Cytoplasmic and Transmembrane Domains of Integrin β_1 and β_3 Subunits Are Functionally Interchangeable

Joanna Solowska,* Jonathan M. Edelman,*‡ Steven M. Albelda,*‡ and Clayton A. Buck*

*The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104; and ‡Pulmonary and Critical Care Section, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Abstract. Integrin β subunits combine with specific sets of α subunits to form functional adhesion receptors. The structure and binding properties of integrins suggest the presence of domains controlling at least three major functions: subunit association, ligand binding, and cytoskeletal interactions. To more carefully define structure/function relationships, a cDNA construct consisting of the extracellular domain of the avian β_1 subunit and the cytoplasmic and transmembrane domains of the human β_3 subunit was prepared and expressed in murine 3T3 cells. The resulting chimeric $\beta_{1/3}$ subunit formed heterodimers with α subunits from the β_1 subfamily, could not interact with α_{IIb} from the β_3 subfamily, was targeted to focal contacts, and formed functional complexes within the focal contacts. A second cDNA construct was prepared that coded for an avian β_1 subunit without a transmembrane or cytoplasmic domain. This subunit was not found in association with an accompanying α subunit, nor was it found expressed on the cell surface. Instead, it accumulated in vesicles within the cytoplasm and was eventually shed from the cell. The results from studies of the behavior of these two cDNA constructs demonstrate that the transmembrane and cytoplasmic domains play no role in α subunit selection, that the cytoplasmic domain of β_3 is capable of functioning in the context of α subunits with which it is not normally paired, and that both integrin subunits must be membrane associated for normal assembly and transport to cell surface adhesive structures.

The integrins are a family of molecules mediating both cell–cell and cell–substratum adhesion (reviewed in Buck and Horwitz, 1987; Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Hemler, 1990). Structurally, all integrins are similar. They are heterodimers consisting of a single β subunit that, in most cases, associates with several different α subunits. It is the $\alpha\beta$ complex that determines the properties of any particular integrin. For example, $\alpha_1\beta_1$ is a promiscuous receptor shown to mediate cell adhesion to type IV collagen and laminin (Kramer and Marks, 1989; Ignatius and Reichardt, 1988; Ignatius et al., 1990) while $\alpha_5\beta_1$ binds exclusively to the cell-binding domain of fibronectin (Pytela et al., 1985). Both the α and β subunits are transmembrane molecules having relatively large extracellular domains, typical transmembrane domains, and with the exception of β_4, fairly short (<50 amino acids) intracellular carboxyl terminal domains (Hemler, 1990). Direct and specific binding of integrins with both extracellular matrix molecules (see above reviews) and cytoskeletal-associated molecules has been demonstrated (Horwitz et al., 1986; Otey et al., 1990). This binding requires the association of both subunits, as neither by itself can bind fibronectin, laminin, or the cytoskeletal-associated molecule talin (Buck et al., 1986).

The structure and binding properties of integrins suggest the presence of domains controlling at least three major functions: subunit association, ligand binding, and cytoskeletal interactions. A careful genetic and biochemical analysis of the extracellular domain of β subunits has led to the identification of regions of the β_3 subunit involved in ligand binding (D'Souza et al., 1988; Smith and Cheresh, 1988) and divalent cation association (Loftus et al., 1990). Similarly, analysis of naturally occurring mutations of the β_2 subunit in patients suffering from leukocyte adhesion deficiency (LAD)[1], a disease in which the response of circulating white cells to infection is greatly compromised (Anderson and Springer, 1987), has shown that point mutations involving Leu^{149}, Gly^{169} (Wardlaw et al., 1990), or mutations leading to the deletion of a 30 amino acid region between Lys^{332} and Asn^{361} (Kishimoto et al., 1989) result in either a total loss of the ability of the affected β subunit to associate with the appropriate α subunits, or a greatly reduced efficiency in heterodimer formation.

The function of the cytoplasmic domain of integrins has been examined by in vitro binding experiments and by the analysis of β_1 cDNA constructs containing deletions in all or part of the cytoplasmic domain. Equilibrium gel filtration experiments have shown that integrins are capable of binding the cytoskeletal-associated protein talin (Horwitz et al., 1986). This talin–integrin interaction requires the presence

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[1] *Abbreviations used in this paper*: CHII, Chickie II; LAD, leukocyte adhesion deficiency; LAM, laminin.

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of both the α and β subunits (Buck et al., 1986) and is prevented by the presence of excessive quantities of a peptide corresponding to portions of the cytoplasmic domain of the β_1 subunit. More recently, Otey et al. (1990) have demonstrated the binding of α-actinin to β_1 cytoplasmic peptides. Expression of cDNA constructs of avian β_1 subunits in murine cells have shown that subunits missing various portions of the cytoplasmic domain either fail to participate in substratum adhesion (Solowska et al., 1989; Hayashi et al., 1990) or participate weakly, depending upon which portion of the cytoplasmic domain is deleted (Marcantonio et al., 1990). Interestingly, this latter study has shown that the presence of the transmembrane domain plus the next 10 amino acids is sufficient for the subunit to regain some of its ability to participate in focal contact formation. In the presence of the next 24 amino acids, however, function is lost and not recovered until nearly the entire remaining cytoplasmic domain is replaced. These observations are particularly interesting in light of the discovery of β_1 and β_3 mRNA coding for subunits with alternative cytoplasmic domains (Altruda et al., 1990; Van Kuppenvelt et al., 1989). In both cases, the affected cytoplasmic domains are shorter by 9 to 13 amino acids and have altered carboxy terminal sequences.

Understanding the functional significance of changes in subunit structure involving truncations or deletions of amino acid sequences can be difficult because of possible changes in secondary or tertiary structure of the molecule. As an alternative, we have begun structure–function analysis of the cytoplasmic and transmembrane domains of chimeric integrin β_1 and β_3 subunits. This approach takes advantage of the fact that the overall structure of the β_1 and β_3 subunits is highly conserved (Tamkun et al., 1986; Zimrin et al., 1988). These two β subunits differ only slightly in size with the mature β_1 subunit consisting of 779 amino acids and the mature β_3 subunit consisting of 762 amino acids. They have an overall amino acid homology of $\sim$47% with certain regions having homologies up to 83%. The positions of the 56-cysteine residues are completely conserved in both subunits, with the majority being located in four cysteine-rich repeating units in the extracellular domain. The cytoplasmic domains, located at the carboxyl terminus of each subunit, consist of 41 to 47 amino acids (depending upon the theoretical length of the transmembrane domain) and have an amino acid homology of 59 to 72%, depending upon whether or not conservative amino acid substitutions are considered. These two subunits share certain functional characteristics. Both bind to cytoskeletal-associated molecules as well as to extracellular molecules. When associated with the proper α subunits, both are able to localize into focal contacts (Albelda et al., 1989), and they can both combine with the α_v subunit (Vogel et al., 1990). However, there are certain important differences. First, despite their close homology, the cytoplasmic domains are serologically distinct, suggesting that there may be structural differences that could affect their function. Second, when present in the same cell, for example, endothelial cells, the β_3 subunit is not found in combination with α subunits from the β_1 subfamily (Albelda et al., 1989). Thus, it is theoretically possible to take advantage of the structural similarities, as well as functional differences, to search for various functional domains by constructing chimeric β subunits.

