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Mapping of the functional determinants of the integrin β_1 cytoplasmic domain by site-directed mutagenesis

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We describe here the expression of deletion mutants of the cytoplasmic domain of the avian integrin β_1 subunit. These mutants, which contain termination codons at positions 767, 776, 791, and 800, were transfected into mouse 3T3 cells to determine which sequences were essential for localization of integrins into focal contact sites. In all cases, high-level expression of the truncated avian integrins was obtained. Heterodimers were formed between the exogenous truncated avian β_1 subunits and endogenous mouse α subunits, and these heterodimers were efficiently exported to the cell surface. The longest truncated β_1 subunit tested, which is only four amino acids shorter than the wild type, does localize to focal contacts. In contrast, β_1 subunits with moderately long truncations of the cytoplasmic domain failed to localize to focal contacts, including one which contains the consensus sequence for tyrosine phosphorylation. Surprisingly, a mutant subunit in which the bulk of the cytoplasmic domain was missing (but the segment nearest the membrane including the dibasic residues (RR) remained) did localize weakly to focal contacts. These results implicate the peptide segment nearest to the transmembrane region in focal contact localization. In addition, mutant subunits that included this segment together with a larger portion of the cytoplasmic domain did not localize as well as the shorter form, suggesting that these cytoplasmic domain segments are defective, presumably because of abnormal folding.

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Introduction

Cellular interactions with the extracellular matrix (ECM) play an important role in many biological systems. ECM proteins provide important cues in many migratory pathways in development and in other processes such as normal wound healing, tumor cell metastasis, and invasion. For cells to utilize the cues provided by the ECM for migration and targetting, there must be receptors that recognize signals from the ECM. Among the receptors that bind ECM proteins are the integrin receptors, a family of transmembrane glycoproteins that interact with a wide variety of ligands, including extracellular matrix glycoproteins, complement, and membrane proteins (Hynes, 1987; Buck and Horwitz, 1987; Ruoslahti and Pierschbacher, 1987; Ruoslahti, 1988; Hemler, 1990). These receptors participate in cell-matrix and cell-cell adhesion in a large number of physiologically important processes, including embryologic development and morphogenesis, hemostasis and thrombosis, wound healing, malignant transformation, and leukocyte helper and killer functions.

These receptors are transmembrane, noncovalently linked heterodimers with distinct α and β subunits. Integrins are found concentrated in focal contacts on the ventral cell surfaces of adherent cells, colocalizing with ECM proteins and with the cytoskeleton (Chen *et al.*, 1985; Damsky *et al.*, 1985; Dejanna *et al.*, 1988; Singer *et al.*, 1988). Integrins bind both to ECM molecules such as fibronectin (FN), laminin, vitronectin, and collagen (see reviews above) and to cytoskeletal proteins such as talin (Horwitz *et al.*, 1986; Tapley *et al.*, 1989). Intact heterodimers are required for binding to both ECM and cytoskeletal molecules (Buck *et al.*, 1986).

There are at least 5 homologous integrin β subunits and 11 different α subunits. All classes of β subunits have a large extracellular domain, and most have a total of 56 conserved cysteine residues, including 4 cysteine-rich repeats. There are single transmembrane segments in all integrin subunits and generally a short cytoplasmic domain. Within the β_1 cytoplasmic

domain, there is a site for tyrosine phosphorylation (Hirst *et al.*, 1986; Tamkun *et al.*, 1986); we have shown that this domain is highly conserved in both vertebrates and invertebrates (DeSimone and Hynes, 1988; Marcantonio and Hynes, 1988). The cytoplasmic domains of integrins are involved in their interactions with the cytoskeleton, because deletion of the β_1 cytoplasmic domain blocks the ability of integrins to organize into focal contacts (Solowska *et al.*, 1989). This domain has been shown to interact with the protein fibulin, which also colocalizes in focal contact sites (Argraves *et al.*, 1989).

