

Expression and Function of Chicken Integrin β 1 Subunit and Its Cytoplasmic Domain Mutants in Mouse NIH 3T3 Cells

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Abstract. Chicken integrin β 1 cDNA and its site-directed mutants were cloned into a mammalian expression vector and introduced into mouse NIH 3T3 cells. Stable transfectants expressing the chicken β 1 subunit or its site-directed mutants were identified by immunostaining with antibodies specific for the chicken integrin β 1 subunit. The chicken β 1 proteins were expressed predominately in the endoplasmic reticulum of transfectants and to a lesser degree in the plasma membrane. Immunoblots and immunoprecipitations, using anti-chicken integrin antibodies, revealed three different sizes of the chicken subunit (90, 95, and 120 kD) and a mouse 140-kD α subunit. Immunoprecipitations of the cell surface receptors showed only two peptides, an 120-kD β 1 and an 140-kD α subunit. Antibodies perturbing mouse and chicken integrin-specific cell adhesions were used to demonstrate that the chimeric receptors functioned in adhesion to both laminin and fibronectin. Immuno-

fluorescent staining with antibodies specific for either the chicken or mouse receptors showed that both the wild type and the chimeric receptors localized in focal contacts. Several mutations in the cytoplasmic domain were synthesized and used in the transfection experiments. In one mutant the tyrosine (Tyr 788) in the consensus sequence for phosphorylation was replaced by a phenylalanine. In another the lysine (Lys 757) at the end of the membrane spanning region was replaced by a leucine. Both of these mutants formed dimers with mouse α subunits, participated in adhesion, localized in focal contacts, and displayed biological properties indistinguishable from the wild-type transfection. In contrast, mutants containing deletions greater than 5–15 amino acids nearest the carboxyl end in the cytoplasmic domain neither promoted adhesion nor localized in focal contacts. They did, however, form heterodimers that were expressed on the cell surface.

THE integrins are a family of heterodimeric cell surface receptors that mediate the interactions between cells and other cells, extracellular matrix molecules, and some serum proteins (Hynes, 1987; Ruoslahti and Piersbacher, 1987; Buck and Horwitz, 1987a). Many, if not all integrins, also appear to interact directly with the cytoskeleton. As dual receptors for both extracellular and intracellular molecules, the integrins participate in diverse cellular phenomena including cell motility, adhesion, cytoskeletal organization, and induction of differentiation (Ruoslahti, 1988). They are also obvious targets for pathological processes which are accompanied by adhesive and morphologic alterations, e.g., transformation, platelet aggregation, and T-cell help (Stoolman, 1989; Cardarelli et al., 1988).

Integrin heterodimers are made up of two distinct glycoprotein subunits, α and β , that generally migrate on reduced SDS-PAGE in the 120–160-kD range. Diversity in the integrin super family is generated by a large number of different α subunits interacting with a small set of (four to five) different β subunits. Each β subunit defines a family and generally interacts with a unique subset of the alpha subunits. The β 1 family has at least six members, is found on diverse cell

types, and functions primarily as receptors for extracellular matrix molecules (Hemler et al., 1987, 1988; Takada et al., 1987, 1988). The β 2 family has three members and is found only on lymphocytes (Anderson and Springer, 1987). The β 3 family has two members: the IIB/IIIa complex found on platelets and the vitronectin receptor, which is more generally distributed (Pytela et al., 1986; Ginsberg et al., 1987). Recently, novel β subunits, which are distinct from previously identified integrin β subunits, have been reported as members of the integrin family of adhesion receptors (Kajiji et al., 1989; Cheresch et al., 1989; Gresham et al., 1989; Holzmann and Weissman, 1989).

The cDNAs encoding the α and β subunits of integrins from several species have been cloned and sequenced (Argaves et al., 1986, 1987; Poncz et al., 1987; Suzuki et al., 1987; Fitzgerald et al., 1987; Kishimoto, 1987). The deduced structures for the α subunits all have large extracellular domains with multiple repeats that are homologous to those seen in the calcium-binding domains of other proteins, a single membrane-spanning domain, and a short (23 AA) cytoplasmic domain. The deduced structures for the β subunits also show some general features: a single trans-

membrane spanning region, a large extracellular domain with four cysteine-rich repeats, and a short cytoplasmic domain. Two of the integrin families, $\beta 1$ and $\beta 2$, have a consensus sequence for tyrosine phosphorylation in their cytoplasmic domains.

The cytoplasmic domain of the $\beta 1$ subunit of integrin seems especially important. Synthetic peptides corresponding to the terminal 21 amino acids or to a tryptic decamer surrounding the tyrosine that lies in a consensus sequence for phosphorylation inhibit the binding of talin, a cytoskeletal-associated protein, to integrin (Buck and Horwitz, 1987b; Tapley et al., 1989). The $\beta 1$ cytoplasmic domain is a substrate for pp60^{v-src} kinase(s) and is phosphorylated after transformation by viruses encoding tyrosine kinases (Marcantonio and Hynes, 1988; Hirst et al., 1986; Tamkun et al., 1986; Tapley et al., 1989). Finally, it is conserved completely in species as distant as humans and *Xenopus* (DeSimone and Hynes, 1988). Thus, it seems likely that this domain plays a key role in the regulatory events and signal transductions mediated by integrin.

To address the role of the $\beta 1$ cytoplasmic domain in subunit interactions and receptor functions, we have constructed avian cDNAs with mutations in different regions of the nucleotide sequence coding for the cytoplasmic domain, and transfected them into mouse fibroblasts. Two of the mutants correspond to single-amino acid substitutions in putative key regions, and the other four represent deletions of portions of the cytoplasmic domain. Several stable transformants were selected and the ability of the mutant receptors to mediate adhesion and participate in cytoskeletal organization was studied. Recently Solowska et al. (1989) described a chimeric receptor, containing a large deletion in the cytoplasmic domain, that does not localize in adhesion plaques.

Materials and Methods

Antibodies

Polyclonal antibodies (Ab 808 and 809) to chicken integrin were generated in rabbits using immunoaffinity-purified antigen (on a CSAT mAb column) isolated from 11-d chicken embryos as described elsewhere (Knudsen et al., 1985; Bozyczko et al., 1989). mAbs to chicken integrin $\beta 1$ (V2E9 and WIB10) were also prepared from immunoaffinity-purified integrin. These mAbs immunoblot the $\beta 1$ subunit of chicken integrin; they stain and immunoprecipitate similarly to the CSAT mAb. Polyclonal antisera to mouse integrin (Ab 3675) and to rat vinculin were provided by S. Akiyama (Akiyama and Yamada, 1987) and R. Bloch (University of Maryland, Baltimore), respectively. The C1 mAb, which was raised against chicken fibroblasts, is described elsewhere (George-Weinstein et al., 1988). FITC- or rhodamine-conjugated antibodies to mouse and rabbit IgG F(ab)₂ were purchased from Cappel Laboratories (Organon Teknika, West Chester, PA).

