Expression of Normal and Mutant Avian Integrin Subunits in Rodent Cells

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Abstract. We describe the expression of the β_1 subunit of avian integrin in rodent cells with the purpose of examining the structure–function relationships of various domains within this subunit. The exogenous subunit is efficiently and stably expressed in 3T3 cells, and it forms hybrid heterodimers with endogenous murine α subunits, including α_3 and α_5. These heterodimers are exported to the cell surface and localize in focal contacts where both extracellular matrix and cytoskeleton associate with the plasma membrane. Hybrid heterodimers consisting of exogenous β_1 and endogenous α subunits bind effectively and specifically to columns of cell-binding fragments of fibronectin. The exogenous avian β_1 subunit appears to function as well as its endogenous murine equivalent, consistent with the high degree of conservation noted previously for integrins. In contrast, expression of a mutant form of avian integrin β_1 subunit lacking the cytoplasmic domain produces hybrid heterodimers which, while efficiently exported to the cell surface and still capable of binding fibronectin, do not localize efficiently in focal contacts. This further implicates the cytoplasmic domain of the β_1 subunit in interactions required for cytoskeletal organization.

C ELLS from a wide variety of both vertebrate and invertebrate species share the ability to adhere to extracellular matrices. Cell adhesion is a property required for cell migration and tissue stability and is central to embryonic development, wound healing, metastasis and other biological processes requiring tethering of a cell to its substratum. Cell adhesion also affects cell shape, cell division, and cell differentiation. For these reasons, the molecules to which cells adhere as well as the constituents of the cell surface involved in the adhesion process have been subjected to intensive investigations (Buck and Horwitz, 1987; Martin and Timpl, 1987; for review, see Ruoslahti, 1988).

Among the receptors playing a major role in cell-substratum adhesion are the members of a family of surface glycoproteins designated integrins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). Integrins are heterodimers consisting of noncovalently associated α and β subunits. Those integrins involved in cell–substratum adhesion are found concentrated in or around focal contacts on the ventral cell surface, colocalizing with extracellular matrix (ECM)[1] molecules and cytoskeleton-associated (CSK) molecules (Chen et al., 1985; Damsky et al., 1985; Singer et al., 1988; Dejanna et al., 1988). Integrins are capable of binding directly to ECM molecules, including fibronectin, vitronectin, or laminin (Pytela et al., 1985a,b; 1986; Horwitz et al., 1985; Akiyama et al., 1985; Gardner and Hynes, 1985; Johansson et al., 1987a,b;

1. *Abbreviations used in this paper*: CSK, cytoskeleton; ECM, extracellular matrix; TBM, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM MnCl$_2$.

Wayner and Carter, 1987; Wayner et al., 1988; Gehlsen et al., 1988; Ignatius and Reichardt, 1988; Sonnenberg et al., 1988), and to CSK molecules such as talin (Horwitz et al., 1986). The integrity of the $\alpha\beta$ complex is required for binding to both ECM and CSK molecules (Buck et al., 1986).

Recent structural and serological data have led to the division of the integrin family into three subfamilies (Hynes, 1987; Anderson and Springer, 1987). Each subfamily is distinguished by a common β subunit that can associate with a limited number of different α subunits. All β subunits share certain structural features (Hynes, 1987; Buck and Horwitz, 1987; Ruoslahti and Pierschbacher, 1987). For example, the major portion of the β subunit is the extracellular domain which contains 56 conserved cysteine residues including four particularly cysteine-rich repeating structures. This is followed by a membrane-spanning domain and a relatively short intracellular domain (Marcantonio and Hynes, 1988; Mueller et al., 1988). Comparisons of the amino acid sequences of β subunits from the three integrin subfamilies reveal a 40–48% identity while β subunits within a single subfamily display over 80% identity among diverse vertebrates (DeSimone and Hynes, 1988) suggesting a molecule whose structure and function are highly conserved. Further evidence for the structural and functional conservation of portions of the β_1 subunit comes from the observation that antibodies against the cytoplasmic domain of the avian β_1 subunit react with β subunits from many phylogenetically diverse sources (Marcantonio and Hynes, 1988).

The β_1 subfamily of integrins includes receptors for such ECM molecules as fibronectin, certain collagens, and laminin. This subfamily contains at least six serologically distinct α subunits each capable of binding to a common β_1 subunit (Hemler et al., 1987, 1988; Hynes, 1987). The substrate specificity of each receptor is determined by the particular combination of α and β subunits. Thus, $\alpha_5\beta_1$ is a fibronectin receptor (Pytela et al., 1985a; Argraves et al., 1987; Wayner et al., 1988), $\alpha_2\beta_1$ is a collagen receptor (Kunicki et al., 1988; Takada et al., 1988) and $\alpha_6\beta_1$ is a laminin receptor (Sonnenberg et al., 1988), while $\alpha_3\beta_1$ is a promiscuous receptor thought to bind to several different ECM molecules (Wayner and Carter, 1987; Wayner et al., 1988).

It is clear from these results that integrins are involved in a variety of interactions and functions, including subunit dimerization, binding of extracellular matrix and cytoskeletal proteins, cell adhesion, and cytoskeletal organization. To begin to dissect the various structure–function relationships of integrin subunits, we have expressed the avian integrin β_1 subunit in rodent cells and assayed its ability to perform various functions in this heterologous context.

Materials and Methods

Plasmid Construction

The restriction enzymes, T4DNA ligase, polynucleotide kinase, *Escherichia coli* DNA polymerase I large fragment, and XbaI linker, were from New England Biolabs (Beverly, MA). Standard recombinant DNA methods (Maniatis et al., 1982) were used.