Because of the functional and structural homologies between the cytoplasmic domains of the β_1 and β_3 subunits, we examined the properties of a chimeric β subunit consisting of the entire extracellular domain of the avian β_1 subunit coupled to the cytoplasmic plus transmembrane domains of the human β_3 subunit. These studies were initiated to determine if functional chimeric subunits can be produced, if the obvious structural homologies translate to functional homologies and to determine what role, if any, the transmembrane and cytoplasmic domains play in subunit association, receptor function, and receptor processing. The role of the transmembrane region is of particular interest since the specificity of the T cell receptor and CD3 association (Manolios et al., 1990), as well as glycophorin aggregation (Bormann et al., 1989), is determined by the structure of the transmembrane domain of these molecules.

Materials and Methods

Site-directed Mutagenesis and Plasmid Construction

We have previously described the construction and expression of the full-length cDNA clone for avian β_1 (Solowska et al., 1989). The full-length cDNA clone for the human β_3 subunit was generously provided by Drs. Morty Poncz and Joel Bennett of the University of Pennsylvania (Philadelphia, PA) (Zimrin et al., 1988). To exchange cDNA fragments for the construction of chimeric cDNA, unique EcoRV restriction sites located between the extracellular and transmembrane regions of chicken β_1 cDNA and human β3 cDNA were created using synthetic mutagenic primers and the Muta-Gene in vitro mutagenesis kit (Bio-Rad Laboratories, Richmond, CA). In both cDNAs, the cytosine nucleotide in the GAC triplet (codon 733 in β_1 cDNA and codon 718 in β_3 cDNA) was replaced by thymidine. These are "silent" mutations since both the GAC and GAT codons code for the amino acid asparagine. The avian β_1 and human β_3 cDNAs with these silent mutations were then inserted into the HindIII cloning site of the SV-40 expression vector pESP-SVTEX (Reddy and Rao, 1986) by blunt-end ligation. The resulting plasmids were designated pCINTβ_1 RV and pHINTβ_3 RV. After digestion with EcoRV and XhoI, fragments containing avian β_1 extracellular region in the SV-40 expression vector and the human β_3 transmembrane and cytoplasmic regions were isolated by agarose gel electrophoresis and ligated to create a chimeric $\beta_{1/3}$ cDNA designated pCHINT$\beta_{1/3}$ RV.

To generate the plasmid pCHINT$\beta_1\Delta$733-803 coding for the mutated chicken integrin β_1 lacking its transmembrane and cytoplasmic domain, the pCINTβ_1 RV DNA was digested with EcoRV and then religated in the presence of a suppressible reading frame termination linker (SMURF; Pharmacia LKB Biotechnology Inc., Piscataway, NJ) containing a new XbaI site. The plasmids containing the linker fragment were identified by the presence of an additional XbaI site.

Transfection of 3T3 Cells

NIH 3T3 cells maintained in DME with 10% FBS were transfected as described previously (Solowska et al., 1989). Briefly, cells ((1×10^5) plated the previous day in 100-mm dishes were cotransfected with 10 μg of pCHINT$\beta_{1/3}$ RV plasmid, 2 μg of pSV2-neo (Southern and Berg, 1982), and 10 μg of salmon sperm DNA as a calcium phosphate precipitate (Wigler et al., 1979). After 2 d, the cells were split 1/15 into DME with 10% FBS and 1 mg/ml Geneticin (G418; Gibco Laboratories; Grand Island, NY). Approximately 2 wk later, G418-resistant clones were picked and screened for truncated avian β_1 or chimeric $\beta_{1/3}$ protein synthesis by indirect immunofluorescence using an anti-avian β_1 subunit specific polyclonal antibody (see below). The positive clones were further enriched by limited dilution subcloning. Clone 4G expressing chimeric $\beta_{1/3}$ protein, and clone ΔTCM expressing β_1 protein lacking the transmembrane and cytoplasmic regions were used for further characterization.

Transfection of COS-7 Cells

Cotransfections were performed in COS-7 cells using standard calcium phosphate–DNA coprecipitation techniques (Sambrook et al., 1989). Confluent T75 flasks of COS-7 cells were split 1/15 and plated onto 100-mm petri dishes in DME with 10% FBS. After 24 h, the cells were transfected singly with either 6 μg of pHINTβ_3 RV, pCHINT$\beta_{1/3}$ RV, or the αIIb subunit cDNA inserted into the mammalian expression vector pMT2ADA (Bonthron et al., 1986) (kindly provided by Drs. Morty Poncz and Joel Bennett of the University of Pennsylvania) or cotransfected with the αIIb subunit cDNA plus either pHINTβ_3 RV or pCHINT$\beta_{1/3}$ RV. All transfectants were harvested for analysis 2 d after transfection.

Antibodies

The following polyclonal antisera were used: (*a*) Anti-rat GP140, raised in rabbits against purified rat integrin preparation (Albelda et al., 1989). This antibody is a "pan" anti-integrin antibody that reacts with β_1 integrins from most mammalian cells; (*b*) a polyclonal rabbit antibody raised against the avian β_1 integrins, Chickie II (CHII) (Damsky et al., 1985) that has minimal cross reactivity with mammalian integrins; (*c*) a polyclonal antiserum #8,275 raised in rabbits directed against the cytoplasmic region of the human β_3 subunit provided by Drs. A. Frelinger III and M. Ginsberg (Scripps Clinic, La Jolla, CA); (*d*) a polyclonal antisera raised against the carboxyl terminal 20 amino acids of the human α_3 subunit; and (*e*) a polyclonal antiserum raised against the carboxyl terminal 20 amino acids of the human α_5 subunit.

The following mAbs were used: (*a*) a rat mAb, GoH3, directed against the α_6 integrin subunit provided by Dr. Arnoud Sonnenberg (Central Laboratory of the Netherlands, Amsterdam) (Sonnenberg et al., 1988); (*b*) SSA6 (Brass et al., 1985), a mAb directed against the platelet glycoprotein IIIa, the β_3 subunit; and (*c*) B1B5 (Silver et al., 1987), a mAb directed against platelet glycoprotein IIb (αIIb). These latter two antibodies were provided by Drs. Joel Bennett and James Hoxie (University of Pennsylvania).