Vector Construction

A vector expressing the chicken integrin $\beta 1$ cDNA was a gift of Dr. C. Buck (Solowska et al., 1989). It is based on the plasmid vector pESV-SVTEXP (pTEX) prepared by Southern and Berg (1982) and modified by Shyam et al. (1986). Integrin cDNA was removed from pGEM1-integrin plasmid (Tamkun et al., 1986) by digestion with Eco RI and Dra I, polished with the Klenow fragment of DNA polymerase I, and purified on agarose gels. The pTEX vector was treated with Hind III, blunt-ended, and ligated with the above integrin cDNA fragment. The resulting vector is designated as pTEX-INTGRN. Mutant integrin cDNAs were cloned into pTEX-INTGRN by digesting the integrin cDNA with Bst E2 and Nde I, isolating the fragment (nucleotides 733–2652, 1.9 kb) and exchanging it with the corresponding fragments of its mutant cDNAs. There are two Nde I sites in the integrin cDNA (positions 2653 and 3009) and one in the intervening sequence derived from SV-40 genome of pTEX. Therefore, pTEX-INTGRN

was partially digested with Nde I, and the vector which was devoid only of an Nde I fragment of the integrin cDNA (nucleotides 2653–3009, 0.4 kb) was isolated and used for exchange with the mutant cDNAs. Thus, pTEX-INTGRN containing mutant cDNAs are ~0.4 kb shorter than that of the wild-type cDNA.

Mutant cDNAs were generated by primer-directed *in vitro* mutagenesis using synthetic oligonucleotides and the Muta-Gene *in vitro* mutagenesis kit (Bio-Rad Laboratories, Richmond, CA). The codons for Tyr 788, Lys 757, and Lys or Tyr 765, 779, 788, and 799 were altered to Phe, Leu, and terminator codons, respectively. These pTEX-INTGRN vectors designated as pTEX-INTGRN.788sY-F, pTEX-INTGRN.757sK-L, and pTEX-INTGRN.765t, pTEX-INTGRN.779t, pTEX-INTGRN.788t, or pTEX-INTGRN.799t. Each mutant sequence was verified by dideoxy sequencing using primers 50–100 bp upstream from each mutated site.

Transfection

Transfections were performed using the calcium phosphate method (Graham and van der Eb, 1973; Wigler et al., 1979). Mouse NIH 3T3 cells were grown in DME containing 10% FCS. 10 μ g of an expression vector containing integrin or its mutant cDNAs were cotransfected for 20 h with 10 μ g of salmon sperm DNA and 1 μ g of pRSV-neo DNA (a plasmid expression vector driven by a Rous sarcoma virus long-terminal repeat; a gift of J. Alwine, University of Pennsylvania) into 1×10^5 NIH 3T3 cells. The cells were allowed to recover for 48 h, replated in five 100-mm tissue-culture dishes, and then selected with 1 mg/ml Geneticin (G418 sulfate) (Sigma Chemical Co., St. Louis, MO). After 15–20 d, individual clones were isolated using cloning rings and then expanded. Positive clones were identified by immunostaining using anti-chicken integrin antibodies. Clones that were not homogeneous were recloned using limiting dilution.

Immunofluorescent Staining

Cultured cells were harvested after treatment with 0.001% trypsin (type XI; Sigma Chemical Co.) and 1 mM EDTA in PBS for 2 min at 20°C. The protease reaction was stopped by adding 0.01% soybean trypsin inhibitor (Sigma Chemical Co.). The cells were centrifuged at 1,000 rpm for 5 min and resuspended in DME containing 2% BSA and soybean trypsin inhibitor (Sigma Chemical Co.). The cells were centrifuged again, resuspended in serum free medium (Bottenstein and Sato, 1979), and seeded onto fibronectin-coated (20 μ g/ml, overnight at 4°C) 35-mm culture dishes. After 6 h incubation at 37°C, the cells were stained either live or after permeabilization as described with 3.7% formaldehyde to fix the cells (Bozyczko et al., 1989). For double staining, antibodies to integrin (Ab-3675 at a dilution of 1 to 100, and 20 μ g/ml V2E9 or WIB10) were incubated with live cells, and antibodies to rat vinculin (5 μ g/ml) were added to the same cells after permeabilization with 0.4% Triton X-100. After incubation with the primary antibodies, the cells were rinsed several times with PBS and then incubated with either rhodamine- or FITC-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (10 μ g/ml; Cappel Laboratories, Malvern, PA). The cells were washed again with PBS, mounted in 0.1 M Tris-HCl (30%), 70% glycerol, and 0.35% propyl gallate at pH = 7.0, and observed in an Axioplan fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

RNA Analysis

RNA was isolated from transfected or nontransfected cells using the guanidine hydrochloride method (Chirgwin et al., 1979; Glisin et al., 1974). 10 μ g of total cellular RNA were electrophoresed in a 1.2% agarose gel containing 2.2 M formaldehyde, transferred to nitrocellulose, and hybridized with a chicken $\beta 1$ integrin cDNA probe that encoded the whole peptide region and part of the 3' noncoding region (Nco I-Puv II fragment, 2.7 kb). The probe DNA was labeled with ³²P-dCTP using a nick translation kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Immunoblotting Analysis

Cultured cells were rinsed twice with PBS and then scraped from the dishes in ice-cold PBS containing 2 mM EDTA and a protease inhibitor mixture containing 2 mM PMSF, 10 μ g/ml leupeptin, 0.1 μ g/ml pepstatin, and 0.1 mM *O*-phenanthroline. The suspended cells were washed twice and resuspended in 10 mM Tris-buffered saline (pH 7.4) containing 0.5 mM CaCl₂, 0.5% NP-40, and the protease inhibitor mixture (TNC).¹ The resul-

1. *Abbreviations used in this paper:* TNC, Tris-buffered saline (pH 7.4) containing 0.5 mM CaCl₂, 0.5% NP-40, and the protease inhibitor mixture.

tant cell lysate was incubated for 30 min on ice and then centrifuged in a microcentrifuge at 10^4 rpm. 40 μ g of each NP-40 extract were separated using SDS-PAGE on 7% gels. The proteins were electrophoretically transferred to nitrocellulose (Towbin et al., 1979) and visualized by immunostaining using an avidin-biotin-peroxidase method (Vector Stain ABC Kit; Vector Laboratories, Burlingame, CA). Protein concentrations were determined using the protein assay reagent (Pierce Chemical Co., Rockford, IL) with BSA as a standard.

Immunoprecipitation

Cells were labeled metabolically with [35 S]methionine (1,000 Ci/mmol; Dupont New England Nuclear, Boston, MA) for 24 h (Bozyczko et al., 1989). Cell surface integrins were immunoprecipitated as follows: after labeling, cell cultures were washed three times with PBS and then incubated for 1 h at 4°C in DME containing WIB10 mAb at a concentration of 50 μ g/ml. The cells were washed gently three times with PBS and then collected mechanically in PBS containing the protease inhibitor mixture. The cells were washed by centrifugation and resuspended in TNC. Total cell integrins were immunoprecipitated as described above except that the mAb WIB10 was added to the NP-40 lysate.

The NP-40 extracts ($1-3 \times 10^6$ cpm) were incubated with 100 μ l of 50% (vol/vol) Protein A-Sepharose or anti-mouse IgG-conjugated agarose. The beads were previously blocked by incubation in an extract from unlabeled cells and suspended in TNC. After incubation in the NP-40 extract containing the primary antibodies, the beads were washed extensively in TNC. The bound proteins were released from the beads by incubation at 60°C for 5 min in 40 μ l of Laemmli sample buffer (Laemmli, 1970) without a reducing agent. The samples were collected by centrifugation at 10^4 rpm for 10 min, and an aliquot (1,500 cpm) from each sample was analyzed by SDS-PAGE on 7% gels. The gels were visualized by autoradiography (Laskey, 1980).

For pulse-chase experiments and subsequent immunoprecipitation, cells were first pulse-labeled with [35 S]methionine for 2 h. The labeling medium was then removed, and the cells rinsed once with DME supplemented with 10% FCS and 5 mM methionine. The cells were then incubated in this methionine containing medium for various times. NP-40 cell extracts were prepared as described above.