A 3.15 kb Eco RI fragment containing the entire coding sequence for chicken integrin β1 was isolated from the cDNA clone 1D described previously (Tamkun et al., 1986). This fragment was then inserted into the Hind III cloning site of the SV40 expression vector pESP-SVTEXP (Reddy and Rao, 1986) by blunt-end ligation. The resulting plasmid is designated pCINTβ_1. A XbaI linker (CTCTAGAG) including an in-frame stop codon was then used to generate the plasmid pCINT$\beta_1\Delta$761-803, which codes for the mutated chicken integrin β_1 lacking its cytoplasmic domain (Fig. 6). Briefly, the chicken integrin β_1 cDNA subcloned in pGEM1 (Promega Biotec, Madison, WI) was propagated in an adenine methylase-deficient *E. coli* strain GM2163 supplied by New England Biolabs. The purified plasmid DNA was then partially digested with restriction enzyme Bcl I and the full-length linear DNA was isolated by agarose gel electrophoresis. After filling in the ends with *E. coli* polymerase I large fragment, the linear DNA was religated in the presence of kinased XbaI linker (molar excess) and transformed into *E. coli* strain HB101. The plasmid having XbaI linker incorporated into the second Bcl I site of integrin β_1 cDNA was identified by restriction analysis and the expected sequence around the junction was confirmed by dideoxy sequencing (sequenase; United States Biochemical Corp., Cleveland, OH). The altered cDNA fragment was then excised from pGEM1 and inserted into the Eco RI cloning site of the SV40 expression vector pECE (Ellis et al., 1986) generating the plasmid pCINT$\beta_1\Delta$761-803.

Transfection of 3T3 Cells

NIH 3T3 cells were maintained in DME supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY). 5×10^5 cells plated the previous day in 100 mm dishes were co-transfected with 20 μg pCINTβ_1 (or pCINTβ_1-Δ761-803) and 2 μg pSV2neo (Southern and Berg, 1982) as a calcium phosphate precipitate (Wigler et al., 1979). Cells were incubated for 20–22 h, washed with PBS, and fresh medium was replaced. Two days later, the transfected cells were split 1:10 and incubated in DME supplemented with 10% FCS and 0.5 mg/ml G418 (Geneticin, Gibco Laboratories). After ∼2 wk, G418-resistant clones were isolated and expanded. The 3T3 cell clones expressing chicken integrin β_1 were identified by indirect immunofluorescence staining using a chicken-specific polyclonal antiserum Chickie II (see below). These positive clones were then subcloned by plating ∼500 cells onto 100-mm dishes coated with 10 mg/ml gelatin. Individual subclones were then isolated and analyzed by immunofluorescence labeling. Subclones 1E encoding wild type chicken integrin β_1 and Δ7E expressing mutant β_1 were used for further characterization.

Antibodies and Peptides

A polyclonal avian-specific antiintegrin antibody designated Chickie II was prepared by injecting CSAT-immunoaffinity-purified avian integrin into rabbits and has been used previously (Damsky et al., 1985). 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 L. Urry (Massachusetts Institute of Technology, Cambridge, MA). CSAT monoclonal antibody was prepared from CSAT hybridomas (Neff et al., 1982) and for immunoprecipitation was covalently coupled to protein A Sepharose (Sigma Chemical Co., St. Louis, MO) by binding in PBS, washing with 100 vol, and coupling with 0.04% glutaraldehyde for 1 h at 37°C, followed by blocking with 0.5 M ethanolamine pH 8.0 (Gyka et al., 1983). Rabbit anti-β_1 cytoplasmic domain antibodies were prepared as described (Marcantonio and Hynes, 1988). Rabbit anti-α_3 and anti-α_5 COOH terminal peptide antibodies were prepared as described (Hynes et al., 1989). Monoclonal antivinculin antibody was a gift of B. Geiger (Weizmann Institute). Rhodamine-labeled phalloidin was purchased from Molecular Probes Inc. (Junction City, Oregon).

GRGESP and GRGDSP were synthesized using a peptide synthesizer (Applied Biosystems Inc., Foster City, CA) using solid phase t-boc chemistry. Peptides were cleaved and deprotected using trifluoromethane sulfonic acid and were desalted on Sephadex G-10. Before use, peptides were purified by reverse phase HPLC chromatography on a vydac C18 semipreparative column (Rainin Instrument Co. Inc., Woburn, MA), eluted with a 0–60% acetonitrile gradient in 0.1% TFA.

Radiolabeling and Immunoprecipitation

For metabolic labeling, cells were incubated for 1 h in DME minus methionine plus 10% FCS, followed by incubation in methionine-free medium plus 10% FCS containing 20 μCi/ml of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) for 6 h. Cells were labeled with Na[^{125}I] (New England Nuclear, Boston, MA) and lactoperoxidase (Sigma Chemical Co., St. Louis, MO) as a monolayer as described (Hynes, 1973). 10^7 cells and 1–2 mCi/ml were used per experiment. Cells were extracted with 0.5% NP-40 and immunoprecipitation was performed as described (Marcantonio and Hynes, 1988).

In some experiments, extracts were immunoprecipitated using CSAT-Sepharose, followed by recovery of the integrin complexes by heating at 100°C for 2 min in 1% SDS. After cooling, a fivefold excess of Triton X-100 was added, and the extracts were reprecipitated using polyclonal antibodies and protein A-Sepharose as described above.

SDS-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-HCl, pH 6.8, 10 mM EDTA, 10% glycerol and bromophenol blue) and boiled for 3 min.

Affinity Chromatography

Purified human plasma fibronectin was purchased from the New York Blood Center (New York, NY). The 120-kD cell-binding fragment of fibronectin was purified from a chymotryptic digest of fibronectin as described by Pierschbacher et al. (1981). Columns were prepared by coupling 1 mg/ml of purified 120-kD fragment to CNBr-activated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.2 M NaHCO$_3$ pH 8.5.

Affinity chromatography of 3T3 cell extracts on 1-ml columns was performed using a modification (Gailit and Ruoslahti, 1988) of the procedures of Pytela et al. (1985a). Briefly, ∼10^7 cells were labeled with [^{125}I] as described above and extracted using 200 mM octyl-β-D-glucopyranoside in 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM MnCl$_2$ (TBM). These extracts were loaded onto the 120-kD fragment columns over 1 h at 4°C, and then washed with 10 vol of TBM. Columns were eluted with 1 vol of TBM containing 1 mg/ml of control peptide (GRGESP), followed by 2 vol of TBM, and then 1 vol of TBM containing 1 mg/ml of GRGDSP. Column fractions were analyzed by immunoprecipitation or directly by SDS-PAGE.

Immunofluorescence

Cells were plated in DME 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 Co., 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 using an axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) and photographed (Tri-X film; Eastman Kodak Co., Rochester, NY).

Quantitative Immunoprecipitation Analysis of Integrin Expression

Quantitative immunoprecipitation of clone 1E ^{125}I-labeled extracts was performed. 10^6 TCA-precipitable cpm of extract were incubated with increasing amounts of 363 or 366 antisera followed by immunoprecipitation and SDS-PAGE as described above to determine the maximum recovery of integrins. Bands corresponding with the β_1 subunit were excised from the gel and counted using a gamma counter.

