), only the \( \beta_3 \) subunit differs from the other \( \beta \) subunits in these clusters. Its cyto-3 cluster is displaced eight amino acid residues farther away from cyto-2. Of the nine amino acid residues implicated in integrin localization to focal adhesions, six are conserved in all of the \( \beta \) families. If one allows conservative substitutions, eight of nine residues are conserved. F768 is the exception, which is replaced by valine. The interpretation of this high degree of conservation is not yet clear as the functions of all of the \( \beta \) subunit cytoplasmic domains have not been characterized. However, a relationship between conservation and localization is evident in \( \beta_1/\beta_3 \) chimeras. Both \( \beta_1 \) and \( \beta_3 \) integrins localize at the ends of actin stress fibers in focal adhesions. Interestingly, transmembrane and cytoplasmic domain chimeras between \( \beta_1 \) and \( \beta_3 \) also localize in focal adhesions (Solowska et al., 1991). The cytoplasmic domains of \( \beta_1 \) and \( \beta_3 \) are only 59% identical while cyto-1, 2, and 3 are 100% identical. Taken together, it appears that these clusters, which are found in most \( \beta \) subunits, are required for localization in focal adhesions.

The four residues in cyto-1 implicated in focal adhesion localization have characteristics suggesting they form an integrin-specific focal adhesion signal. Computer-generated predictions of the structure of the \( \beta_1 \) cytoplasmic domain, using the algorithms of Chou and Fasman (1978) and Garnier et al. (1978), show that cyto-1 forms an \( \alpha \) helix with high probability (data not shown; DNAAstar, Madison, WI). The four identified residues, D764, F768, F771, and E774, constitute only 36% of this region. However, these four identified residues cluster on one side of the helix, when drawn using a helical wheel with 100° spacing (data not shown, Schiffer and Edmundson, 1967). This suggests that the cyto-1 signal is composed of these four residues present on one face of an \( \alpha \) helix. A database search for proteins with sequences containing cyto-1 residues found only integrin \( \beta \) subunit sequence matches (Pearson and Lipman, 1988). Since these residues are also highly conserved among \( \beta \) subunits, these findings point to the cyto-1 residues as a signal required for focal adhesion localization.

Cyto-1 may also be involved in \( \alpha \beta \) dimerization. Transfectants expressing the K770R \( \beta_1 \) mutant showed enhanced surface expression, reduced vesicular staining, and enhanced ratios of mature to pre-\( \beta_1 \), relative to wild-type (data not shown). In addition, the F771L/N797I double mutant failed to form a dimer with one of the murine \( \alpha \) subunits. A similar observation has been previously described for a \( \beta_4 \) termination deletion mutant (7651), which lacked residues 765–803 and only formed dimers with a lower molecular weight \( \alpha \) subunit (Hayashi et al., 1990). Taken together, these observations implicate this part of cyto-1 in heterodimer affinity and specificity.

The cyto-2 cluster is comprised of the sequence, NPIY. Mutations of either the asparagine (N), proline (P), or tyrosine (Y) alone results in reduced localization to focal adhesions, while triple (N, P, and Y) or quadruple mutations of these four residues resulted in trace localization (manuscript in preparation). The general NPYX structure has been identified as one of a group of tyrosine-bearing internalization sequences (Chen et al., 1990). The NPYX sequence forms tight turns in four of five proteins with known three-dimensional crystallographic structures (Collawn et al., 1990). The effects of removal or addition of proline in and adjacent to this region suggests that this sequence in integrin also forms a tight turn that is important for function. This region of \( \beta_4 \) has also been demonstrated to interact with pp60-src, which phosphorylates Y788 both in vivo and in vitro, and is implicated in integrin-talin interaction in vitro. Therefore, cyto-2 is a recognized sequence. With only conservative substitutions for isoleucine (I787) and tyrosine (Y788) among the different \( \beta \) subunits, cyto-2 likely plays a consistent role throughout the integrin families. Taken together, we conclude that cyto-2 provides a folded conformation and might itself act as a signal directly involved in integrin localization to focal adhesions.