Immunofluorescence

Cells were plated in DME containing 10% FBS onto coverslips coated with human plasma fibronectin (10 μg/ml) to facilitate adhesion. After 24 h, cells were fixed for 20 min with 3% paraformaldehyde and permeabilized with an ice-cold solution of 0.15 M NaCl containing 0.5% NP-40 for 1 min. The coverslips were then incubated for 1 h at room temperature with a mixture of antibodies containing the polyclonal anti-avian integrin antibody (CHII) and a monoclonal anti-vinculin antibody. The cells were then washed and stained for 1 h at room temperature with fluorescein-conjugated goat anti-rabbit IgG and rhodamine conjugated goat anti-mouse IgG (Organon Teknica Corp., Malvern, PA). The cells were examined using a phase-epi-fluorescent microscope (Zeiss, Oberkochen, Germany) with 63× planachromat oil-immersion lens and photographed using TMAX 400 film (Eastman Kodak Co., Rochester, NY).

Radiolabeling

24 h after transfection, the COS cells were labeled metabolically with 200 μCi of ^{35}[S]methionine (New England Nuclear, Boston, MA) in methionine-free DME containing 10% dialyzed FBS. After 24 h of labeling, nonionic detergent extracts were prepared by treating the cells with 0.5 ml of 0.01 M Tris acetate, pH 8.0, 0.5% NP-40, 0.5 mM Ca^{2+} (TNC) with 2 mM PMSF. After pipetting on ice for 15 min, the extracts were centrifuged for 40 min at 12,000 rpm in a microcentrifuge. To surface label cells, intact monolayers in 75 cm^2 tissue culture flasks were washed with PBS and exposed sequentially to 100 U/ml of lactoperoxidase (Sigma Chemical Co., St. Louis, MO), 1 mCi of carrier-free ^{125}I (Amersham Corp., Arlington Heights, IL), and three 40-μl aliquots of 0.06% hydrogen peroxide. The cells were harvested and extracted as described above.

Immunoprecipitation

For immunoprecipitation, the nonionic detergent cell extracts were preadsorbed with Protein A-conjugated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) for 30 min at 4°C. 100 μl of this antigen solution was reacted with 50 μl of antibody solution for 18 h at 4°C. Immune complexes were adsorbed to Protein A-conjugated Sepharose beads for 1 h at 4°C and were washed five times with a buffer containing 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5% deoxycholate, and 0.1% SDS. The complexes were then eluted in electrophoresis sample buffer (62.5 mM Tris base, 2% SDS, 10% glycerol, pH 6.8). Samples were electrophoresed on 6% polyacrylamide gels under nonreducing conditions and processed for autoradiography as described previously (Laemmli, 1970; Albelda et al., 1989).

In some experiments, labeled chimeric receptor was prepared by passing ^{125}I-labeled nonionic detergent extracts of the 4G cell line (expressing the $\beta_{1/3}$ chimeric protein) over an antibody affinity column prepared by linking the anti-avian integrin polyclonal antibody (CHII) to Sepharose. The chimeric $\beta_{1/3}$ integrin complex that adhered to the column was recovered by elution with 50 mM diethylamine, pH 11.5, followed by immediate neutralization. After extensive dialysis against TNC, the column eluates were immunoprecipitated with anti-α and -β integrin subunit antibodies as described above.

To identify secreted integrin proteins, cells containing the truncated or intact integrin constructs were labeled with ^{35}[S]methionine overnight. The radioactive media was then discarded and replaced with fresh media. After 8 h, this media, containing the labeled, secreted proteins was removed and reacted with Protein A-conjugated Sepharose beads that had been preincubated with anti-chicken integrin (CHII) antibody. After overnight incubation, the beads were washed and processed as described above.

Antibody-mediated Inhibition of Adhesion

Nontissue culture plastic 24-well plates (Costar, Cambridge, MA) were coated with laminin (LAM) (20 μg/ml) as previously described (Albelda et al., 1989). To block nonspecific binding, DME containing 2% BSA was then added to each well for 1 h at 37°C. Cells were labeled overnight with 3[H]thymidine (1 μCi/ml medium), trypsinized, treated with soy bean trypsin inhibitor (Worthington Biochemical Corp., Freehold, NJ), resuspended in serum-free DME, and plated at a density of 4 × 10^4 cells per well in the presence of preimmune serum, anti-rat GP140 diluted 1/30, 200 μg of purified anti-avian integrin (CHII) immunoglobulin, or a mixture of anti-rat GP140 and CHII IgG. After 18 h at 37°C, the cells were washed twice in PBS and the attached cells lysed in 1 ml of a solution containing 1% SDS/0.5% Triton X-100. The lysed cells were transferred to scintillation vials and radioactivity was measured. The results were expressed as the percentage of the total number of cells that adhered. Each experimental point represents the average of four samples.

Results

Construction and Expression of the $\beta_{1/3}$ Chimeric Subunit

A chimeric $\beta_{1/3}$ subunit consisting of the complete extracellular domain of avian β_1 and the transmembrane and cytoplasmic domains of human β_3 was constructed as outlined in Materials and Methods. Expression of the chimeric subunit was evaluated by immunoprecipitating nonionic detergent extracts from ^{125}I-labeled transfected 3T3 cells using antibodies specific for either the avian β_1 subunit or the cytoplasmic domain of the human β_3 subunit. The immunoprecipitates were analyzed by SDS-PAGE. 3T3 cells transfected with the vector carrying the intact avian β_1 subunit (Solowska et al., 1989) were used as positive controls throughout these experiments. Material from cells transfected with chimeric cDNA and immunoprecipitated with avian β_1 specific antibodies (Fig. 1, lane *3*) behaved identically to material precipitated from 3T3 cells transfected with control avian β_1 cDNA upon SDS-PAGE analysis (Fig. 1, lane 2). Both immunoprecipitates contained bands characteristic of integrin αβ complexes. Control 3T3 cells contained no material which cross reacted with the anti-avian β_1 antibody used in these experiments (Fig. 1, lane *1*). When immunoprecipitations were repeated on the same extracts using an antibody specific for the cytoplasmic domain of the human β_3 subunit, no material was precipitated from the cells expressing the wild type avian β_1 subunit (Fig. 1, lane *4*), indicating that this antibody did not cross react with the cytoplasmic domain of the transfected avian β_1 subunit. In contrast, this antibody precipitated a heterodimer consisting of both α and β subunits from cells expressing the chi-