Quantitation of the ratio of α/β subunit and the relative amounts of the chicken and mouse integrin subunits was performed by integration of peaks obtained from scans of the autoradiographs using an LKB ultrascan XL laser densitometer (LKB Instruments, Gaithersburg, MD).

Results

Expression of Avian Integrin β_1 Subunit

The cDNA sequence of avian integrin β_1 subunit has been described (Tamkun et al., 1986). A full length cDNA clone, 1D, was used for the analysis reported here. A 3.15-kb Eco RI fragment containing the entire coding region was isolated and subcloned into an SV40-based expression vector (Reddy and Rao, 1986) to generate pCINTβ_1 (see Materials and Methods for details). This plasmid was cotransfected with pSV2neo (Southern and Berg, 1982) into murine 3T3 cells and clones resistant to G418 were selected and expanded as described in Materials and Methods.

To analyze the expression of avian β_1 integrin, we used the CSAT monoclonal antibody specific for this subunit (Buck et al., 1986). Fig. 1 shows CSAT immunoprecipitates from $[^{35}S]$methionine-labeled transfected 3T3 cells. SDS-PAGE analysis of immunoprecipitates from four independent clones of cells transfected with pCINTβ_1 are shown in lanes B–E. The immunoprecipitates contain heterodimers typical of members of the integrin family. The lower molecular mass 110-kD band migrates in about the same position on nonreduced SDS-PAGE as the β_1 subunit found in a control immunoprecipitate from avian cells (Fig. 1, lane A). No material was immunoprecipitated from 3T3 cells transfected with vector containing insert in the reverse orientation (Fig. 1, lane G) or from control 3T3 cells transfected only with PSV2neo (Fig. 1, lane F). That the 110-kD band contained the avian β_1 subunit was confirmed by reaction in immunoblots with a second monoclonal antibody, G (Buck et al., 1986), which is also specific for the avian β_1 subunit (data not shown). These results clearly demonstrate the expression of the avian β_1 subunit in the cloned transfected 3T3 cells. Subsequent experiments concentrated on one of these clones, 1E (Fig. 1, lane E).

The Exogenous β_1 Subunit Forms Heterodimers with Endogenous α Subunits

The presence of additional polypeptides in the CSAT immunoprecipitates from transfected cells suggested that the avian β_1 subunit could combine with endogenous murine α subunits. To document this, and to examine whether or not such complexes could be transported to the cell surface, clone 1E and control cells were surface labeled with ^{125}I. Detergent extracts were then immunoprecipitated with sev-
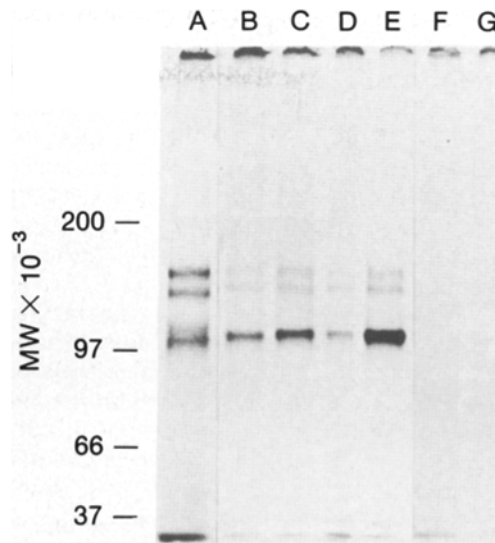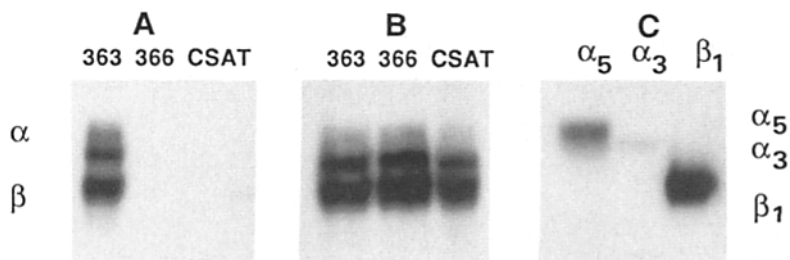

Figure 1. SDS-PAGE analysis of immunoprecipitates from transfected and control cells. NP-40 extracts from $[^{35}S]$methionine cells were immunoprecipitated with the monoclonal antibody CSAT specific to the avian β_1 subunit. Immune complexes were subjected to SDS-PAGE under nonreducing conditions. Lane A, immunoprecipitates from control chick fibroblasts. The 110-kD protein is the avian β_1 subunit. Lanes B to E, four independent clones of 3T3 cells transfected with pCINTβ_1. Lane F, control 3T3 cells transfected with pSV2neo only. Lane G, 3T3 cells transfected with the vector carrying insert in the reverse orientation.

eral different antibodies (Fig. 2). Antiserum 363 was raised against a β_1 cytoplasmic domain peptide (Marcantonio and Hynes, 1988). This antiserum reacts exclusively with the cytoplasmic domain of β_1 subunits regardless of species. Fig. 2, A and B, shows that this antibody precipitates β_1 subunits together with at least two α subunits from both control and transfected 3T3 cells. A second antiserum, 366, was raised against SDS gel-purified avian β_1 subunit and reacts only with the avian subunit (Fig. 2, A and B; Urry, L., and R. O. Hynes, unpublished observations). It immunoprecipitates the avian β_1 subunit and associated α subunits from cells transfected with pCINTβ_1 (Fig. 2 B), but not from control 3T3 cells (Fig. 2 A) or from cells transfected with pSV2neo alone (data not shown). Immunoprecipitates using the monoclonal antibody CSAT were included in these experiments as a control for specificity as well as for comparison of integrin subunit behavior on SDS-PAGE. Comparison of the relative intensities of the α and β bands in the different immunoprecipitates suggests that the avian β_1 subunit associates with the murine α subunits as efficiently as does the endogenous murine β_1 subunit. Quantitative comparisons of the total surface level of all β_1 integrins (363 immunoprecipitates) with that of avian β_1 integrin (366 or CSAT immunoprecipitates) shows that in stably transfected clone 1E cells, integrins containing the avian subunit make up 40–60% of the total β_1 integrins expressed by these cells. In several experiments, no consistent differences were detected in the ratios of α to β labeling in total integrins and in integrins containing the chicken β_1 subunit.