To study the structure-function relationships of various domains within the avian β_1 subunit, we expressed this subunit in mouse 3T3 cells under the control of an SV40 early promoter (Solowska *et al.*, 1989). This exogenous subunit was efficiently and stably expressed and formed heterodimers with the endogenous mouse α subunits. Furthermore, these heterodimers are exported to the cell surface where they bind FN and localize to focal contact sites where both the extracellular matrix and the cytoskeleton associate with the plasma membrane. Thus, the exogenous avian β_1 subunit appears to function together with the endogenous murine α subunits, consistent with the high degree of sequence homology between murine and avian β_1 subunits. Next we produced and expressed a mutant form of the avian β_1 subunit lacking the entire cytoplasmic domain (Δ1; Figure 1 below). This truncated β_1 subunit formed heterodimers with endogenous α subunits and was efficiently exported to the cell surface. These heterodimers bound to the cell-binding fragment of FN in an RGD-dependent manner, but did not localize to focal contact sites when the cells were plated on FN (Solowska *et al.*, 1989). The results show that interaction with the extracellular matrix is not sufficient to maintain integrin in focal contact sites, but that cytoskeletal interaction, which is dependent on the β_1 cytoplasmic domain, is required. This is not in conflict with earlier data from other laboratories, which show that the precise nature of the ECM proteins to which cells attach determines which integrins enter focal contact sites (Singer *et al.*, 1988; Dejanna *et al.*, 1988). Rather, our data showed that the cytoplasmic domain and, presumably, cytoskeletal interactions are also necessary. Using site-directed mutagenesis, we now extend those studies with further mapping of the site necessary for focal contact localization within the integrin β_1 subunit. A recently published paper used a similar approach (Hayashi *et al.*, 1990), but our data lead us to significantly different conclusions.

Results

Expression of avian integrin β_1 mutants

We decided to map the functional site within the integrin β_1 cytoplasmic domain by oligonucleotide-mediated site-directed mutagenesis. Mutants with termination codons placed at various sites within the β_1 cytoplasmic domain (Figure 1) were generated by the use of standard techniques (see Materials and methods). These mutants included deletions from residues 767

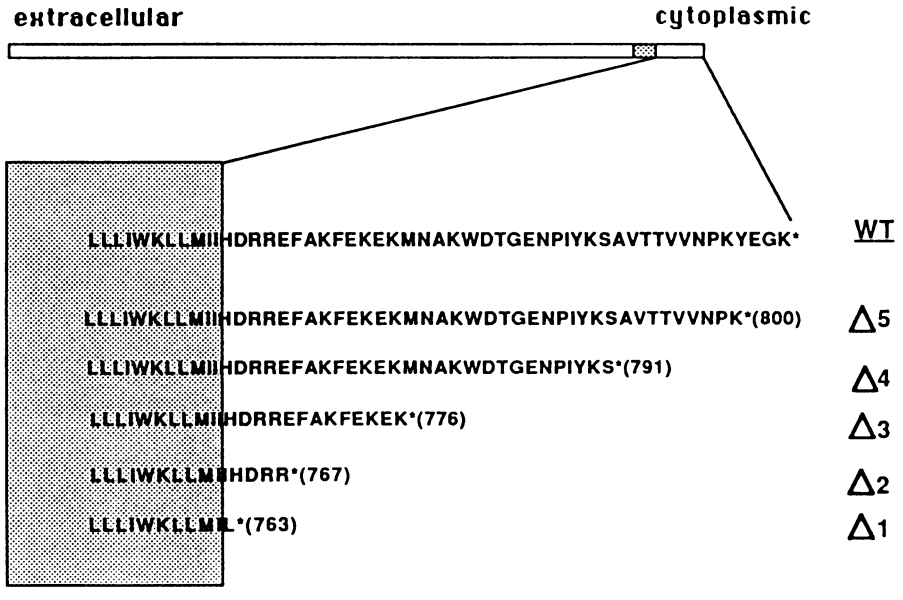

***Figure 1.* Deletions of the cytoplasmic domain of integrin β_1.** Portions of the amino acid sequence of the transmembrane region and the cytoplasmic domain are shown. The amino acid position of the termination codon (*) is shown in parentheses.