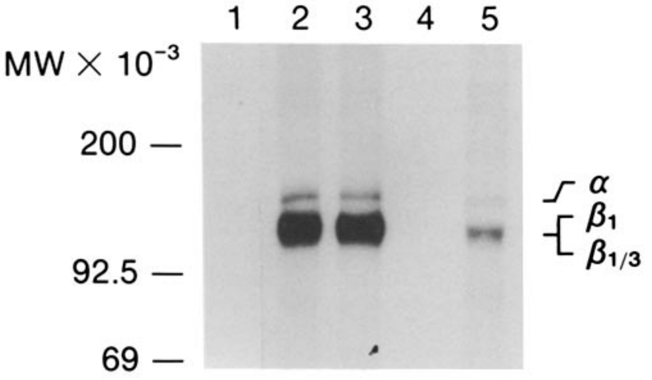

Figure 1. Expression of avian β_1 and chimeric $\beta_{1/3}$ subunits in transfected 3T3 cells. Immunoprecipitates of nonionic detergent extracts from transfected, ^{125}I-surface-labeled 3T3 cells were subjected to SDS-PAGE and autoradiography. Lane *1*, extract from control 3T3 cells immunoprecipitated with anti-avian β_1 polyclonal antibody CHII. Lane *2*, extract from 3T3 cells transfected with avian β_1 cDNA and immunoprecipitated with anti-avian β_1 polyclonal antibody CHII. Lane *3*, extract from 3T3 cells transfected with chimeric $\beta_{1/3}$ cDNA and immunoprecipitated with anti-avian β_1 polyclonal antibody CHII. Lane *4*, extract from cells transfected with avian β_1 cDNA and immunoprecipitated with anti-human β_3 cytoplasmic domain polyclonal antibody. Lane *5*, extract from cells transfected with chimeric $\beta_{1/3}$ cDNA and immunoprecipitated with anti-human β_3 cytoplasmic domain polyclonal antibody. Positions on gel where α subunits and β_1 as well as $\beta_{1/3}$ subunits migrate are designated in the right margin. Molecular weight markers in kD are on the left.

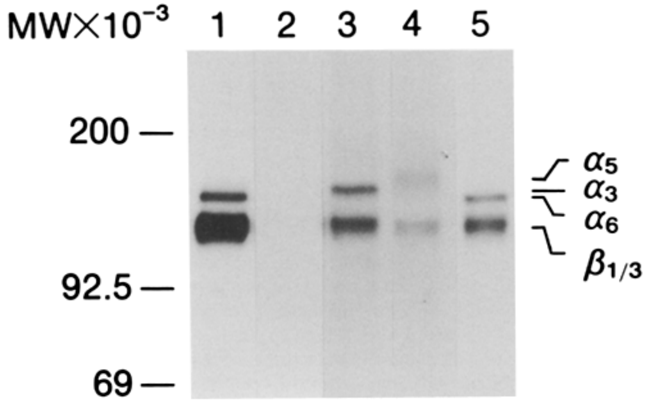

Figure 2. Association of chimeric $\beta_{1/3}$ subunit with α subunits of the mouse β_1 subfamily. An extract from ^{125}I-surface-labeled 3T3 cells transfected with $\beta_{1/3}$ cDNA was applied to a Sepharose affinity column prepared from anti-avian β_1 polyclonal antibody CHII. The eluted material was then immunoprecipitated with antibodies specific for various α subunits and the immunoprecipitates analyzed by SDS-PAGE and autoradiography. Lane *1*, extract immunoprecipitated with anti-avian β_1 polyclonal antibody CHII before affinity chromatography. Lane *2*, the same extract immunoprecipitated with polyclonal anti-avian β_1 antibody CHII after passage over the antibody affinity column. Material eluted from the affinity column was subsequently immunoprecipitated with: lane *3*, anti-α_3 polyclonal antibody; lane *4*, anti-α_5 polyclonal antibody; lane *5*, anti-α_6 mAb. The positions of the various subunits are designated in the right margin. Molecular weight standards are shown on the left in kD.

meric subunit (Fig. 1, lane *5*). Thus, the protein expressed in cells carrying the $\beta_{1/3}$ chimeric cDNA was indeed a chimeric protein containing the cytoplasmic domain of β_3.

The Chimeric $\beta_{1/3}$ Subunit Forms Heterodimers with Murine α Subunits of the β_1 Subfamily

To identify the α subunits with which the chimeric subunit was associated, ^{125}I-labeled extracts of the transfected cells were applied to an immunoglobulin affinity column prepared from the CHII antibody which reacts with proteins carrying all or part of the avian β_1 subunit. Bound material was eluted from the column and subsequently immunoprecipitated with polyclonal antibodies specific for the cytoplasmic domains of α_3 or α_5 or a monoclonal anti-α_6 antibody. Fig. 2, lane *1* shows that the immunoprecipitates obtained with anti-avian β_1 antiserum before affinity chromatography contained two bands, one in the 140-kD region of the gel typical of α subunits and one in the 120-kD region of the gel typical of β subunits. The immunoaffinity column isolated all integrins containing the chimeric $\beta_{1/3}$ subunit, as anti-avian β_1 antibodies precipitated no material from extracts that had been passed over the antibody affinity column (Fig. 2, lane *2*). Heterodimers containing the chimeric subunit in association with either murine α_3 (Fig. 2, lane *3*), α_5 (Fig. 2, lane *4*), or α_6 (Fig. 2, lane *5*) subunits could be immunoprecipitated from the column eluate using antibodies specific for each of these subunits. Immunoprecipitation with a mixture of antibodies to α_3, α_5, and α_6 resulted in nearly complete removal of all the integrins containing the chimeric subunit (not shown). Quantitation of these immunoprecipitates showed that >90% of the $\beta_{1/3}$ chimeric subunit expressed on the cell surface was associated with these three α subunits.

The Chimeric $\beta_{1/3}$ Subunit Does Not Associate with α_{IIb}

To determine if the chimeric subunit was capable of associating with the α_{IIb} subunit (which is found exclusively complexed with β_3), COS cells were cotransfected with cDNA coding for human α_{IIb} and either the human β_3 subunit or the chimeric $\beta_{1/3}$ subunit. After transfection, the cells were labeled with ^{35}[S]methionine and nonionic detergent extracts were immunoprecipitated with antibodies specific for the appropriate β subunit or for the cotransfected α_{IIb} subunit. SDS-PAGE analyses of the immunoprecipitates are shown in Fig. 3. Extracts of cells transfected with the human β_3 cDNA and immunoprecipitated with a monoclonal anti-β_3 antibody revealed a single band of radioactivity at ∼92 kD characteristic of the β_3 subunit (Fig. 3 *A*, lane *1*), accompanied by what appears to be a nonspecifically precipitated protein of ∼210 kD. COS cells alone contained no material which reacted with this antibody (Fig. 3 *A*, lane *2*). A 140-kD band of radioactivity plus some nonspecific peptides were immunoprecipitated from extracts of cells transfected with human α_{IIb} cDNA alone using antibodies specific for this α subunit (Fig. 3 *A*, lane *3*). Only nonspecifically reacting material was found in extracts of control COS cells immunoprecipitated with the same antibody (Fig. 3 *A*, lane *4*). When extracts from COS cells cotransfected with vectors carrying cDNA for the human β_3 and α_{IIb} subunits were immunoprecipitated with a mAb against the β_3 subunit, two proteins were noted (Fig. 3 *A*, lane *5*): a protein in the vicin-