The identity of the accompanying α subunits was determined by sequential immunoprecipitations. Clone 1E cells were iodinated, extracted and immunoprecipitated with the

Figure 2. Immunoprecipitation of mouse and chicken integrins. *A* and *B*, extracts of ^{125}I-surface-labeled control 3T3 cells (*A*) and clone 1E cells expressing chicken integrin β_1 subunit. (*B*) were incubated with broad spectrum anti-β_1 peptide serum (363), anti–chicken β_1 serum (366) or monoclonal anti–chicken β_1 (*CSAT*) Sepharose. Immunoprecipitates were recovered directly (*CSAT*) or indirectly using protein A-Sepharose (363, 366) and analyzed by SDS-PAGE. *C*, A nondenatured extract of ^{125}I-surface–labeled clone 1E cells was immunoprecipitated using *CSAT* monoclonal anti–chicken β_1-Sepharose. The recovered complexes were denatured in SDS, after which a fivefold excess of Triton X-100 was added. The denatured integrins were then incubated with anti-α_5 peptide serum, anti-α_3 peptide serum, or anti-β_1 peptide serum. The samples were immunoprecipiated using protein A-Sepharose and analyzed by SDS-PAGE. Thus, in the transfected cells, chicken β_1 is present at the cell surface, and associates with the endogenous mouse α subunits, predominantly α_3 and α_5.

avian-specific monoclonal antibody CSAT. The resulting precipitates were dissolved in SDS and then reprecipitated with rabbit antisera raised against peptides from specific integrin α subunits (Hynes et al., 1989; see Materials and Methods). Results from such an experiment are shown in Fig. 2 *C*. The three bands immunoprecipitated by CSAT can be reprecipitated by antisera to α_5, α_3, and β_1 after SDS denaturation (Fig. 2 *C*). The α_3 and α_5 subunits are the major β_1 subfamily α subunits expressed in 3T3 cells (Marcantonio, E., unpublished observations). These data prove that the avian β_1 subunit expressed in transfected 3T3 cells is transported to the cell surface and associates with the appropriate murine α_3 and α_5 subunits.

Hybrid Integrin Heterodimers Bind Fibronectin

To assay the function of hybrid receptors, we analyzed the ability of the complexes to bind to columns containing the 120-kD cell-binding fragment of fibronectin. The $\alpha_5\beta_1$ complexes from a variety of species bind specifically to such columns and can be eluted with peptides containing the RGD sequence (Pytela et al., 1985*a*, 1986; Wayner and Carter, 1987; Wayner et al., 1988; Gailit and Ruoslahti, 1988; Hynes et al., 1989). Clone 1E cells were iodinated and extracted with β-octylglucoside in buffer containing MnCl$_2$ (see Materials and Methods). The extracts were passed over

columns of fibronectin cell-binding fragment and eluted sequentially with GRGESP and GRGDSP peptides. Total integrin content of the eluate was demonstrated by immunoprecipitation with antiserum 363 (Fig. 3 *A*). The fraction of the eluted integrins consisting of hybrid receptors was identified by immunoprecipitation with antiserum 366 specific for the avian β_1 subunit (Fig. 3 *B*). As can readily be seen, integrins were eluted specifically with GRGDSP. The eluate included integrins containing the avian β_1 subunit (Fig. 3 *B*). The doublet form of the α bands in the eluted fractions is frequently observed (Hynes et al., 1989). Both portions of this doublet react with antisera raised against α_5 peptides (data not shown). The hybrid heterodimers consisting of avian β_1 and murine α subunits are clearly able to bind to columns containing fibronectin cell-binding fragment. Quantitation shows that the hybrid heterodimers bind to the columns as efficiently as do the endogenous murine integrins. That is, the ratio of avian β_1 to total β_1 is the same in the bound material as in the total extract, and the ratios of α and β are the same in the total integrins and the integrins containing avian β_1.

The Avian β_1 Integrin Subunit Becomes Localized in Focal Contacts

We next examined whether the exogenous avian β_1 subunit could be correctly localized in focal contacts in the same
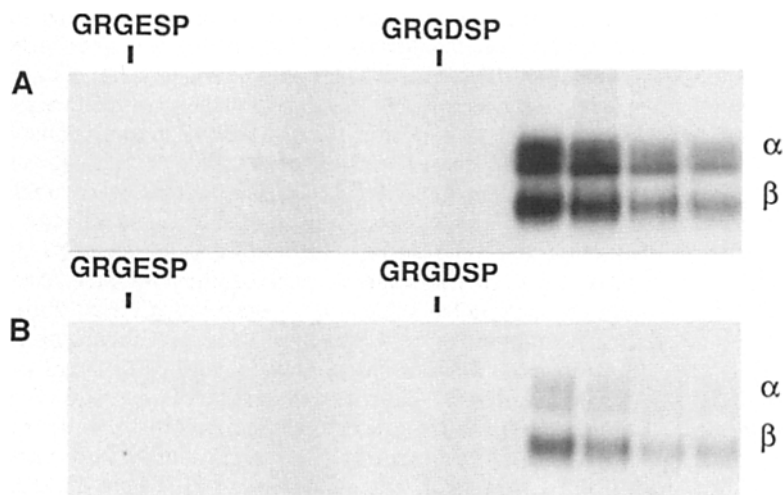