(Δ2), 776 (Δ3), 791 (Δ4), and 800 (Δ5) and were isolated and subcloned into the expression vector pLEN as described in Materials and methods. The resulting plasmids were cotransfected with pSV2neo (Southern and Berg, 1982) into mouse NIH 3T3 cells; clones resistant to G418 were isolated and analyzed by indirect immunofluorescence with the use of a chicken-specific antiserum as described previously (Solowska *et al.*, 1989). Positive clones were subcloned, and usually one subclone expressing a high level of avian β_1 integrin, as determined by immunofluorescence, was expanded and further analyzed. In the case of mutant Δ2, multiple transfections were carried out and multiple positive subclones were analyzed.

The expression of the mutant avian integrins was further analyzed by surface labeling of transfected cells with ^{125}I. Detergent extracts were immunoprecipitated with several different antibodies (Figures 2 and 3). Antiserum raised against an integrin β_1 cytoplasmic domain peptide (Marcantonio and Hynes, 1988) reacts with the cytoplasmic domain of β_1 subunits regardless of species. Figures 2, a and f, and 3, a and f, show that this antibody precipitates β_1 subunits together with α subunits from all clones expressing mutant proteins. These β subunits represent the endogenous mouse subunits, because the avian subunits lack most of the cytoplasmic domain (see below). Immunoprecipitation with either a chicken-specific polyclonal antiserum (Figures 2, b and g, and 3, b and g) or with the chicken-specific monoclonal antibody CSAT (Figures 2, c and h, and 3, c and h) results in the precipitation of mutant avian β_1 subunits with associated mouse α subunits. The ratios of α to β subunits in the hybrid heterodimers involving chicken β_1 were similar to those involving murine β_1 (Figures 2 and 3). Thus, in all cases, heterodimers were formed between the exogenous mutant avian β_1 subunits and endogenous α subunits, and these hybrid heterodimers were efficiently exported to the cell surface. In addition, sequential immunoprecipitations were performed to prove that the mutant proteins were truly lacking cytoplasmic domain sequences. Cells expressing the deletion mutants Δ2, Δ4, and Δ5 were tested by

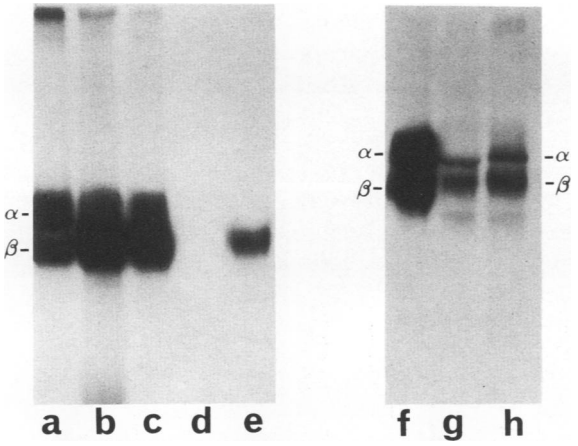

Figure 2. **Immunoprecipitation of mouse and chicken integrins.** Extracts of [^{125}I]-surface-labeled Δ2 cells (left panel) and Δ3 cells (right panel) were incubated with broad-spectrum anti-β_1 cytoplasmic peptide serum (a and f), anti-chicken β_1 serum (b and g), or monoclonal anti-chicken β_1 (CSAT) Sepharose (c and h). Immunoprecipitates were recovered either directly (CSAT) or indirectly by the use of protein A-Sepharose. In addition, a nondenatured extract of [^{125}I]-surface-labeled Δ2 cells (d and e) was immunoprecipitated with CSAT monoclonal anti-chicken β_1-Sepharose. The recovered complexes were denatured in SDS, after which an excess of Triton X-100 was added. The denatured integrins were then incubated with anti-β_1 cytoplasmic peptide serum (d) or anti-chicken β_1 serum (e). The samples were immunoprecipitated with the use of protein A-Sepharose and analyzed with the use of SDS-PAGE. Thus, the mutant integrins efficiently form heterodimers with endogenous mouse α subunits and are expressed on the cell surface.