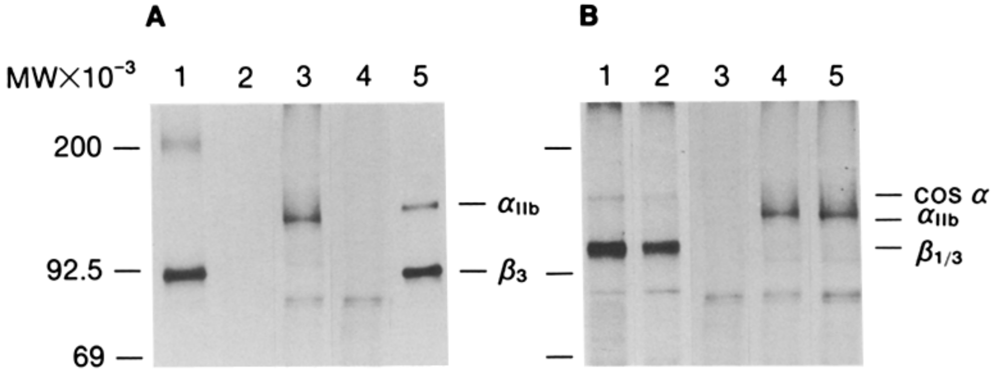

Figure 3. Inability of the chimeric $\beta_{1/3}$ subunit to combine with the human α_{IIb} subunit. Nonionic detergent extracts from ^{35}[S]methionine transfected and control COS cells were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. In *A*, lane *1*, extract from COS cells transfected with β_3 cDNA and immunoprecipitated with an anti-β_3 mAb; lane *2*, extract from control COS cells immunoprecipitated with the same anti-β_3 mAb; lane *3*, extract from COS cells transfected with human α_{IIb} cDNA and immunoprecipitated with an anti-human α_{IIb} mAb; lane *4*, control COS cells immunoprecipitated with the same anti-human α_{IIb} mAb; lane *5*, extract from COS cells cotransfected with human β_3 cDNA and human α_{IIb} cDNA, immunoprecipitated with the anti-human β_3 mAb. Note coprecipitation of both α_{IIb} and β_3 by the same antibody. In *B*, lane *1*, extract from COS cells transfected with chimeric $\beta_{1/3}$ cDNA immunoprecipitated with a polyclonal anti-avian β_1 antibody (*CHII*); lane *2*, extract from COS cells cotransfected with chimeric $\beta_{1/3}$ cDNA and human α_{IIb} cDNA immunoprecipitated with a polyclonal anti-avian β_1 antibody (*CHII*); lane *3*, control COS cells immunoprecipitated with the polyclonal anti-avian β_1 antibody CHII; lane *4*, extract from COS cells cotransfected with chimeric $\beta_{1/3}$ cDNA and human α_{IIb} cDNA immunoprecipitated with a monoclonal anti-α_{IIb} antibody; lane *5*, extract from COS cells transfected with α_{IIb} cDNA and immunoprecipitated with a monoclonal anti-α_{IIb} antibody. Positions of endogenous COS α subunits, the α_{IIb} and β_3 or chimeric $\beta_{1/3}$ subunits are designated in the right hand margins. Molecular weight standards in kD are on the left.

ity of the 92.5-kD marker corresponding to the β_3 subunit and a 140-kD band characteristic of the accompanying α_{IIb} subunit. Similarly, anti-α_{IIb} antibodies immunoprecipitated the same two proteins from extracts of these cells (data not shown). Thus, the transfected α_{IIb} subunit was capable of combining with the appropriate β_3 subunit if it was present in COS cells, and this complex could be immunoprecipitated with antibodies against either subunit.

In contrast, when COS cells were cotransfected with a vector carrying the cDNA coding for the $\beta_{1/3}$ chimeric subunit along with a vector carrying the cDNA for α_{IIb}, immunoprecipitates using antibodies reactive against the α_{IIb} subunit failed to coprecipitate any associated β subunit (Fig. 3 *B*, lane *4*). The anti-avian β subunit antibody precipitated the $\beta_{1/3}$ subunit in association with endogenous α subunits from extracts of cells transfected with the $\beta_{1/3}$ subunit alone (Fig. 3 *B*, lane *1*) or from cells cotransfected with both the $\beta_{1/3}$ subunit and the α_{IIb} subunit (Fig. 3 *B*, lane *2*). This antibody did not coprecipitate α_{IIb} from extracts of the cotransfected cells even though the subunit was readily detected by the anti-α_{IIb} antibody. Immunoprecipitates from extracts of control cells contained only nonspecifically precipitated material at ~80 kD (Fig. 3 *B*, lane *3*). Thus, unlike the native β_3 subunit, the $\beta_{1/3}$ chimeric subunit was not able to form heterodimers with α_{IIb} even though both subunits were present in the cell. These results, together with the results from transfected 3T3 cells, demonstrate that the chimeric $\beta_{1/3}$ subunit functions as a wild type β_1 subunit with respect to α subunit selection and that the β_3 transmembrane and cytoplasmic domains have no effect upon α subunit selection.

Chimeric $\beta_{1/3}$ Subunit Is Localized in Focal Contacts

The cellular distribution of the chimeric $\beta_{1/3}$ subunit was determined by immunofluorescence. Control and transfected 3T3 cells were plated on coverslips coated with fibronectin to hasten adhesion and spreading. 24 h later they were fixed and stained with a polyclonal antibody specific for the avian β_1 subunit. Control 3T3 cells contained no material that reacted with this antibody (Fig. 4 *A*) despite the fact that focal contacts were readily detectable in these same cells using an antibody specific for the cytoskeletal-associated molecule vinculin (Fig. 4 *B*). In contrast, double immunofluorescence staining of cells expressing the chimeric subunit showed that material reacting with the anti-avian β_1 antibody was found colocalized with vinculin in what appeared to be focal contacts on their ventral surface (Fig. 4, *C* and *D*). Thus, the chimeric subunit was targeted to adhesive structures in the transfected cells.