Figure 3. Binding of hybrid integrins to fibronectin. Clone 1E cells were labeled with ^{125}I and extracts were prepared as described in Materials and Methods. One milliliter of extract was incubated with one milliliter of 120-kD fibronectin cell-binding fragment-Sepharose for 1 h at 4°C. After washing, the column was sequentially eluted using GRGESP and GRGDSP as indicated at the top of *A* and *B*. 0.5-ml fractions were collected, and 100-μl aliquots were immunoprecipitated with 363 antiserum (*A*) or 366 (*B*) antiserum as described in Materials and Methods. Both the endogenous mouse and the chicken–mouse hybrid integrin complexes bind to fragments of fibronectin and are specifically eluted using the cell-binding site peptide GRGDSP.
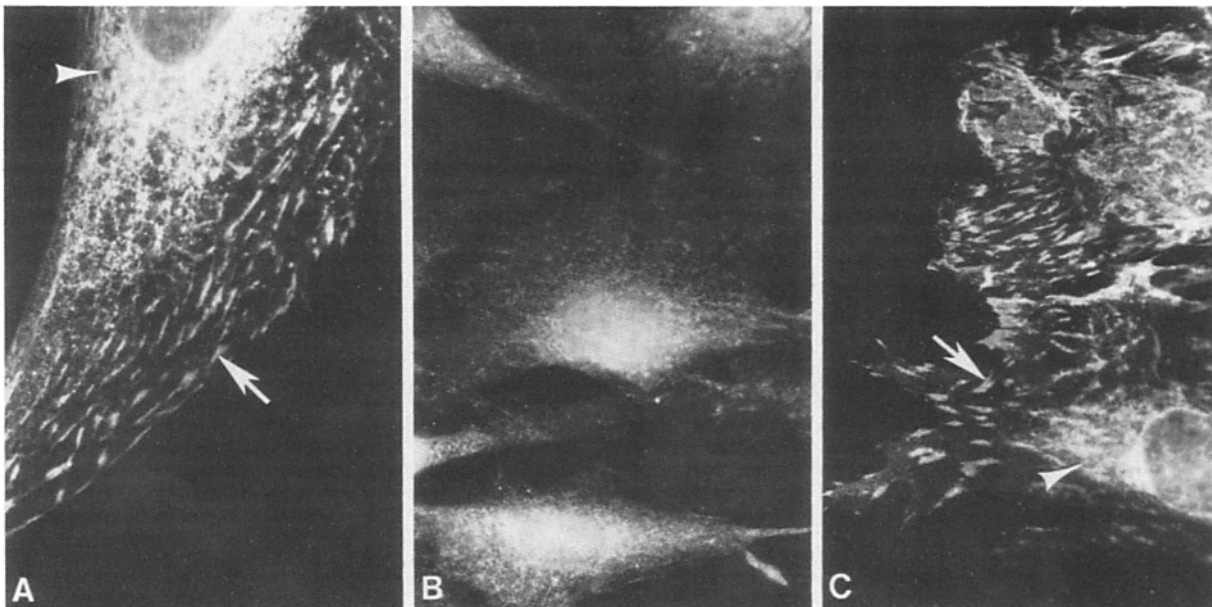
Figure 4. Localization of avian β_1 subunit in transfected 3T3 cells by immunofluorescence. Transfected and control 3T3 cells were processed for indirect immunofluorescence as described in Materials and Methods. An avian-specific polyclonal antibody, Chickie II, was used to localize the avian β_1 subunit. *A* and *C*, two independent clones of 3T3 cells transfected with pCINTβ_1. *B*, 3T3 cells transfected with pSV2neo only. Regions of intense fluorescence result from the presence of the avian β_1 subunit (*arrowheads*). Concentrated fluorescence is obvious in focal contactlike structures as well as in the cytoplasmic membranes surrounding the nucleus. Magnification of 1,600.

fashion as endogenous integrins (Chen et al., 1985; Damsky et al., 1985; Singer et al., 1988; Dejanna et al., 1988; Marcantonio and Hynes, 1988). To examine the distribution of the receptor, cells were grown on coverslips and stained for immunofluorescence using a polyclonal antibody that will react with avian integrin, but not with integrins normally found in 3T3 cells. The results are shown in Fig. 4. Control 3T3 cells, as well as pSV2neo-transfected 3T3 cells not expressing the avian β subunit, show only background fluorescence; no typical focal contactlike structures are evident (Fig. 4 *B*). In contrast, two independent clones, 1D and 1E, expressing the avian subunit exhibit strong immunofluorescence in brushstrokelike patterns on the ventral surface of cells (Fig. 4, *A* and *C*). This staining pattern is characteristic of focal contacts and closely resembles that seen in chicken cells stained with this same antibody or with monoclonal antibodies specific for the avian β subunit (Damsky et al., 1985; Chen et al., 1985). Similar results are obtained if the cells are stained with the CSAT monoclonal antibody (Solowska, J., unpublished observations). Double immunofluorescence experiments, in which clone 1E cells are exposed to rhodamine-labeled phalloidin (to mark microfilament bundles) and the avian integrin-specific antibody, show that the actin-containing microfilaments terminate in the structures stained by the antiintegrin antibody (Fig. 5), confirming the identity of these structures as focal contacts.

Deletion of the Cytoplasmic Domain Produces Partially Functional Hybrid Integrins

To begin the analysis of the function of specific domains of the β subunit, we deleted a major portion of the cytoplasmic domain by in vitro mutagenesis. Fig. 6 shows a comparison between the avian integrin β_1 subunit and the mutated form.

The altered sequence contains a termination codon close to the beginning of the COOH terminal cytoplasmic domain. Plasmid pCINT$\beta_1\Delta$761-803, which encodes mutagenized avian β_1, was transfected into 3T3 cells together with pSV2neo. G418-resistant clones were isolated as described above and a stably expressing subclone Δ7E was further analyzed.