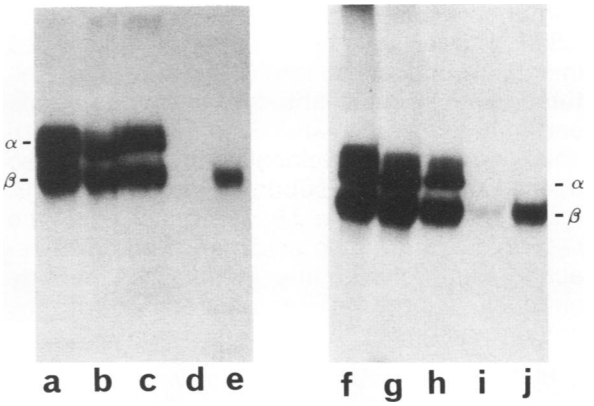

Figure 3. **Immunoprecipitation of mouse and chicken integrins.** Extracts of [^{125}I]-surface-labeled Δ4 cells (left panel) and Δ5 cells (right panel) were incubated with broad-spectrum anti-β_1 cytoplasmic peptide serum (a and f), anti-chicken β_1 serum (b and g), or monoclonal anti-chicken β_1 (CSAT) Sepharose (c and h). Immunoprecipitates were recovered either directly (CSAT) or indirectly with the use of protein A-Sepharose. In addition, nondenatured extracts of [^{125}I]-surface-labeled Δ4 cells (d and e) or Δ5 cells (i and j) were immunoprecipitated with the use of CSAT monoclonal anti-chicken β_1-Sepharose. The recovered complexes were denatured in SDS, after which an excess of Triton X-100 was added. The denatured integrins were then incubated with anti-β_1 cytoplasmic peptide serum (d and i) or anti-chicken β_1 serum (e and j). The samples were immunoprecipitated with protein A-Sepharose and analyzed by SDS-PAGE. Thus, the mutant integrins efficiently form heterodimers with endogenous mouse α subunits and are expressed on the cell surface.

immunoprecipitation of detergent extracts with CSAT. The resulting precipitates were dissolved in sodium dodecyl sulfate (SDS) and reprecipitated with either anti-cytoplasmic peptide serum (Figures 2d and 3, d and i) or with chicken-specific polyclonal antibody prepared against the *entire* β_1 subunit (Figures 2e and 3, e and j). Only deletion Δ5, which lacks only the C-terminal four amino acids, showed cross-reacting material with the cytoplasmic peptide antibody, whereas all subunits reacted with the antiserum against the entire subunit.

Cellular localization of deletion mutants

We next examined the localization of mutant avian integrins by indirect immunofluorescence. Cells were grown on fibronectin-coated coverslips, and stained in double-label immunofluorescence with anti-vinculin to visualize focal contacts and a chicken-specific polyclonal antiserum to mark the location of hybrid integrins. Figure 4 shows the results of these experiments. Mutant proteins Δ3 (c, c′) and Δ4 (d, d′), with moderately long truncations of the cytoplasmic domain, did not localize to focal contacts in 3T3 cells. The Δ4 mutant protein includes the tyrosine, which can be phosphorylated in transformed cells, yet does not localize to focal contacts. The longest truncated form, Δ5 (e, e′), which is only four amino acids shorter than the wild type, does localize to focal contacts but less well than the wild-type chicken β_1 (a, a′). Interestingly, the mutant protein Δ2 (b, b′)—which lacks the bulk of the cytoplasmic domain but retains the segment nearest the membrane, including the dibasic residues (RR)—did localize to focal contacts, but also not as well as the wild-type (a, a′). Wild-type chicken β_1 and both mutant forms (Δ2 and Δ5) localize both in focal contacts and along stress fibers as previously reported for chicken integrins. The pattern of localization of the Δ2 mutant protein was a consistent finding seen with multiple primary clones isolated from the initial transfection. This integrin is indeed truncated because it does not react with antiserum against a cytoplasmic peptide (Figure 2). Furthermore, the mutagenized region of the plasmid pCINT Δ2 was sequenced several times, and the position of the termination codon at residue 767 was confirmed. Last, we have repeated the transfection with this plasmid and have again isolated cells in which the Δ2 integrin is weakly localized in focal contacts.