Chimeric $\beta_{1/3}$ Subunit Functions to Promote Adhesion in Transfected 3T3 Cells

To determine if the presence of integrins carrying the chimeric subunit in focal contacts contributes to the adhesive properties of transfected 3T3 cells, cells expressing the chimeric subunit were compared to control 3T3 cells with respect to their resistance to the adhesion disrupting activity of antibodies specific for the mammalian β_1 subunit. These experiments are similar to those of Hayashi et al. (1990) in which the presence of integrins carrying the avian β_1 subunit in focal contacts of transfected 3T3 cells was able to confer resistance to the adhesive disrupting effect of antibodies against the endogenous murine β subunit. Both control and transfected cells adhered and spread equally well on laminin coated surfaces (Fig. 5, *A* and *B*). Antibodies specific for the avian β_1 subunit had no effect on the adhesion of either transfected or control cells (Fig. 5, *C* and *D*), presumably because the focal contacts from both cells contained integrins with mammalian β_1 subunits that were not reactive with the anti-avian subunit antibodies. In contrast, the antibody reactive with the mammalian β_1 subunit prevented adhesion of up to 70% of the control 3T3 cells while having a much smaller effect upon the adhesion of transfected cells (Fig. 5, *E* and *F*). This difference was presumably because of the presence of integrins carrying a chimeric $\beta_{1/3}$

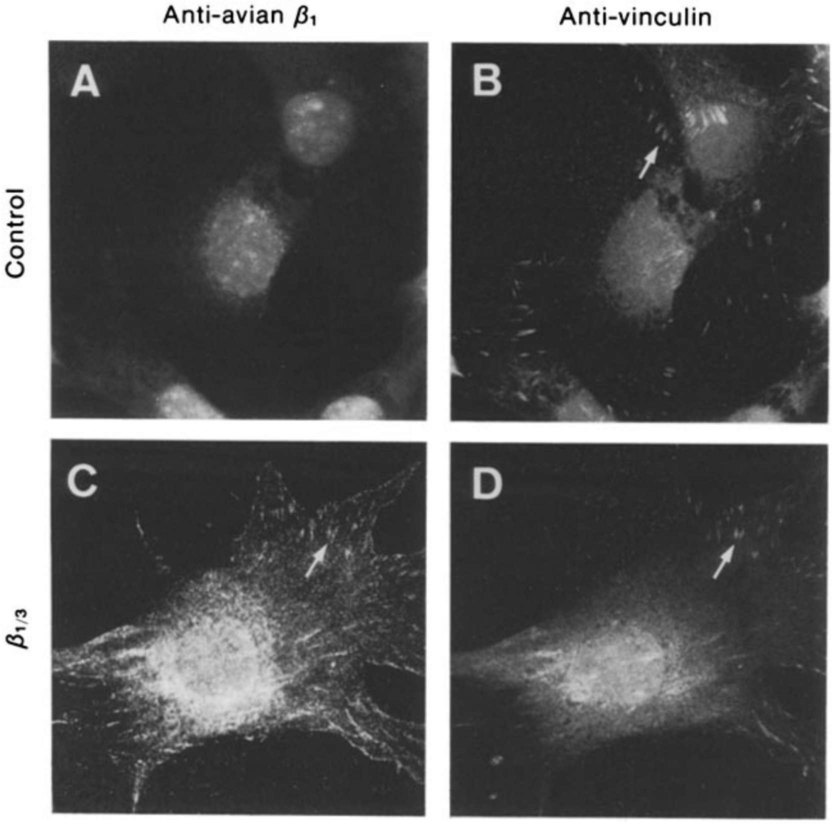

Figure 4. Localization of chimeric $\beta_{1/3}$ subunit in adhesion plaques. Control 3T3 cells (*A* and *B*) or 3T3 cells transfected with chimeric $\beta_{1/3}$ cDNA ($\beta_{1/3}$, *C*, and *D*) were plated on fibronectin coated coverslips and stained with a mixture of an anti-avian β_1 polyclonal antibody (*CHII*) and a monoclonal anti-vinculin antibody. The coverslips were counterstained using fluorescein-conjugated goat anti-rabbit IgG (*A* and *C*) and rhodamine conjugated goat anti-mouse IgG (*B* and *D*). Typical focal contacts are indicated by arrowheads.

subunit. This was confirmed by the fact that the combination of anti-avian β_1 and anti-mammalian integrin antibodies markedly diminished the adhesion of the transfected cells as well as that of the control 3T3 cells (Fig. 5, *G* and *H*). It would appear that not only does the chimeric β subunit serve to target integrins to focal contacts, but integrins carrying this subunit are able to participate in appropriate adhesive interactions of the cell.

Transmembrane Domain Is Required for Subunit Participation in Normal Integrin Maturation

The preceding results show that the cytoplasmic and transmembrane domains of β_1 and β_3 integrin subunits are interchangeable with respect to subunit association and function. The question arises, however, as to whether membrane insertion of both integrin subunits is required for heterodimer formation and receptor processing during the normal synthesis and transport of integrins to the cell surface or if membrane association of only one of the pair of subunits is sufficient. To address this, an avian β_1 subunit was constructed that was missing its transmembrane and cytoplasmic domains by inserting a "stop" codon just before the transmembrane domain. 3T3 clones expressing this construct were selected and either surface labeled with ^{125}I or metabolically labeled with ^{35}[S]methionine. Extracts of these cells or their culture media were immunoprecipitated with a polyclonal antiserum (CHII) specific for the avian β_1 subunit. The results are shown in Fig. 6. The SDS-PAGE profile of immunoprecipitates from 3T3 clones transfected with the intact β_1 subunit revealed three specific bands in the 100- to 140-kD regions of the gel (Fig. 6, *^{35}[S]Methionine Extract*, lane *1*). The material at $\sim$110 kD represents the β subunit precursor while the mature β subunit is visible just above it at 120 kD. The associated α subunits are seen in this case as a narrow band at $\sim$140 kD. The material at the bottom of the gel was the result of nonspecific reactivity as it is also found in nontransfected 3T3 cells (Fig. 6, *^{35}[S]Methionine Extract*, lane *3*). A similar analysis of extracts from a 3T3 clone expressing the β_1 subunit with the cytoplasmic and transmembrane domains deleted shows that the subunit was indeed synthesized and migrated on SDS-PAGE at a position just below that of the β_1 precursor (Fig. 6, *^{35}[S]Methionine Extract*, lane *2*). No similar material was present in immunoprecipitates from control, nontransfected 3T3 cells (Fig. 6, *^{35}[S]Methionine Extract*, lane *3*).

When these same clones were surface labeled with ^{125}I, however, no integrins containing the mutated β_1 subunit were detected on the cell surface (Fig. 6, *^{125}I Surface*, lane *2*) even though the intact avian β_1 subunit could be readily detected when expressed (Fig. 6, *^{125}I Surface*, lane *1*). In this case, the β subunit precursor was not iodinated (note the absence of a band at $\sim$110 kD), and there was no material on the surface of control 3T3 cells that reacted with the anti-avian β_1 antibody (Fig. 6, *^{125}I Surface*, lane *3*). Interestingly, when the media from the ^{35}[S]methionine cells was immunoprecipitated with the anti-avian β_1 integrin antibody, the mutated β subunit, lacking the transmembrane and cytoplasmic domains, was present at clearly detectable levels (Fig. 6, *[^{35}S]Methionine Conditioned Media*, lane *2*). No such material was present in the medium from 3T3 cells transfected with the intact β_1 subunit (Fig. 6, *[^{35}S]Methionine Conditioned Media*, lane *1*) or from nontransfected 3T3 cells (Fig. 6, *[^{35}S]Methionine Conditioned Media*, lane *3*).