Fig. 7 shows immunoprecipitation analysis of surface-labeled Δ7E cells. As before, antiserum 363 precipitates a set of integrins comprising at least two α subunits and a β_1 subunit (Fig. 7 *A*). In this case, since the mutated β_1 cDNA encodes a truncated form of the chicken β_1 subunit lacking the cytoplasmic domain recognized by this antiserum, 363 precipitates only the endogenous murine integrins (see also below). Immunoprecipitation with antiserum 366 or monoclonal antibody CSAT, both of which are avian-specific, selects only those integrins containing the avian β_1 subunit. The fact that these integrins also contain α subunits is confirmed by reprecipitation of CSAT-selected integrins with antisera specific for different subunits (Fig. 7 *B*). As expected, antiserum 363 fails to precipitate the avian β_1 subunit confirming the fact that the cytoplasmic domain is missing. The truncated β_1 subunit is, however, precipitated by the avian-specific antibodies (Fig. 7, *A* and *B*). α subunits from these hybrid integrins are immunoprecipitated by antibodies specific for α_5 (Fig. 7 *B*) and α_3 (data not shown). Thus, the truncated β_1 integrin can form heterodimers with endogenous α subunits and be exported to the cell surface. Quantitation shows that the ratio of α subunits to mutant β_1 subunits is lower than that for the wild type avian β_1 subunit (Fig. 2). It is unclear whether this lower ratio of α to β subunits reflects a defect in assembly or in stability of the complex or the high level of expression of avian β_1 integrin subunit in these cells. Nonetheless, it is clear that the
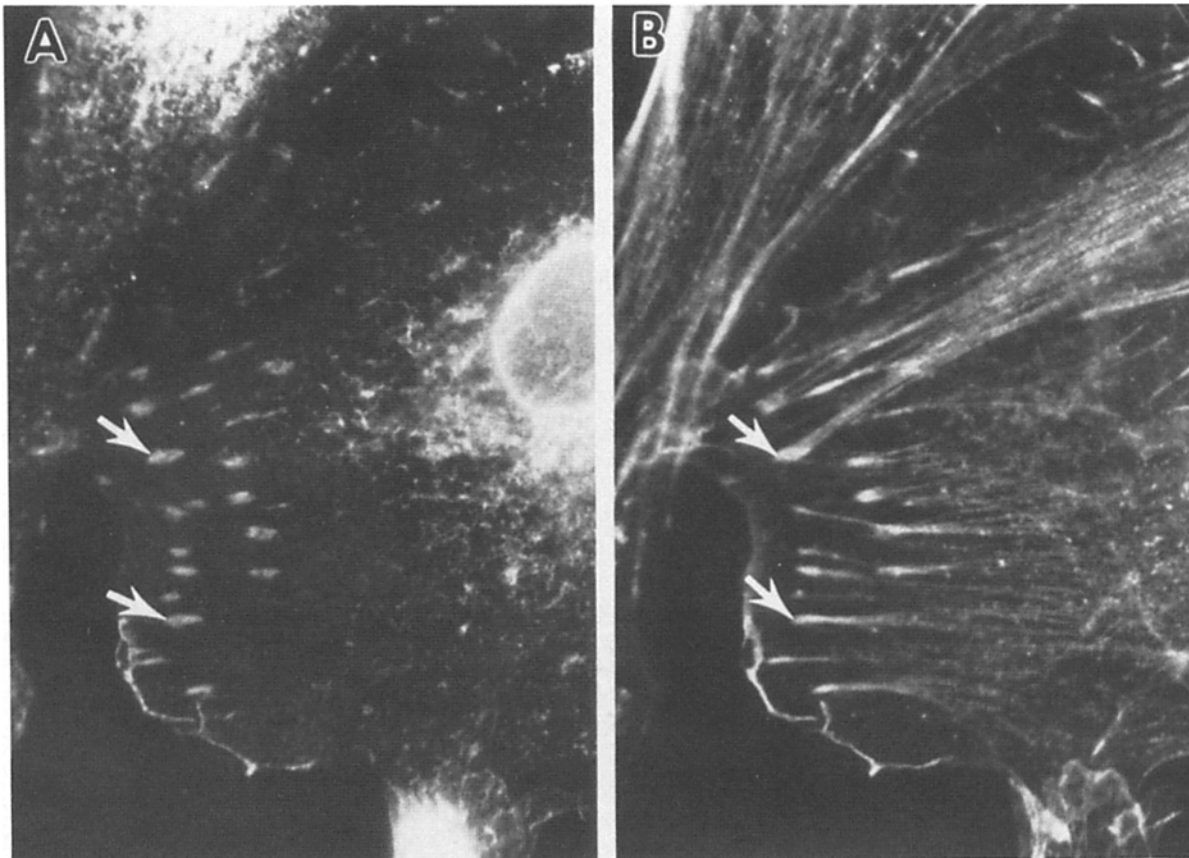
Figure 5. Double label immunofluorescence analysis of transfected 3T3 cells. 3T3 cells transfected with pCINTβ₁ were stained with a polyclonal antibody against avian integrin (Chickie II) to localize the avian β₁ subunit, and subsequently exposed to rhodamine-labeled phalloidin to mark actin-containing microfilaments. *A*, fluorescein-marked avian β₁ subunit distribution; *B*, same field showing rhodamine-marked actin-containing microfilaments. Note the termination of microfilament bundles in the focal contactlike structures stained with the antiavian β₁ (*arrowheads*). Magnification of 1,600.

presence of the β₁ cytoplasmic domain is not essential either for dimerization or for processing and export to the cell surface (see Discussion).

Analysis of the binding to fibronectin affinity columns of hybrid receptors is shown in Fig. 8. Extracts of surface-labeled Δ7E cells were analyzed as described previously. Heterodimers containing the truncated avian β₁ subunit
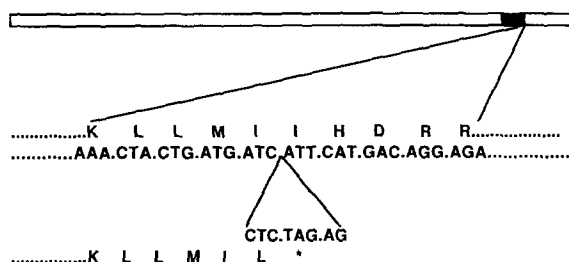
Figure 6. Deletion of the integrin β₁ cytoplasmic domain. Parts of the amino acid and nucleotide sequences of the chicken integrin β₁ cytoplasmic domain are shown. Insertion of the Xbal linker (CTCTAGAG) containing the in-frame termination codon results in the truncated molecule Δ761-803 shown below. The mutant protein lacks 42 of the 47 residues in the wild type cytoplasmic domain and contains an extra leucine at the COOH terminus.

were identified using antiserum 366 (Fig. 8 *B*) and elute in the same fractions as the endogenous murine heterodimers detected by antiserum 363 (Fig. 8 *A*). It is clear that the deleted form of the avian β₁ subunit can form functional heterodimers with endogenous murine α subunits that bind to fibronectin in an RGD-sensitive manner.

However, immunofluorescence analysis of the distribution of the mutant avian β₁ subunit shows that the truncation does produce defects in localization of the integrin. Fig. 9 shows double label immunofluorescence analysis using antivinculin to mark focal contacts and antibodies specific for the avian β subunit to mark the location of hybrid receptors. In clone 1E cells expressing the unaltered avian β subunit, hybrid receptor and vinculin colocalize on the ventral cell surface (Fig. 9, *A* and *B*). In contrast, Δ7E cells expressing the truncated form of the avian β subunit display little, if any, hybrid receptor in the focal contacts marked by the antivinculin antibody (Fig. 9 *C* and *D*). Endogenous murine integrin in these same cells is localized in focal contacts (data not shown). Therefore, deletion of the β₁ cytoplasmic domain interferes with localization of the hybrid heterodimers into focal contacts.