The similarity between the \( \beta_4 \) cyto-2 and cyto-3 signals, NPYV vs NPKY, would suggest that these are redundant NPYX signals, perhaps with both forming tight turns and both involved in integrin–cytoskeletal interactions. However, differences in conservation and relative importance of residues in cyto-3 and cyto-2 point to distinct functions for each. Only the asparagine and tyrosine residues of cyto-3 are conserved among the \( \beta \) families, while the proline and lysine residues show no conservation at all. Internalization studies on the LDL receptor demonstrate the importance of the proline residue for NPYX signal function (Chen et al., 1990). In this study, we have found that proline in cyto-2 is also important for focal adhesion localization, while the cyto-3 proline residue can be replaced with no apparent effect on localization in focal adhesions. The insensitivity of this region to the removal of proline and lysine and the lack of conservation of this residue, implies that the function of this region does not require a strict structural motif. However, the specific location of this sequence may be necessary for its function. The \( \beta_4 \) subunit, which contains a cyto-3-like sequence, shifted eight amino acids away from its location in the other \( \beta \) subunits (McLean et al., 1990; Ramaswamy and Hemler, 1990), does not localize in focal adhesions when dimerized with \( \alpha_6 \) (Wayner et al., 1991). However, the \( \alpha_6/\beta_4 \) integrin in the same cells does localize in focal adhesions, presumably because the \( \beta_4 \) cyto-3 signal is identical with that of \( \beta_4 \). Taken together, we conclude that cyto-3 is an NXX.Y signal, which contributes to integrin localization in focal adhesion structures.

Integrin phosphorylation has been linked to the regulation and loss of integrin function in normal and oncogenically...
transformed cells. Both threonine and serine have also been implicated in integrin regulation. Threonine phosphorylation of platelet β₁ integrin subunits (GPIIb), has been related to structural or functional alterations (Hillery et al., 1991). All β₁c threonine substitutions in this study resulted in normal integrin localization, demonstrating that individual threonine residues are not required for focal adhesion localization. Serine phosphorylation has been linked to both negative regulation of β₁ integrins (Dahl and Grabel, 1989) and positive regulation of β₁ integrins (Chatilla et al., 1989). The β₁c serine to methionine (S790M) mutant in this study resulted in normal focal adhesion localization, demonstrating that serine phosphorylation does not positively regulate β₁ integrin localization in focal adhesions. An acidic substitution for the β₁c serine (S790D) did reduce integrin localization, supporting the hypothesis that phosphorylation negatively regulates β₁c integrin function. Tyrosine phosphorylation by pp60⁻⁰⁻⁻⁻⁻ is linked to reduced integrin organization and overall morphological changes observed in RSV-transformed cells (Hirst et al., 1986; Horvath et al., 1990). Both Y788A and Y788E inhibited localization. Y788E resulted in the most significant reduction in focal adhesion staining of all single point mutations, suggesting that negative charge played a role in this effect. Taken together our findings demonstrate that neither individual serine, threonine, nor tyrosine residues are required for integrin localization in focal adhesions and that serine and tyrosine phosphorylation could act negatively to regulate integrin function.

In summary, the integrin β₁c subunit cytoplasmic domain has three regions implicated in integrin localization to focal adhesions. A depiction of the putative integrin signals for focal adhesion localization is shown in Fig. 8. Cyto-1 is unique, not found in any protein sequences reported to date, and contains four key residues that appear to form a signal on one side of an α helix. Cyto-2 shows homology to a tight turn motif recognized by proteins involved in receptor internalization and by pp60⁻⁰⁻⁻⁻⁻. In addition to its structural role, it may also interact with components of the cytoskeleton. This sequence is also a candidate for negative regulation through serine and tyrosine phosphorylations. Cyto-3, although similar to cyto-2 and the NPYX motif, appears to be distinct based on the lack of conservation and sensitivity to changes of the proline and lysine residues.

How then, do these three regions, removed in sequence, form an active complex? The available evidence points to a folded structure that may juxtapose cyto-1 and cyto-3. Together these signals affect both heterodimer formation and localization. Cyto-2 forms a turn, is subject to regulation and, perhaps, is also a part of the signal.