To quantitate the relative amounts of chicken β 1 and mouse integrin, immunoprecipitations were performed using AB-3675 (at a dilution of 1 to 100) or WIB10 (50 μ g/ml). The x-ray film (Kodak X-Omat AR; Eastman Kodak Co., Rochester, NY) was exposed to the gels for various lengths of time to insure that each band was in the linear range of the film. Band densities were quantitated on a scanning densitometer (UltroScan XL; LKB Instruments, Bromma, Sweden).

Inhibition Assays for Cellular Attachment and Spreading

Cell attachment and spreading assays were performed in Terasaki plates coated with fibronectin or laminin as described elsewhere (Duband et al., 1986) with a slight modification. In brief, cells were harvested after treatment with 0.001% trypsin and 1 mM EDTA in PBS for 2 min at 20°C. The trypsin was inhibited by adding DME supplemented with 2% BSA and 0.01% soybean trypsin inhibitor. The cells were then washed by centrifugation and resuspended with the same solution. A 20- μ l aliquot of cell suspension containing 2,000 cells was mixed with 20 μ l of PBS containing the specified amount of WIB10 and/or Ab-3675. Control experiments used preimmune rabbit serum or an unrelated mAb (C1) (George-Weinstein et al., 1988). After a 5-min incubation, 20 μ l of each mixture was dispensed into the microwells that were then incubated at 37°C for 1 h. For the initial cell adhesion assays, the wells were rinsed three times in PBS to remove nonadherent cells, and the attached cells were then fixed in PBS containing 3.7% formaldehyde. Cell adhesion is expressed as the ratio of cells present in the tested wells to that present in control experiments. Cell spreading was assayed by observing the adherent cells directly using phase-contrast microscopy. The fraction of spread cells to total cells was scored.

Results

Generation of Integrin β 1 Subunits with Mutations in the Membrane-Spanning and Cytoplasmic Domains

Mutations in the cytoplasmic and membrane-spanning domains of integrin β 1 cDNAs were prepared in vitro using site-directed mutagenesis (Fig. 1). Two amino acid substitu-

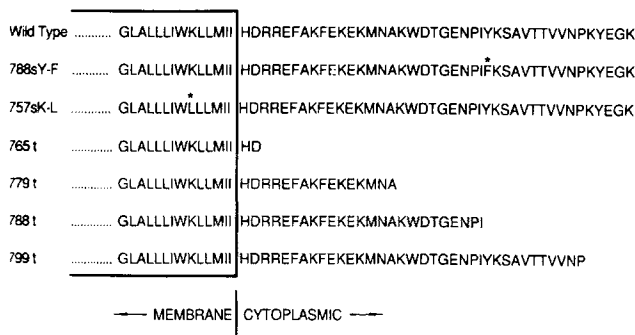


Figure 1. Amino acid sequences around the mutated regions of the chicken integrin β 1 subunits prepared for these studies. Part of the putative membrane spanning regions along with the entire cytoplasmic domain is depicted. The membrane spanning regions of the mutants are aligned with that of wild-type protein. Asterisks denote the amino acid replaced.

tions were prepared. In one, the tyrosine at position 788 was replaced with a phenylalanine; and in the other the lysine at position 757 was replaced with a leucine. The tyrosine lies in a consensus sequence for phosphorylation by tyrosine kinases (Tamkun et al., 1986). It is phosphorylated in vivo after transformation by viruses encoding tyrosine kinases and in vitro by pp60^{v-src} kinase (Hirst et al., 1986; Tapley et al., 1989). The lysine lies near the end of the membrane-spanning domain (or possibly at the beginning of the cytoplasmic domain). It is surrounded by stretches of hydrophobic amino acids and thus may be involved in subunit interactions and possibly transduction of signals through the membrane (Engelman and Zaccari, 1980). Deletions in the cytoplasmic domain were made by placing termination codons at positions 765, 779, 788, and 799. These positions correspond to deletions ranging from 12 to 95% of the cytoplasmic domain. The first position (765) results in a truncation of essentially the entire cytoplasmic domain. The other three correspond to lysines or a tyrosine where folding algorithms predict turns or other features of potential structural interest (Chou and Fasman, 1978; Garnier et al., 1978; Kyte and Doolittle, 1982; Hopp, 1985). The wild-type cDNA and these mutants were cloned into the pTEX expression vector and used to transfect mouse NIH 3T3 cells.

Expression of Chicken Integrin β 1 Subunits in Transfectants

Several cell lines expressing either the wild-type or a mutant β 1 subunit were screened by immunostaining with either polyclonal or mAbs to chicken integrin. One or two representatives from each type of clone were examined in detail.

Northern blot analysis of the RNAs derived from each transfectant showed a single band at 3.8 kb in a transfectant with pTEX-cINTGRN and at \sim 3.3 kb in cells transfected with pTEX containing the mutant cDNAs (Fig. 2). These sizes were those predicted from the size of the cDNAs in the pTEX vector (see Materials and Methods). The level of chicken integrin mRNA expressed in the transfected NIH 3T3 cells depended on the particular clone studied. It varied between 0.5 and 10 times that measured in normal (untransfected) chicken embryo fibroblasts.

Immunoblots of the transfected cells using a chicken

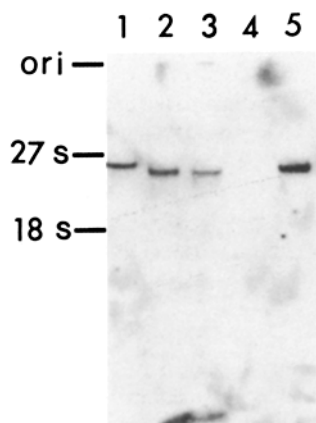


Figure 2. Northern blot analysis of chicken integrin $\beta 1$ subunit in NIH 3T3 cells transfected with pTEX-INTGRN containing wild-type or mutant cDNAs. 10 μ g of total RNA from each cell population was separated on an agarose/formaldehyde gel and blotted onto nitrocellulose prior to hybridization with 32 P-labeled chicken integrin $\beta 1$ cDNA. RNAs were extracted from 3T3-cINTGRN (lane 1), 3T3-cINTGRN.788sY-F (lane 2), 3T3-cINTGRN.757sK-L (lane 3), and 3T3-cINTGRN.765t (lane

5), respectively. Lane 4 contains RNAs from untransfected cells. Positions of 27S and 18S avian rRNA are shown.

specific polyclonal antibody (Ab-809) (not shown) showed predominantly three different sizes of integrin $\beta 1$ proteins (90, 95 and 120 kD). The integrin $\beta 1$ subunit in chicken embryo fibroblasts migrates at 110 kD. The presence of a single band on Northern analysis indicates that the three proteins arise from a single transcript and likely represent posttranslational modifications.