Immunofluorescence studies of the cells containing the

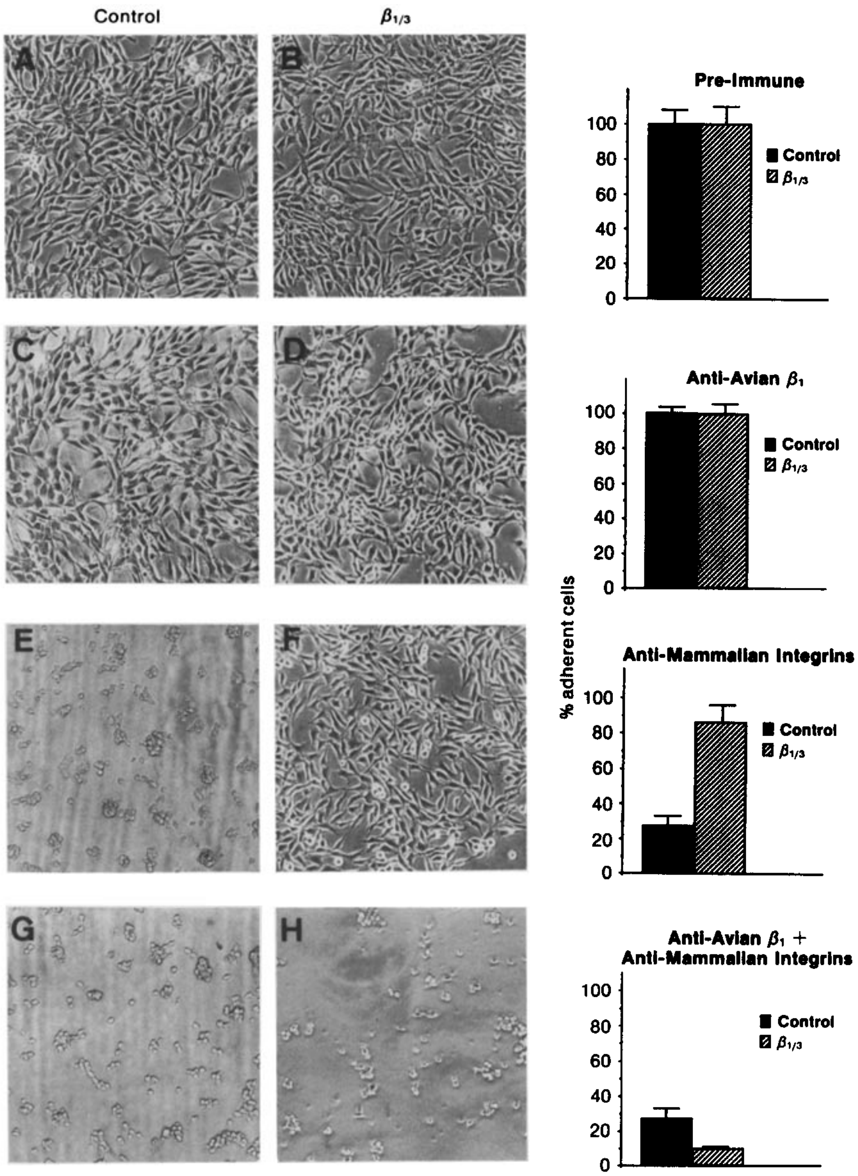

Figure 5. Effect of anti-integrin antibodies on the adhesion of control and transfected cells to laminin. ^{3}[H]thymidine control 3T3 cells (*3T3*; *A*, *C*, *E*, and *G*), or 3T3 cells expressing chimeric $\beta_{1/3}$ subunit (*B*, *D*, *F*, and *H*) were plated into laminin-coated wells either in the presence of pre-immune serum (*A* and *B*); anti-avian β_1 polyclonal antibody, CHII (*C* and *D*); anti-mammalian integrin polyclonal antibody (*E* and *F*); or a mixture of the two antibodies (*G* and *H*). After 18 h the cells were photographed, washed, the radioactivity of attached cells determined, and the percent of adherent cells calculated. The results are shown as bar graphs beside the photograph of the appropriate cell line. (■) control 3T3 cells; (▨) 3T3 cells expressing the $\beta_{1/3}$ chimera.

mutated β_1 subunit using antiserum specific for the avian β_1 subunit (Fig. 6, *lower panel*) revealed fluorescence dispersed, in what appear to be vesicles (*arrow*), throughout the cytoplasm of the cell with no sign of participation in focal contact formation. Thus, the β subunit unable to be inserted in the membrane was also not able to pair with membrane associated α subunits and participate in normal integrin biosynthesis. Interestingly, although heterodimer formation is required for normal integrin biosynthesis and expression on the cell surface, it is not required for the shedding of subunits from the cell.

Discussion

As an alternative to the introduction of mutations or deletions to identify integrin subunit functional domains, we have begun the construction of chimeric proteins by interchanging homologous portions of β_1 and β_3 subunits. This approach takes advantage of both the structural similarities, as well as the functional differences between these two proteins. Our initial chimeric integrin has been constructed by inserting the transmembrane plus cytoplasmic domains of the human β_3 subunit in place of the homologous regions of the avian β_1 subunit. These regions of each molecule were chosen because they contain no cysteine residues; hence, proper disulfide bond formation should not present a problem in producing a functional chimeric subunit. In addition, the cytoplasmic domains of the two molecules are serologically distinct, and with the exception of α_v, each functions in the context of different α subunits. Finally, such chimeric constructs allowed us to examine the importance of these regions of the molecule in subunit selection, as well as cytoskeletal organization.