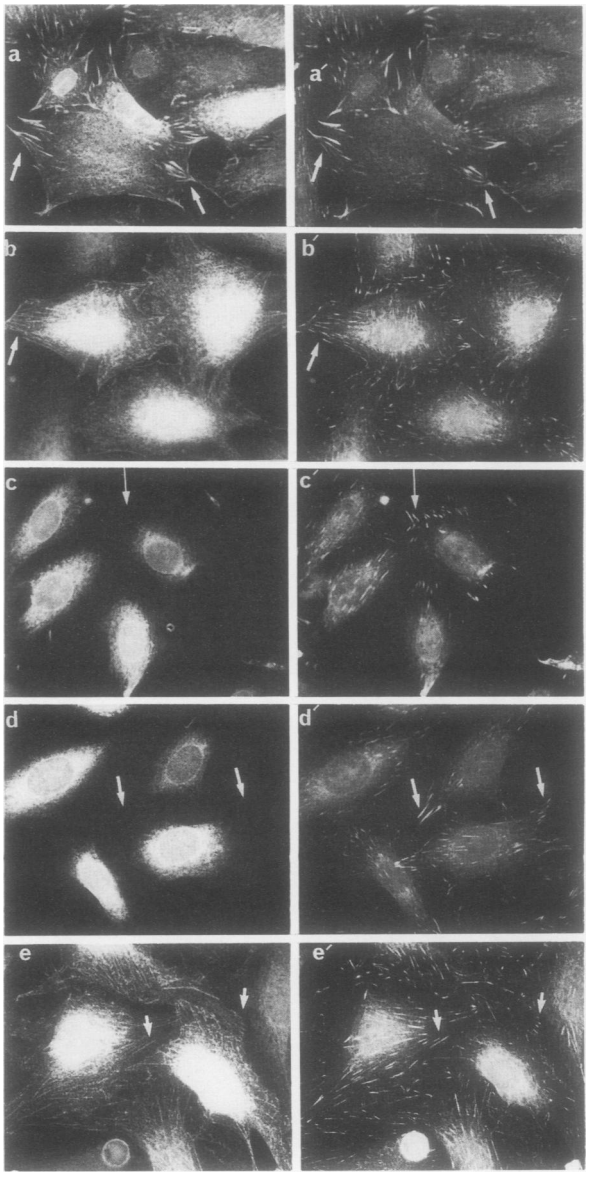

Figure 4. **Double-label immunofluorescence of cells expressing full-length avian integrin β_1 subunit (a and a′) and of cells expressing truncated avian β_1 subunits Δ2 (b and b′), Δ3 (c and c′), Δ4 (d and d′), and Δ5 (e and e′).** Cells were stained with a mixture of mouse antivinculin antibody (a′–e′) and rabbit anti-chicken β_1 serum (a–e) followed by visualization by the use of rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated goat anti-mouse IgG. Note that Δ2 and Δ5 show weak focal contact localization of avian integrin, whereas Δ3 and Δ4 do not.

Discussion

The experiments described here were designed to map the sequences within the avian integrin β_1 cytoplasmic domain that are essential for the inclusion of this subunit in focal contacts. The aim was to determine the sequences that include the binding sites for cytoskeletal proteins within the focal contact, such as talin, which have been shown to interact with integrin in vitro

(Horwitz *et al.,* 1986). Previously, we have characterized the heterologous expression in mouse 3T3 cells of both the wild-type avian integrin β_1 subunit and a truncated avian integrin β_1 with a termination codon at position 763, which lacked the entire cytoplasmic domain (Δ1; Figure 1; Solowska *et al.,* 1989). These exogenous subunits were efficiently expressed, formed heterodimers with endogenous mouse α subunits, and were exported to the cell surface. Although both hybrid integrins bound to the cell-binding fragment of FN in an RGD-dependent manner, only the wild-type avian integrin β_1 localized to focal contact sites where both the extracellular matrix and the cytoskeleton associate with the plasma membrane; the truncated form did not. In the current study, we have transfected mouse 3T3 cells with integrin cDNAs containing deletion mutants of the cytoplasmic domain. These mutants contain termination codons at positions 767, 776, 791, and 800 (Figure 1). In all cases, high-level expression of the truncated avian integrins was obtained. Furthermore, heterodimers were formed between the exogenous truncated avian β_1 subunits and endogenous mouse α subunits, and these heterodimers were efficiently exported to the cell surface (Figures 2 and 3).