A chicken-specific mAb (WIB10) immunoprecipitated bands at 95 and 120 kD, corresponding to two of the $\beta 1$ subunits and a new polypeptide at 140 kD (Fig. 3 A). To identify the integrins expressed on the cell surface, intact cells were preincubated with chicken-specific antibodies and washed carefully before lysis and immunoprecipitation. In contrast to the results from whole cell extracts, two major bands, at 120 and 140 kD, respectively, and some faint minor bands were observed (Fig. 3 B). The 120-kD band and the minor band at 95 kD both blotted with antibodies against the chicken $\beta 1$ subunit, whereas the 140-kD band did not. Antibodies (Ab-3675) specific for the mouse integrin complex also precipitated at 140-kD alpha subunit (data not shown and Akiyama et al., 1987). Pulse-chase analyses using pulses of [35 S]methionine followed by chases with cold methionine and immunoprecipitation using anti-chicken inte-

grin antibodies showed that the 95-kD (and 140-kD α chain) peptide is labeled prominently at the earliest time point studied (3 h chase) (Fig. 4). The 120-kD band was only weakly labeled. Over the next 9 h, the label became more prominent in the 120-kD band and was chased almost completely from the 95-kD band.

The label in the 120-kD band persists for at least 18 h and appeared to chase in parallel with the 140-kD α subunit. The 90-kD peptide seen on immunoblots was not detected in these experiments. Together, these data suggest that the 95-kD peptide is a precursor of the 120-kD peptide that is found on the cell surface associated with the α subunit (Hirst et al., 1986; Hemler et al., 1987; Akiyama and Yamada, 1987).

The transfected cell lines express the native mouse receptor, $\alpha_m\beta_m$, as well as the chimeric receptor, $\alpha_m\beta_c$. The ratio of the chimeric integrin to mouse integrin expressed in the transfectants was determined by immunoprecipitation using anti-mouse and -chicken integrin antibodies and comparing the intensities of the 140-kD bands. In general, the ratios of the two receptor complexes correlated with the ratios of the different $\beta 1$ subunits synthesized by the cells. The latter were estimated by comparing the intensities of the mouse and chicken $\beta 1$ subunits. The cell surface ratio of receptor complexes varied between 0.5 and 1.4 among the different clones. The corresponding ratio of total chicken to native mouse $\beta 1$ subunit varied between 1.5 and 4.9. These observations suggest that the chicken $\beta 1$ subunit has a lower affinity for the mouse α subunit than does the mouse $\beta 1$ subunit.

Chicken integrin $\beta 1$ subunit was also expressed in NIH 3T3, PC-12, and rat L8 cells using a plasmid vector that contains a RSV-LTR linked to the dexamethasone-inducible MMTV-LTR promoter (data not shown).

Adhesive Properties and Cellular Localization of the Chimeric Receptors

The adhesive activities of the chimeric receptors were determined by assaying the adhesion of NIH 3T3 cells transfected with pTEX-cINTGRN to fibronectin and laminin-coated substrates. Antibodies specific for the mouse or chicken integrins were used to dissect the role of each receptor system in the transfected cells. Fig. 5 shows the adhesion of mouse and chick cells in the presence of antibodies specific for

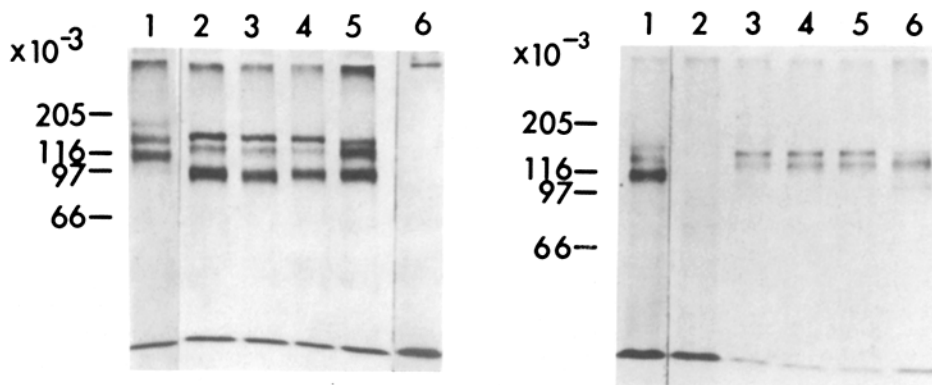


Figure 3. Immunoprecipitation of integrin from NIH 3T3 cells transfected with cDNA encoding chicken integrin $\beta 1$ subunit. NIH 3T3 cells were labeled metabolically for 24 h with [35 S]methionine and the cell extracts were then immunoprecipitated with WIB10, an mAb to chicken integrin $\beta 1$ subunit. (A) Total cell extract. Lane 1, chicken embryo fibroblasts, lane 2, 3T3-cINTGRN; lane 3, 3T3-cINTGRN.788sY-F; lane 4, 3T3-cINTGRN.757sK-L; lane 5, 3T3-cINTGRN.765t; lane 6, untransfected NIH 3T3 cells. (B) labeled cells exposed to WIB10 before solubilization

to select for surface receptors. Lane 1, chick embryo fibroblasts; lane 2, untransfected 3T3 cells; lane 3, 3T3-cINTGRN; lane 4, 3T3-cINTGRN.788sY-F; lane 5, 3T3-cINTGRN.755sK-L; lane 6, 3T3-cINTGRN.765t.

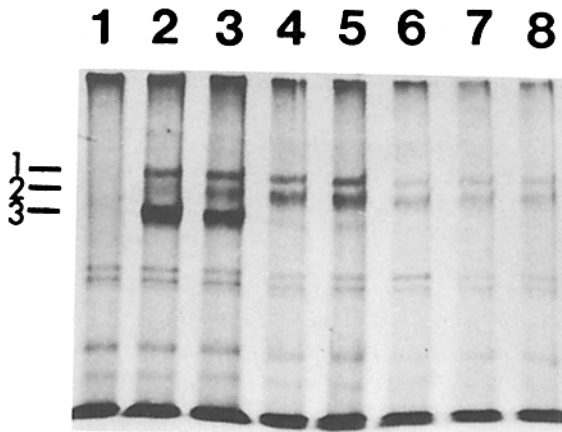


Figure 4. Pulse-chase analysis of the synthesis and turnover of chimeric integrin. 3T3-cINTGRN cells were labeled with a 2-h pulse of [³⁵S]methionine, chased with cold methionine for the indicated times, and immunoprecipitated with WIB10 mAb. Lane 1 was immunoprecipitated with no primary antibody after a 3-h chase. Chase times: 3 (lane 2); 6 (lane 3); 12 (lane 4); 18 (lane 5); 26 (lane 6); 32 (lane 7); and 48 h (lane 8). The makers indicate the migration positions of 140-kD mouse α subunit (1); 120 kD chicken β 1 subunit (2); and 95 kD chicken β 1 subunit (3).

chick and mouse integrins. The adhesion of chicken embryo fibroblasts to FN was inhibited, in a dose-dependent manner, by an adhesion perturbing monoclonal antibody specific for chicken integrin (WIB10) (data not shown). Polyclonal antibodies raised against mouse integrins (Ab 3675) did not inhibit the adhesion of the chicken cells (Fig. 5 A). Likewise, the adhesion of untransfected NIH 3T3 cells to fibronectin was not inhibited by the WIB10 mAb (Fig. 5 B) but was in-

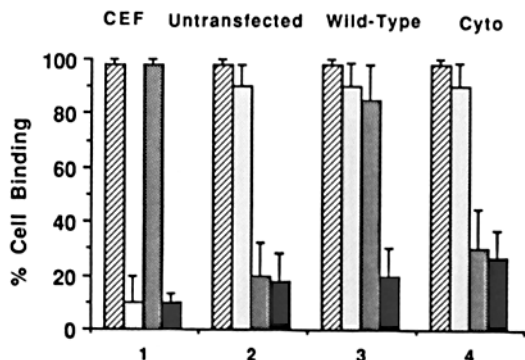


Figure 5. Inhibition of wild-type and chimeric NIH 3T3 cell adhesion to fibronectin coated substrates by anti-mouse and anti-chicken integrin antibodies. Cultures of cells were collected by brief trypsin/EDTA treatment and suspended for 5 min in PBS in the presence or absence of the indicated concentrations of a mAb to chicken integrin (WIB10) and polyclonal antibodies to mouse integrin (Ab 3765). The cells were seeded onto fibronectin coated wells and incubated for 1 h at 37°C. The wells were washed, fixed with formaldehyde, and the number of bound cells was scored. (1) chicken embryo fibroblasts; (2) 3T3 cells (untransfected); (3) 3T3-cINTGRN (mild); and (4) 3T3-cINTGRN.765t. (Column A) No antibody addition; (B) mAb to chicken integrin (WIB10 300 µg/m); (C) Antibody to mouse integrin (Ab-3675; at a dilution of 1:100); and (D) both anti-chicken and anti-mouse antibodies. The data are representative of five to six independent determinations.

hibited in a dose dependent manner by the mouse antibodies (data not shown).