Because the truncated form of the avian β₁ subunit appeared to be somewhat deficient in its ability to form stable hybrid heterodimers, we quantitated the level of functional heterodimers in Δ7E cells. The integrins eluted from
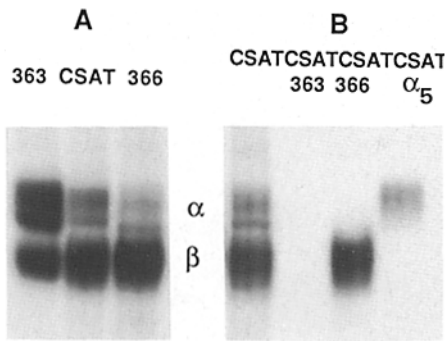
Figure 7. Immunoprecipitation of integrins from clone Δ7E cells. A, Extracts of ^{125}I-surface-labeled clone Δ7E cells were incubated with anti-β_1 cytoplasmic domain serum (363), monoclonal anti–chicken β_1-Sepharose (CSAT) or polyclonal anti–chicken β_1 serum (366). Immunoprecipitates were recovered either directly (CSAT) or indirectly using protein A-Sepharose (363, 366) and analyzed by SDS-PAGE under nonreducing conditions. B, a nondenatured extract of ^{125}I-surface-labeled clone Δ7E cells was immunoprecipitated using monoclonal anti–chicken β_1-Sepharose (CSAT). The recovered complexes were either analyzed directly (lane 1) or were denatured in SDS. After addition of Triton X-100, the extracts were then incubated with anti-β_1 cytoplasmic domain serum (363), anti–chicken β_1 serum (366) or anti-α_5 peptide serum (α_5), followed by immunoprecipitation using protein A-Sepharose and analysis by SDS-PAGE under nonreduced conditions. The truncated chicken β_1 subunit is expressed on the surface and forms heterodimers with the endogenous mouse α subunits.

fibronectin affinity columns (Fig. 8) are, by definition, both surface located and functional in ligand binding. Quantitation showed that 25.4% of the β_1 integrin subunits eluted from the FN columns are avian and 74.6% are murine. This should be compared with a proportion of 40–60% avian β_1-containing integrins in 1E cells expressing the intact avian β_1 subunit. Comparison of the pattern of avian integrins in 1E cells (Fig. 9 A) with that in Δ7E cells (Fig. 9 C) shows clearly that the hybrid heterodimers in Δ7E cells do not localize in focal contacts in anywhere near the proportions detected by binding to FN columns. We cannot rule out a small fraction localizing in focal contacts, but it is clear that the truncated avian β_1 subunit, while largely competent to form hybrid heterodimers that are exported to the surface and will bind ligand, is severely compromised in its ability to assemble into focal contacts.

Discussion

The experiments reported here show that an exogenous avian integrin β_1 subunit can be functionally expressed in mouse 3T3 cells. We have also observed successful expression in rat, hamster, and monkey cells (Guan and Marcantonio, unpublished data). When expressed in heterologous cells, the exogenous β_1 subunit forms heterodimers with endogenous α subunits. These hybrid integrins can bind directly to fibronectin, are exported efficiently to the cell surface and are correctly localized in focal contacts. These data suggest that the heterologous subunit participates in the formation of fully functional integrins capable of interaction with molecules of both the extracellular matrix and the cytoskeleton. Thus, the high degree of sequence conservation between

avian and mammalian β subunits (DeSimone and Hynes, 1988) is reflected in conservation of function.

This conclusion focuses attention on segments of the β_1 sequence that are most highly conserved. One of these is the cytoplasmic domain that is virtually identical in avian, human, frog (DeSimone and Hynes, 1988), and also murine integrins (DeSimone, D., V. Patel, H. F. Lodish, and R. O. Hynes, unpublished data). The cytoplasmic domain is thought to interact with elements of the cytoskeleton. Avian integrin has been shown to bind to the cytoskeletal protein, talin, in equilibrium gel filtration experiments (Horwitz et al., 1986). This binding is competed by synthetic peptides containing the consensus tyrosine kinase phosphorylation site of the avian β_1 cytoplasmic domain (Tapley et al., 1989). These results implicate the β_1 cytoplasmic domain in interactions with the cytoskeleton.

The behavior of the mutant form of avian β_1 subunit that we have expressed is consistent with this supposition. The truncated form lacking a β_1 cytoplasmic domain is efficiently expressed and exported to the cell surface. It is found in heterodimers with endogenous α subunits that are still competent to bind fibronectin. These results suggest that the β_1 cytoplasmic domain plays only a minor role, if any, in dimerization, processing or binding to the extracellular matrix, although we do not rule out subtle effects on affinity. In contrast, the mutant heterodimer fails to localize normally in focal contacts where the cytoskeleton is associated with the ventral membrane of cells (Fig. 9). Therefore, it appears that the β_1 cytoplasmic domain is indeed involved in interaction with the cytoskeleton. Furthermore, it appears that interaction with the extracellular matrix may not be sufficient to maintain integrins in focal contacts. Several recent papers have shown that the nature of the external ligand plays a key role in or-
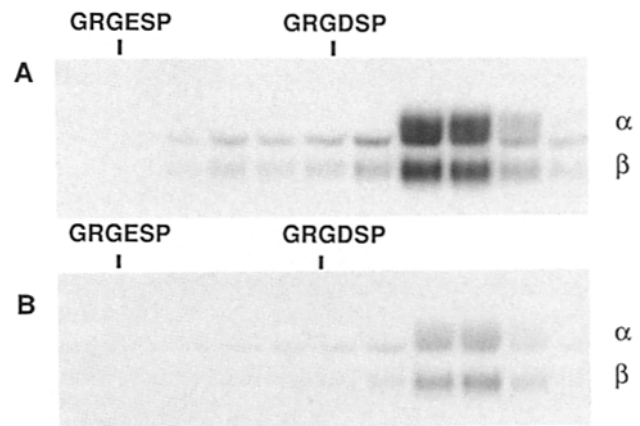