The cellular localizations of the truncated integrins (Figure 4) show an interesting but somewhat confusing finding. The longest mutated β_1 subunit, Δ5, which is only four amino acids shorter than the wild type, does localize to focal contacts, albeit not as well as the intact form. Mutants Δ3 and Δ4, with moderately long truncations of the cytoplasmic domain, did not localize to focal contacts; these results were not unexpected. However, the finding that the truncated mutant protein encoded by Δ2, in which the bulk of the cytoplasmic domain is missing but the segment nearest the membrane, including the RR, remains, did localize to focal contacts was quite surprising. The fact that the truncated mutant protein Δ1 does not appear in focal contacts and Δ2 does suggests that the peptide segment nearest to the transmembrane region is important in proper integrin-cytoskeletal interaction.

The data do not distinguish among several possible roles for the membrane-proximal segment. It could be necessary as a stop transfer signal ensuring proper insertion of the subunit in the membrane, for efficient $\alpha\beta$ dimerization, or for interactions with other, perhaps cytoskeletal components. It is, in fact, not certain exactly where the chain emerges from the lipid bylayer.

Recently Hayashi *et al.* (1990) described a similar study of the expression of deletion mutants of the avian integrin β_1 cytoplasmic domain. Virtually all of their truncated integrins failed to localize to focal contact sites, with the exception of a truncation of the C-terminal five amino acids. These authors tentatively concluded that sequences between residues 788 and 798 (YKSAVTTVVNP) are required for focal contact localization and presumably for talin binding. Because in vitro binding studies have shown inhibition of talin binding to integrin by a decamer between residues 780 and 789 (WDTGENPIYK) (Tapley *et al.,* 1989), they suggested that the YK and surrounding sequences are crucial, because a truncation at residue 788 (WDTGENPI) did not localize to focal contacts (Hayashi *et al.,* 1990). Our results lead to quite different conclusions. The Δ4 truncated protein terminates at residue 791 and includes the entire sequence of the decapeptide plus one additional amino acid (WDTGENPIYKS), yet does not localize to focal contacts. This sequence includes the consensus sequence for tyrosine phosphorylation (Tamkun *et al.,* 1986), and indeed this tyrosine is phosphorylated in transformed cells (Hirst *et al.,* 1986; Tapley *et al.,* 1989). If the primary sequence were the main consideration, the data shown here would imply that the tyrosine and the surrounding region are not sufficient for proper focal contact localization, despite the fact that the Δ4 mutant protein includes the putative talin binding site.

Table 1 summarizes our data and those of Hayashi *et al.* (1990). Taking each set of data in isolation, one comes to different conclusions. Hayashi *et al.* (1990) concluded from their data that the crucial segment lies between 788 and 798 based largely on the comparison of 788t and 799t. Similarly, based on a comparison between Δ4 and Δ5, one might conclude that the crucial segment lies between 791 and 799, i.e., not including the tyrosine residue that is phosphorylated. However, a comparison between the results for Δ1 or 765t and those for Δ2 leads to the conclusion that the dibasic sequence RR (residues 765 and 766) is crucial and indeed is sufficient for some localization in focal contacts. How then can one explain the observation that the truncated integrins Δ3 and Δ4, with longer cytoplasmic domains than Δ2, fail to localize to focal contacts? One possible explanation is that partial cytoplasmic domains (such as those of Δ3 and Δ4) are defective, presumably because of abnormal conformations, and interfere with the localization of the avian integrin in focal contacts. This domain becomes functional (or

Table 1. Properties of mutant β_1 integrin subunits

β_1 Subunit	Termination codon	Heterodimer formation	Focal contact localization	Conclusion
Δ1*	763	+	−	Cytoplasmic domain is necessary
765t†	765	+	−	
Δ2	767	++	+	RR is necessary
Δ3	776	++	−	Partial cytoplasmic domains interfere
779t†	779	ND	−	
788t†	788	ND	−	
Δ4	791	++	−	
799t†	799	ND	+	Last 4–5 residues not essential but do enhance localization
Δ5	800	++	+	
Y-F‡	804	++	++	Y hydroxyl not necessary
WT*	804	++	++	

* Data from Solowska *et al.* (1989).