The adhesion of NIH 3T3 cells transfected with chicken integrin (3T3-cINTGRN) to fibronectin was not inhibited significantly by either the chicken (WIB10) or mouse (Ab-3675) antibodies when added alone (Fig. 5 C). The simultaneous presence of both antibodies inhibited completely the adhesion of the transfected cells to fibronectin. These observations demonstrate that both the native and the chimeric receptors participate in adhesion of 3T3-cINTGRN to fibronectin.

There is evidence suggesting that some integrins also mediate adhesion to laminin. The properties of the normal and chimeric receptors in mediating adhesion to laminin were also examined (Fig. 6). In chicken cells, this adhesion is inhibited by anti-chicken integrin antibodies (Horwitz et al., 1985). Both untransfected and 3T3-cINTGRN cells spread on laminin after incubation at 37°C for 1 h. The anti-mouse integrin antibody (Ab-3675) inhibited this initial adhesion of the untransfected NIH 3T3 cells, and the chicken-specific mAb (WIB10) had no effect (data not shown). In contrast, the adhesion of 3T3-cINTGRN on laminin was inhibited by WIB10 but not by the mouse antibodies. These surprising observations show that both native and chimeric receptors can mediate adhesion to laminin; however the chimeric receptor, when present, is dominant.

The cellular distribution of the chimeric and native mouse integrin was visualized by immunofluorescence microscopy using the antibodies specific for the chicken β 1 subunit or the mouse integrin complex. In permeabilized cells, the staining was consistent with a predominant localization of chicken integrin in the endoplasmic reticulum (Fig. 7 A). In areas of well-spread cells separated from the heavy endoplasmic reticulum staining, focal contact staining was also apparent. The focal contact staining was particularly striking on unpermeabilized cells (Figs. 8 B and 9 A). Double staining of cells

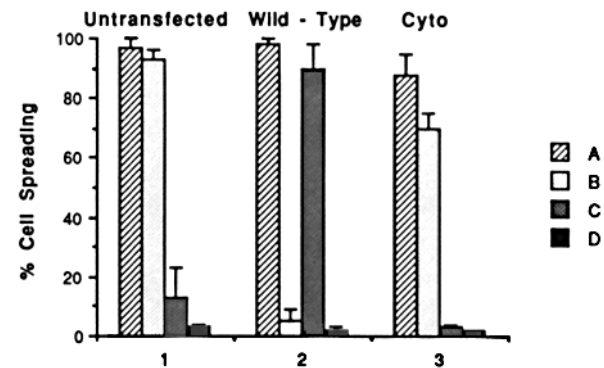


Figure 6. Inhibition of wild-type and chimeric NIH 3T3 cell adhesion to laminin by the antibodies to mouse and chicken integrin. Cultures of cells were collected as described in Fig. 5. The cells were then seeded onto laminin coated wells and incubated for 1 h at 37°C. The percent of spread cells in the total cell population was then scored. (1) 3T3 cells (untransfected); (2) 3T3-cINTGRN (wild type); (3) 3T3-cINTGRN.765t (cyto). (Column A) No antibody addition; (B) mAb to chicken integrin (WIB10 200 µg/ml); (C) antibody to mouse integrin (Ab-3675 at a dilution of 1:100); and (D) both anti-chicken and anti-mouse antibodies. The data are representative of five to six independent determinations.