Figure 8. Binding of mutant integrin to fibronectin. Clone Δ7E cells were labeled with ^{125}I and extracts were prepared as described in Materials and Methods. 1 ml of extract was incubated with 1 ml of 120-kD fibronectin cell-binding fragment Sepharose for 1 h at 4°C. After washing, the column was sequentially eluted using GRGESP and GRGDSP as indicated at the top of A and B. 0.5-ml fractions were collected and 100-μl aliquots were immunoprecipitated with 363 antiserum (A) or 366 antiserum (B) as described in Materials and Methods. Both the endogenous mouse (A) and truncated chicken β_1-mouse hybrid (B) integrin complexes bind to fragments of fibronectin and are specifically eluted with GRGDSP.
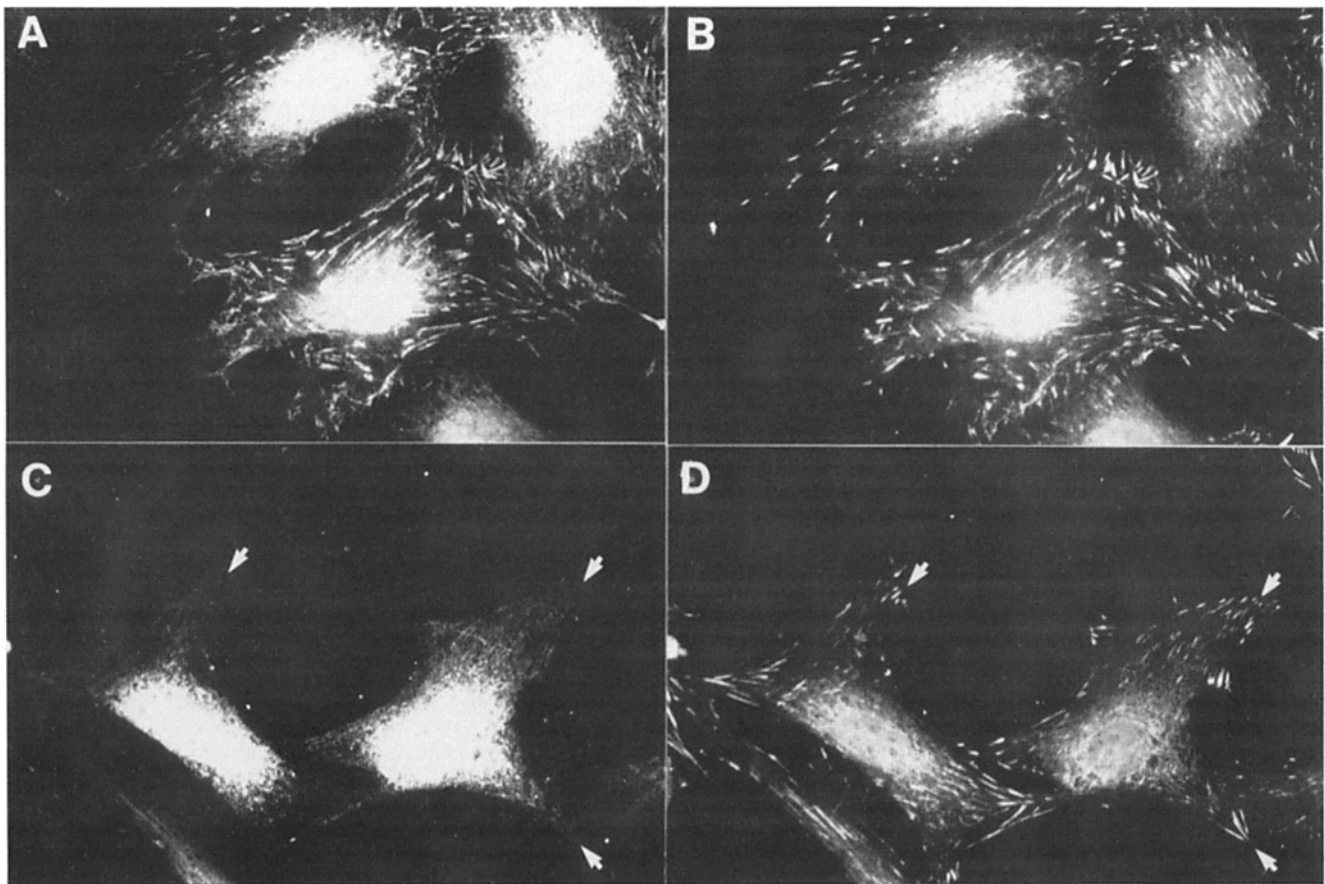
Figure 9. Double label immunofluorescence of clone 1E expressing full length avian integrin β_1 subunit (*A* and *B*) and clone Δ7E cells expressing truncated avian β_1 subunit (*B* and *C*). Cells were stained with mixtures of mouse antivinculin antibody (*B* and *D*) and rabbit anti–chicken β_1 serum (*A* and *C*) followed by visualization using rhodamine-conjugated goat anti–rabbit IgG and fluorescein-conjugated goat anti–mouse IgG. The vinculin stain marks the focal contacts some of which are indicated by arrowheads. The intact avian integrin colocalizes with vinculin (*A* and *B*) while the truncated avian integrin does not (*C* and *D*). In both cell types, murine integrins do colocalize with the vinculin (data not shown).

ganizing specific integrins into these structures (Singer et al., 1988; Dejanna et al., 1988; Albelda et al., 1989). Our data suggest that, in addition, interaction of the cytoplasmic domain with the cytoskeleton or some other cytoplasmic component is necessary for correct localization and/or maintenance of the integrins in focal contacts.

Photobleaching and recovery experiments (Duband et al., 1988) have demonstrated that integrins within focal contacts are extremely stable and replaced slowly. In contrast, those found outside the focal contact appear more mobile within the cell membrane. The data suggest a simple model in which the integrins exist within the plasma membrane as free heterodimers that undergo a conformational change upon occupancy by an extracellular ligand. This change favors the interaction of the receptor with the cytoskeleton. Once adhesion has been initiated, this interaction can lead to the stabilization of integrins within the focal contact or to the recruitment of more receptors into the region of the extracellular matrix. Presumably the deletion we have studied interferes with some step in this pathway, be it propagation of a signal, a conformational change or interaction with the cytoskeletal complex. More subtle alterations in the cytoplasmic domain (and elsewhere) of both α and β subunits will be necessary to elucidate these details. Such experiments are now in progress.

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