† Data from Hayashi *et al.* (1990).

‡ Data from Hayashi *et al.* (1990) and our unpublished data.

+, partial; −, not detectable; ++, normal; RR, dibasic residues; ND, not done.

noninterfering) only when all but the last four or five amino acids are included (799t and Δ5 truncations). This explanation of these results also leads to the conclusion that one cannot rule out a role for the sequences between 767 and 791, because one would predict that the Δ3 and Δ4 truncated integrins should localize as well as Δ2 if primary sequence were the only consideration.

Taking all the data together (Table 1) we come to the following conclusions.

1) The dibasic sequence R_{765} R_{766} is important for correct localization of integrins to focal contacts. It may also be important for efficient and/or stable dimer formation because the Δ1 truncated form produced a lower α/β ratio than wild-type chicken β_1 (Solowska *et al.*, 1989), whereas the Δ2 truncation gave a normal ratio (Figure 2).

2) The hydroxyl residue of the tyrosine is not necessary for efficient dimer formation, export, or localization in focal contacts.

3) The last four or five amino acids do contribute somehow to focal contact localization, perhaps merely by promoting correct folding of the cytoplasmic domain.

4) Partially truncated forms (Δ3, Δ4, 779t, and 788t) are defective, presumably because of abnormal conformations of their partial cytoplasmic domains.

5) Therefore, one cannot definitively rule in or out other segments of the cytoplasmic domain as being important contributors to focal contact localization.

The difficulties in interpreting the properties of the various truncated forms are typical of deletion mutants and suggest that further analyses will require more subtle perturbations such as site-specific changes or generation of chimeric subunits.

Materials and methods

Site-directed mutagenesis and plasmid constructions

Restriction enzymes, T4 DNA ligase, Klenow fragment of DNA polymerase, and polynucleotide kinase were from New England Biolabs (Beverly, MA). Standard recombinant DNA methods were used (Maniatis *et al.*, 1982). Expression vectors encoding cytoplasmic domain deletion mutants of chicken integrin β_1 were constructed as follows. A 3.15-kb *Eco*RI fragment containing the entire coding sequence for chicken integrin β_1 was isolated from the cDNA clone 1D described previously (Tamkun *et al.*, 1986). The ends were filled by the use of Klenow fragment of DNA polymerase and *Bam*HI linkers were attached. This fragment was then digested with *Bam*HI restriction enzyme and inserted into the expression vector pLEN (Neufeld *et al.*, 1988) at the *Bam*HI site. The 5′ *Bam*HI site was subsequently eliminated by partial digestion with *Bam*HI, filling in of sticky ends, and religation of the linear form to generate plasmid pLEN XBCINTβ_1. This plasmid contains an internal *Bam*HI site located 5′ to the transmembrane domain and the 3′ *Bam*HI at the cloning site. It encodes a fully functional chicken integrin β_1, as tested in transfection experiments similar to those described previously for plasmid pCINTβ_1 based on pESP–SVTEXP vector (Solowska *et al.*, 1989).