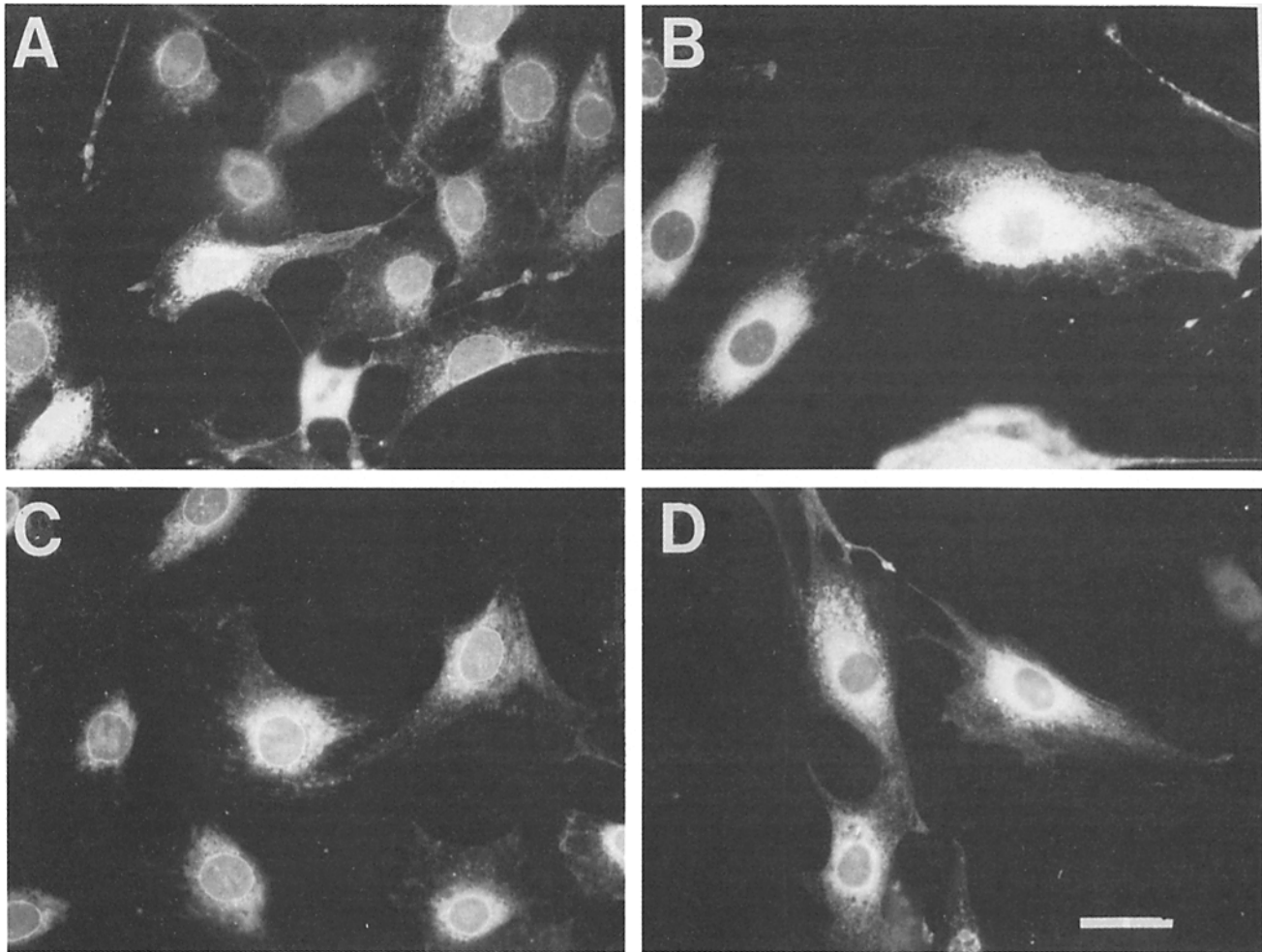


Figure 7. Distribution of chicken integrin in transfected NIH 3T3 cells. Freshly trypsinized cells were plated in serum-free medium onto fibronectin-coated dishes and allowed to attach and spread for 6 h. Permeabilized cultures were first fixed for 10 min with 3.7% formaldehyde, permeabilized with 0.4% Triton X-100, stained with Ab-808, a rabbit polyclonal antibody to chicken integrin, and then labeled with a rhodamine-conjugated goat anti-rabbit antibody. (A) 3T3-cINTGRN; (B) 3T3-cINTGRN.788sY-F, (C) 3T3-cINTGRN.757sK-L; and (D) 3T3-cINTGRN.765t. Bar, 30 μ m.

containing chimeric receptors using both anti-mouse and -chicken integrin antibodies, showed that both native and chimeric receptors colocalized, along with vinculin, in the same focal contacts (Fig. 8, A, B, and C).

Effect of Amino Acid Substitutions and Deletions on Integrin Receptor Properties

As described above for 3T3-cINTGRN, all of the transfectants containing mutant integrins also adhered and spread on fibronectin-coated substrates. In addition, with two exceptions, neither the anti-mouse nor -chicken integrin antibodies, when added alone, inhibited adhesion. In contrast the simultaneous presence of both antibodies inhibited of the cell adhesion by 75% (Fig. 5 D). Two of the cytoplasmic deletions, 3T3-cINTGRN.765t and 3T3-cINTGRN.775t deviated from the above generalizations. Both cell types expressed chimeric receptors and adhered to fibronectin-coated substrates; however, their adhesion was inhibited by the presence of antibodies directed against the mouse integrin complex (Ab-3675) alone (Fig. 6 D). These results show that the

chimeric receptors with large deletions in the cytoplasmic domain are not fully functional.

As described above for 3T3-cINTGRN, 3T3-cINTGRN.788sY-F and 3T3-cINTGRN.757sK-L spread on laminin. The initial adhesion was inhibited by the chicken-specific mAb (WIB10), but not by the mouse-specific antibodies (Ab-3675) (data not shown). 3T3-cINTGRN.765t cells also spread on laminin after incubation at 37°C for 1 h. The chicken-specific mAb WIB10 had no effect on this initial spreading, whereas Ab-3675 did. These observations differed substantially from those for adhesion to fibronectin. It appeared that the wild-type, chimeric receptor, when present, was dominant for adhesion to laminin. Chimeric receptors with large cytoplasmic deletions produced a switch in dominance to the native mouse receptor for adhesion to laminin.

Immunoprecipitation of transfectants containing the mutant integrins showed that they also formed dimers (Fig. 3 B). However, the peptides precipitated from cells transfected with the largest cytoplasmic deletion, 3T3-cINTGRN.765t, appeared to differ from the others. The α subunit migrated

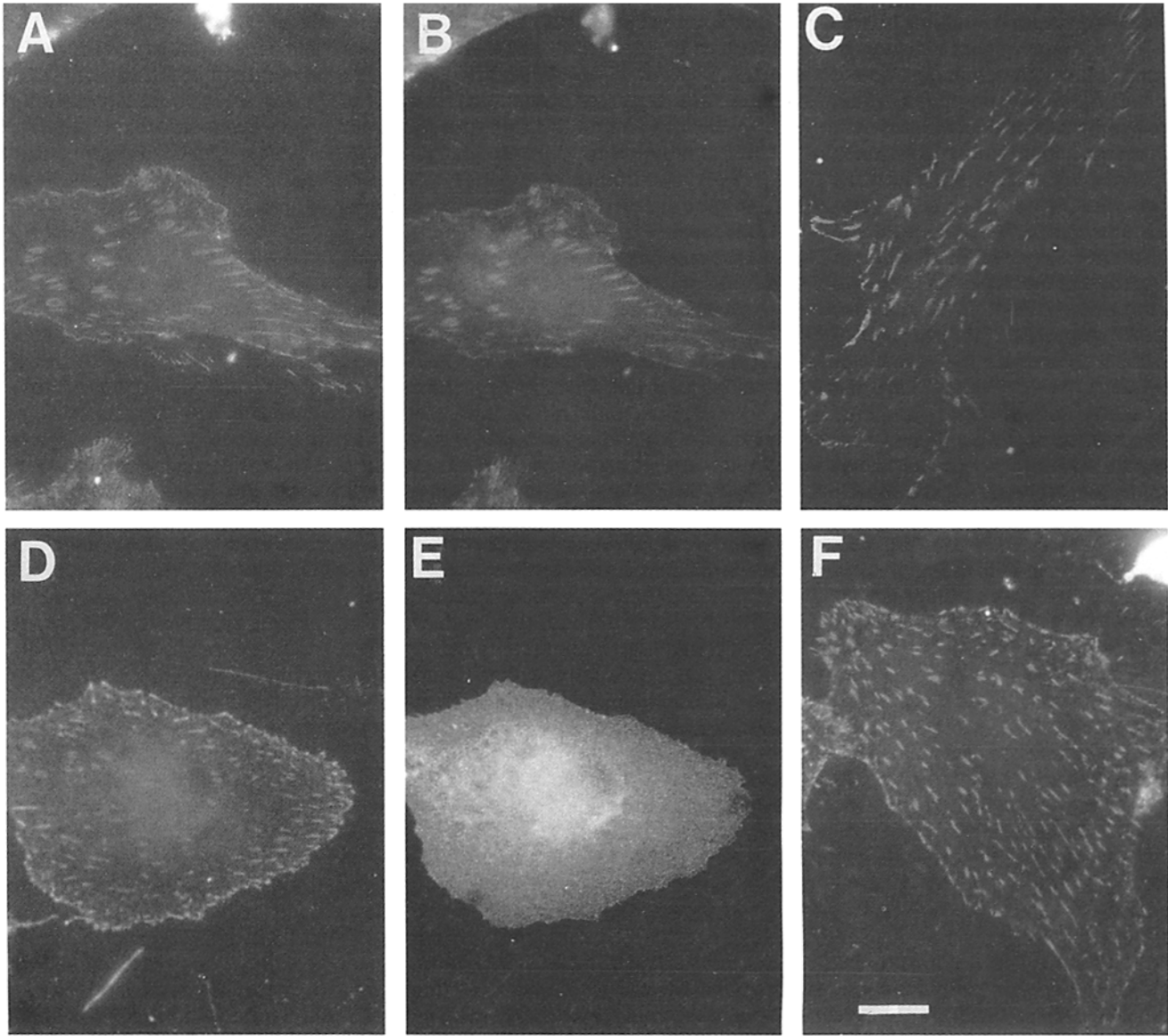


Figure 8. Distribution of mouse and chicken integrins and vinculin in transfected NIH 3T3 cells. Freshly trypsinized cells were plated in a serum free medium onto fibronectin-coated dishes and allowed to attach and spread for 6 h. Cell cultures were first labeled with antibodies to mouse integrin (Ab-3675) and a mAb to chicken integrin (V2E9), fixed with 3.7% formaldehyde, and then labeled with a mixture of rhodamine-conjugated goat anti-rabbit antibody and FITC-conjugated goat anti-mouse antibody. Vinculin staining was performed on permeabilized cells as described in Fig. 7 using anti-rat vinculin antibody as a primary antibody and a rhodamine-conjugated goat anti-rabbit secondary antibody. (A and B) 3T3-cINTGRN cells double-labeled with anti-mouse integrin (A) and anti-chicken integrin (B). (D and E) 3T3-cINTGRN.765t cells double-labeled with anti-mouse integrin (D) and anti-chicken integrin (E). (C and F) 3T3-cINTGRN (C) and 3T3-cINTGRN.765t (F) labeled with anti-rat vinculin. Bar, 15 μ m.