pLEN XBCINTβ_1 was used to generate deletion mutants in the cytoplasmic domain of the chicken integrin β_1. The 1.1-kb *Bam*HI fragment was isolated from the plasmid pLENXBCINT β_1 and inserted into M13 vector MP18 for mutagenesis. Site-directed mutagenesis was performed by the method of Eckstein (Nakamaye and Eckstein, 1986) using an oligonucleotide-directed in vitro mutagenesis kit (Amersham, Arlington Heights, IL). Oligonucleotides were purchased from Research Genetics (Birmingham, AL) and were purified with reverse-phase chromatography using OPC cartridges (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. After mutagenesis,

the mutant DNA was confirmed by dideoxy sequencing (Sequenase; United States Biochemical, Cleveland, OH) using internal oligonucleotide primers. Termination codons were placed at residues 767 (Δ2), 776 (Δ3), 791 (Δ4), and 800 (Δ5). The mutagenized inserts were recovered by digestion with *Bam*HI followed by preparative gel electrophoresis and religated into pLENXBCINTβ_1, from which the corresponding *Bam*HI fragment had been removed, to reconstitute full-length cDNAs. The resulting plasmids, designated pCINT Δ2, pCINT Δ3, pCINT Δ4, and pCINT Δ5, were isolated by banding twice on CsCl gradients and were used for transfections.

Transfection of 3T3 cells

NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) (GIBCO Laboratories, Grand Island, NY). Cells (5×10^5) plated the previous day were co-transfected with 20 μg of chicken integrin plasmids and 2 μg pSV2neo (Southern and Berg, 1982) as a calcium phosphate precipitate, as described previously (Solowska *et al.*, 1989). After 3 d, the cells were split 1:20 into DMEM supplemented with 10% FCS and 0.5 mg/ml G418 (Geneticin, GIBCO Laboratories). After 10–14 d, G418-resistant clones were isolated and screened by indirect immunofluorescence staining using chicken-specific anti-β_1 antiserum, as described previously (Solowska *et al.*, 1989). These positive clones were further subcloned on gelatin-coated dishes and analyzed by immunofluorescence as described (Solowska *et al.*, 1989).

Antibodies

A polyclonal avian-specific anti-integrin antibody designated Chickie II was a gift of Clayton Buck (Wistar Institute) and has been described previously (Solowska *et al.*, 1989). A second chicken-specific rabbit anti-β_1 (366) serum was prepared by injection of SDS-gel purified chicken integrin complex and was kindly provided by Lisa Urry (Massachusetts Institute of Technology). CSAT monoclonal was prepared from hybridomas, and was coupled to protein A Sepharose (Sigma Chemical, St. Louis, MO) as described (Solowska *et al.*, 1989). Rabbit anti-β_1 cytoplasmic domain antibodies were prepared as described (Marcantonio and Hynes, 1988). Monoclonal anti-vinculin antibodies were purchased from Sigma Chemical.

Radiolabeling and immunoprecipitation

Cells were labeled with Na[^{125}I] (New England Nuclear, Boston, MA) and lactoperoxidase as a monolayer as described (Hynes, 1973). In each experiment, 10^7 cells and 1–2 mCi/ml were used. The cells were washed three times with cold phosphate-buffered saline (PBS) followed by extraction. Cells were extracted with 0.5% NP-40, and immunoprecipitation was performed as described (Marcantonio and Hynes, 1988).

SDS–polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (1970). Separation gels were 7.0% acrylamide with a 3% stacking gel. Samples were prepared in sample buffer (5% SDS, 100 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 6.8, 10 mM EDTA, 10% glycerol, and bromphenol blue) and boiled for 3 min.

Immunofluorescence

Cells were plated in DMEM with 0.5% FCS overnight on coverslips previously coated with human plasma fibronectin (0.02 mg/ml). Cells were rinsed twice in PBS and fixed for 15 min in a freshly prepared 4% solution of paraformaldehyde (Fluka Chemical, Buchs, Switzerland) in PBS, rinsed and permeabilized with 0.5% NP-40 in PBS for 15 min. Cells were stained with primary antiserum in 10% normal goat serum in PBS for 30 min at 37°C. After three washes with PBS, the second antibody mixture (rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated goat anti-mouse IgG in 10% normal goat serum in PBS; Organon Teknika-Cappel, Malvern, PA) was added and incubated for 30 min at 37°C. After three washes, coverslips were mounted in gelvatol and examined with an Axiophot microscope (Carl Zeiss, Thornwood, NY) and photographed (Tri-X film, Eastman Kodak, Rochester, NY).

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