slightly, but reproducibly, faster than that in any of the other transfectants. This behavior was seen in both of the independent clones studied but was not seen in any of the other transfectants. This shift may reflect a difference in α subunit preference produced by the deletion. Alternatively, it may represent differences in glycosylation.

Immunofluorescence localization studies revealed further differences among the cytoplasmic mutants and the other mutant integrins (Fig. 9). The chimeric receptors in the transfected cells with pTEX-cINTGRN.765t, pTEX-cINTGRN.779t, or pTEX-cINTGRN.788t were distributed dif-

fusely in the plasma membrane (Figs. 8 and 9), although the mouse integrin receptor in the same cells localized, along with vinculin, in typical focal contacts (Fig. 8). In cells transfected with pTEX-cINTGRN.799t, the chimeric receptors, although present in the focal contacts, stained relatively weakly (Fig. 9 D) and somewhat more diffusely than that of the mouse heterodimers. NIH-3T3 cells transfected with pTEX-cINTGRN, pTEX-INTGRN.788sY-F and pTEX-cINTGRN.757sK-L showed typical focal contacts (Fig. 9 A, B, and C).

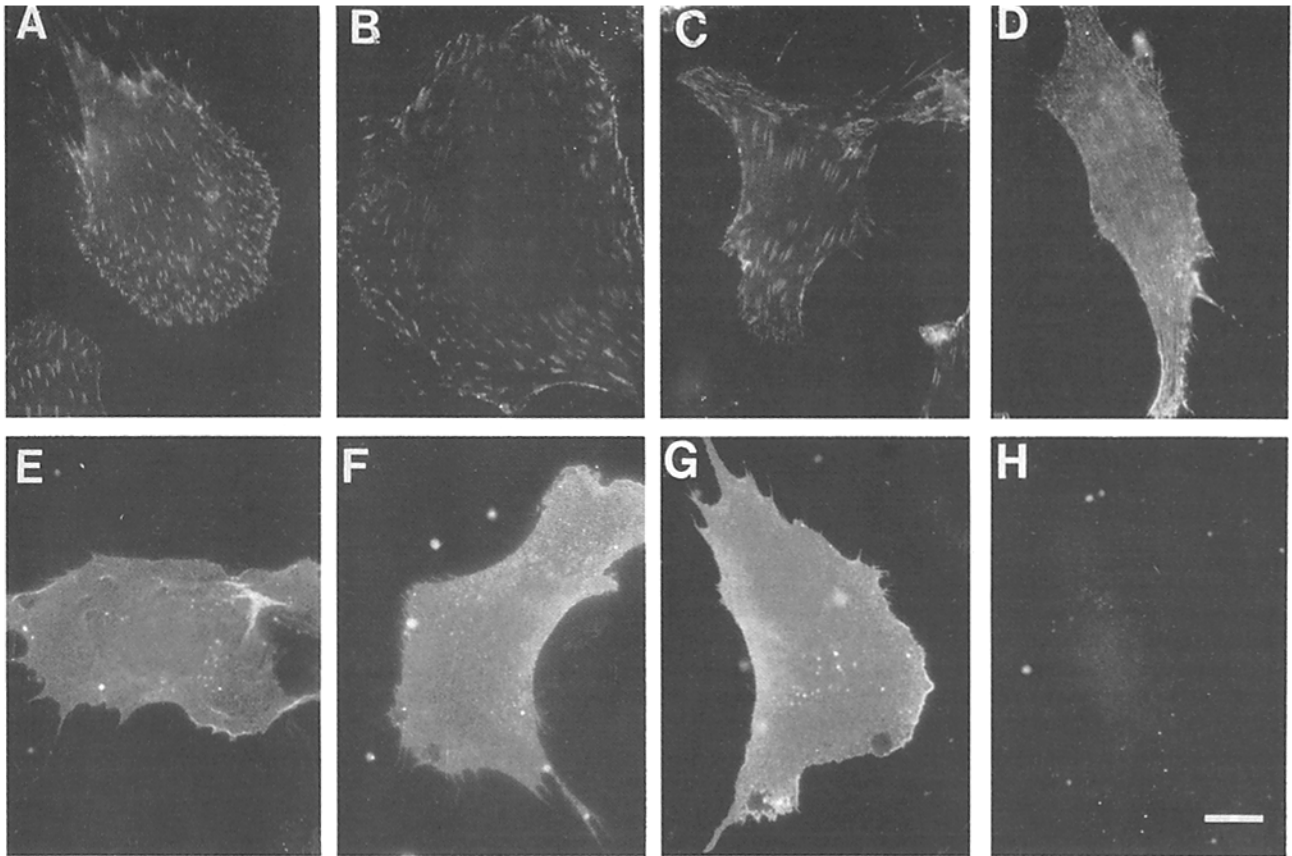


Figure 9. Distribution of chicken integrin on the cell surfaces of NIH 3T3 cells containing chimeric integrins with deletions in the cytoplasmic domain. The cultured cells were first labeled with Ab 808, fixed with 3.7% formaldehyde for 10 min, and then labeled with a rhodamine-conjugated goat anti-rabbit polyclonal antibody. (A) 3T3-cINTGRN; (B) 3T3-cINTGRN.788sY-F; (C) 3T3-cINTGRN.757sK-L; (D) 3T3-cINTGRN.799t; (E) 3T3-cINTGRN.788t; (F) 3T3-cINTGRN.779t; (G) 3T3-cINTGRN.765t; (H) 3T3 (untransfected). Bar, 15 μ m.

Discussion

We have addressed the function of the integrin β 1 cytoplasmic domain by transfection of NIH 3T3 cells with integrin cDNAs containing mutations in the cytoplasmic domain. The wild-type and mutant chicken β 1 subunits studied here were expressed at high levels and modified posttranslationally. They localized on the cell surface as well as in the endoplasmic reticulum and were detected by immunostaining with anti-chicken integrin antibodies.

Our results indicate that the chicken integrin β 1 subunit in transfected cells interacts with mouse α subunits to form chimeric receptors. These receptors are biologically active and mediate both cell adhesion and cytoskeletal organization. This interpretation derives from the following observations: (a) immunoprecipitation of chicken integrin β 1 using antibodies specific for the chicken β 1 subunit coprecipitates a mouse peptide that is very similar, if not identical, to the mouse α subunit precipitated with mouse anti-integrin antibodies. (b) The chicken/mouse chimeric receptor colocalizes with the native mouse integrin and vinculin on the cell surface in adhesion plaques. (c) Inhibition of the adhesion to fibronectin of cells containing the chimeric receptors requires the presence of anti-chicken β 1 integrin antibodies. These properties of the chimeric receptors are comparable

to those of native integrin receptors. There was one apparent difference between the native and transfected chicken β 1 subunit: the mouse α subunit appeared to have a lower affinity for the chicken than the mouse β 1 subunit.

The single mRNA, transcribed from the integrated plasmid vector containing the chicken cDNA, produced three proteins (90, 95, and 120 kD). The largest protein, 120 kD, was the only species used to form chimeric receptors that localized on the cell surface. The 95 kD protein appears to be a precursor and storage form of the 120-kD species. It labeled strongly after only a short pulse of metabolic label with [³⁵S]methionine, localized primarily inside the cell, was the predominant β 1 subunit species seen on immunoblots, and was chased into the 120-kD subunit. The role of the 90 kDa protein is unclear. It is detected as only a minor protein band on immunoblots with anti-chicken integrin β 1 antibodies and is not readily detected in pulse chase experiments.

The ratio of chimeric to native mouse heterodimer receptor expressed in the transfected cells varied from 0.5 to 1.5 among the different clones. The corresponding ratio of chicken to native mouse β 1 subunit varied from 2 to 5. This latter ratio of subunits was similar to the ratio of the mRNA coding for the integrin β 1 subunit in chicken fibroblasts to that in the mouse fibroblast transfectants. Although the chicken β 1 subunit is expressed at higher levels than the

mouse $\beta 1$ subunit, the ratio of native and chimeric heterodimers suggests that the mouse α subunit associates preferentially with the native mouse $\beta 1$ subunit. This preference could reflect a higher affinity of the mouse α subunit for the mouse than the chicken $\beta 1$ subunit. Alternatively, the pool of α subunits might not be in equilibrium with the total pool of (chick and mouse) $\beta 1$ subunits. The apparent proportionality between the ratio of chimeric to native receptors and the ratio of chick to mouse $\beta 1$ subunits favors an equilibrium situation. Preliminary data indicate a lower affinity for the mutants with large deletions in the cytoplasmic domain (unpublished data).

Immunolocalization experiments showed that the wild-type and several mutant receptors localized in adhesion plaques. Evidence has been reported showing that talin, a cytoskeletal associated protein found in adhesion plaques, binds to the cytoplasmic domain of integrin (Horwitz et al., 1986; Burn et al., 1988). Talin also binds, (at a different domain) to vinculin, another prominent component of adhesion plaques in fibroblasts (Burrige and Mangeat, 1984). These proteins are believed to anchor actin filaments to the cell surface (Burrige et al., 1988). This model, based largely on immunofluorescent colocalization and *in vitro* binding data, predicts that mutations in the cytoplasmic domain of the integrin $\beta 1$ subunit may alter its binding to talin and therefore not localize in adhesion plaques. Three mutants (3T3-cINTGRN.765t, 3T3-cINTGRN.779t, and 3T3-cINTGRN.788t), which have deletions of 15 or more amino acids, did not localize in focal contacts. Only the smallest deletion that we have prepared (3T3-cINTGRN.799t), a termination five amino acids from the carboxyl end, resulted in chimeric receptors that did localize in adhesion plaques. In contrast, the native mouse integrin, along with vinculin, localized in adhesion plaques in all of these cells. The mutant receptors with large cytoplasmic deletions form large patches when incubated with polyclonal anti-integrin antibodies at room temperature, further supporting the contention that they are dissociated from the cytoskeleton (unpublished observations).

The experiments using chimeric integrins with deletions in the cytoplasmic domain are a first step in identifying amino acid sequences required for localization in focal contacts, and presumably talin binding. The data presented here suggest that such a sequence includes amino acids between residues 788 and 799 (YKSAVTTVVNP). Previous *in vitro* studies demonstrated inhibition of talin binding to integrin by a decamer containing the amino acids between residues 780 and 789 (WDTGENPIYK) (Tapley et al., 1989). These amino acids include part of the consensus sequence for tyrosine phosphorylation. Together, the *in vitro* and *in vivo* data point to the amino acids YK, including tyrosine 788 and its surrounding regions as potentially important and deserving further investigation. This region of the cytoplasmic domain appears to correspond to a turn where there is a change in secondary structure and hydrophobicity (Chou and Fasman, 1978; Garnier et al., 1978; Kyte and Doolittle, 1982; Hopp, 1985).

The chimeric receptors also participated in adhesion to fibronectin and laminin. Adhesion to fibronectin used both the native and chimeric receptors; in contrast, however, the chimeric receptor was the major system used for adhesion to laminin. The origin of this dominance is not clear. It could

reflect either a difference in specificity of the native mouse and chicken $\beta 1$ subunit for α subunits or a difference in the ligand affinity of the native and chimeric receptors for laminin.

Deletions in the cytoplasmic domain of the chicken integrin $\beta 1$ subunit reduced the contribution of the chimeric receptors for adhesion to both fibronectin and laminin. The native mouse heterodimer was the dominant adhesive receptor in these cells. There are several possible explanations for the loss of adhesion resulting from alterations in the cytoplasmic domain: (a) deletions in the cytoplasmic domain inhibit the ability of the chimeric receptors to form the aggregates that might be required for high avidity interactions with ligands. (b) Deletions in the cytoplasmic domain might alter the conformation of the heterodimeric receptor complex, resulting in a lowered affinity for ligand. (c) The cytoplasmic domain might contain key residues that serve regulatory functions which, when modified, alter the affinity for ligands. The recent paper of Solowska et al. (1989) demonstrates *in vitro* binding, in the presence of Mn^{2+} between fibronectin and a chimeric integrin containing a cytoplasmic deletion. This observation, in the absence of any evidence for high-level posttranslational modification of the cytoplasmic domain in normal cells, favors the first hypothesis.

None of the substitution mutations had a detectable effect on either adhesion or the ability of the chimeric integrin to localize in adhesion plaques. The tyrosine (788) in the cytoplasmic domain is a residue of particular potential importance as it lies in a consensus sequence for phosphorylation by tyrosine kinases and is phosphorylated in cells transformed by viruses encoding oncogenes with tyrosine kinase activity (Hirst et al., 1986; Tamkun et al., 1986). In this context, it is interesting that the substitution of a phenylalanine for a tyrosine in the cytoplasmic domain had no effect on the ability of the chimeric receptors to associate with adhesion plaques or mediate adhesion. In preliminary experiments, time-lapse video phase-contrast microscopy was used to watch the morphology of transfected cells as they entered mitosis and underwent cytokinesis and subsequent migration. No detectable differences were observed between wild-type and phenylalanine substituted chimeras growing on either fibronectin or laminin (Regen, C., E. Crowley, and Y. Hayashi, unpublished observations). In addition, the phenylalanine substituted chimeras, when transformed in NIH 3T3 cells by Rous sarcoma virus, showed adhesion plaques and morphologies similar to those of the wild-type chimeras (Haimovich, B., and Y. Hayashi, unpublished observations). The lack of obvious biological or structural effects of substituting the phenylalanine for tyrosine at position 788 are somewhat surprising. Roughly half of the integrin receptors in these cells are chimeric; they localize in adhesion plaques and mediate substrate adhesion. If phosphorylation of integrin on tyrosine were primarily responsible for the altered morphology, adhesion, and cytoskeletal organization seen during these processes, one would expect residual adhesion plaques and other features of normal, quiescent cells. Clearly the role of integrin phosphorylation, if any, in these processes needs careful examination.

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