The $\beta_{1/3}$ chimeric subunit behaved essentially as an intact β_1 subunit in its selection of α subunits. More than 90% of the surface expressed $\beta_{1/3}$ chimera was found by immunoprecipitation to be associated with either α_3, α_5, or α_6 in transfected 3T3 cells (Fig. 3). The remaining fraction was associated with an unidentified subunit, perhaps α_v, since it has been recently shown that α_v can combine with β_1 to

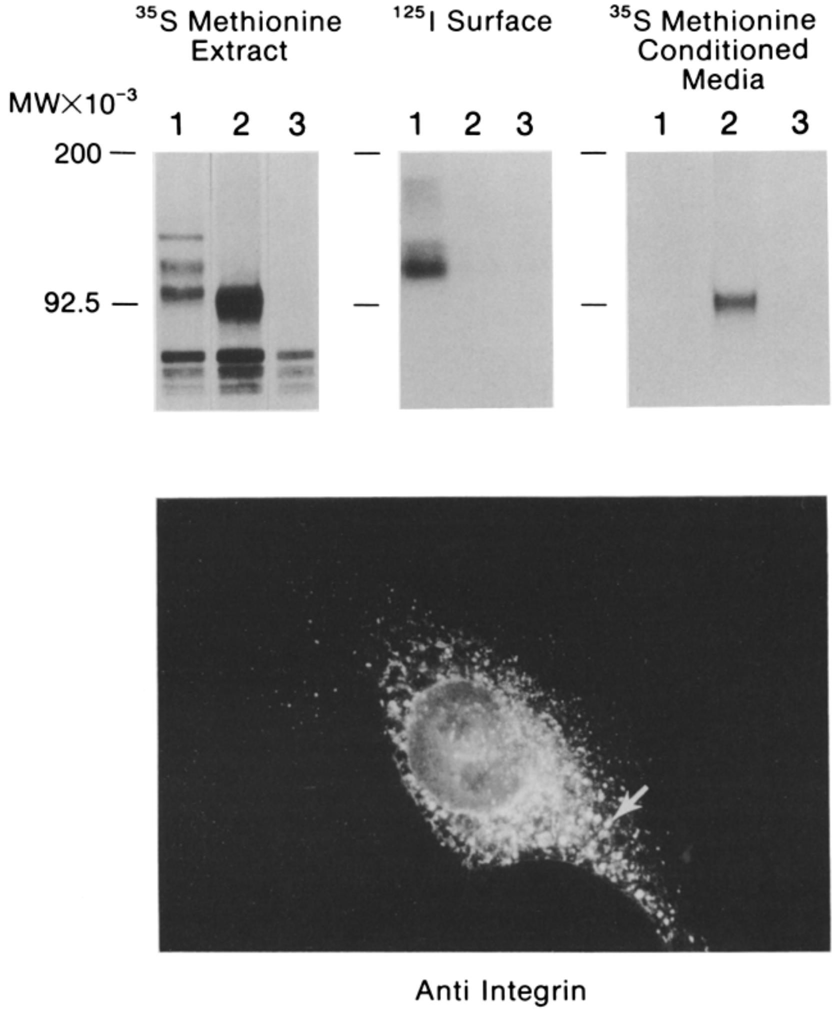
^{35}S Methionine Extract
^{125}I Surface
^{35}S Methionine Conditioned Media
MW×10^{-3}
1 2 3
1 2 3
1 2 3
200
92.5
Anti Integrin

Figure 6. Synthesis and distribution of a β_1 subunit missing its transmembrane and cytoplasmic domains. 3T3 cells were transfected with cDNA coding for either an intact avian β_1 subunit, or an avian β_1 subunit missing its transmembrane and cytoplasmic domain. An extract from cells labeled with ^{35}[S]methionine (*^{35}[S]methionine extract*) or ^{125}I (*^{125}I Surface*) or conditioned medium (*^{35}[S]methionine-conditioned media*) from each cell line was subjected to immunoprecipitation using polyclonal anti-avian β_1 antibody (*CHII*) and the results analyzed by SDS-PAGE autoradiography. In each panel, lane *1*, immunoprecipitates from cells expressing the intact β_1 subunit; lane *2*, immunoprecipitates from cells expressing the β_1 subunit missing its transmembrane and cytoplasmic domain; lane *3*, immunoprecipitates from control, nontransfected 3T3 cells. (Photograph) A typical 3T3 cell transfected with the mutated β_1 subunit stained with the polyclonal anti-avian β_1 antibody CHII and FITC-labeled goat anti–rabbit antiserum. Arrow designates a typical cytoplasmic vacuole.

form an integrin which binds to both fibronectin and vitronectin (Vogel et al., 1990). Even if the residual, unidentified α subunit found in the transfected 3T3 cells was α_v, it is doubtful that it was present in sufficient quantities to account for all the functional aspects of the chimeric subunit reported here.

The chimeric subunit did not associate with α_{IIb}, one of the two α subunits known to associate with β_3, when vectors carrying cDNA coding for both subunits were cotransfected into the same cells under conditions in which wild type β_3 could clearly associate with the cotransfected α_{IIb} subunit (Fig. 3). Thus, neither the β_3 transmembrane or cytoplasmic domain had any effect on the ability of the chimeric subunit to select appropriate α subunits. The domain(s) controlling α subunit selection must therefore reside entirely in the extracellular portion of the molecule.

The chimeric subunit was able to form fully functional integrins. Integrins containing the chimeric subunit could localize to focal contacts (Fig. 4) and were also able to render transfected 3T3 cells resistant to the adhesion disruptive properties of anti-mammalian integrin antibodies (Fig. 5). Both these properties required avian β subunits with functionally intact cytoplasmic domains, as avian subunits missing all or parts of specific regions of their cytoplasmic domains were unable to form focal contacts (Solowska et al., 1989; Hayashi et al., 1990) or to render the transfected cells resistant to anti-mammalian integrin antibodies (Hayashi et al., 1990; Solowska, J., unpublished observations). This suggests that the integrin binding site for cytoskeletal associated proteins may be "generic" in nature. That is, despite the fact that serological evidence suggests clear structural differences between the cytoplasmic domains of the β_1 and β_3 subunits, they are each able to form a functional cytoskeletal binding site in association with a variety of different α subunits. Such a binding site may be composed of regions of the cytoplasmic domains of both the α and β subunit. If this is the case, then at least two different β subunits are able to associate with nine different α subunits to form a protein pocket into which a common cytoskeletal associated molecule will fit. Consistent with this idea is the observation that α/β subunit association was shown to be required for talin binding to isolated integrins in solution (Buck et al., 1986). On the other hand, these data, and the fact that integrins having β subunits missing their cytoplasmic domain will not localize to focal contacts, are also consistent with a model in which only the cytoplasmic domain of the β subunit interacts with cytoskeletal elements required for positioning integrins into the appropriate adhesive structures. This model is supported by the observation that peptides from the cytoplasmic domain of the β_1 subunit are capable of binding the cytoskeletal-associated protein α-actinin (Otey et al., 1990). This latter example could be a special case involving only

α-actinin, suggesting the existence of multiple binding sites for cytoskeletal-associated proteins in the cytoplasmic domain of integrins. It will be important to determine the role of the α subunit cytoplasmic domains in this process before these alternatives can be evaluated effectively.

The data presented here demonstrate that the transmembrane domain of integrin β subunits is not a determining factor for α subunit selection. Neither is it required for subunit association (Dana et al., 1991). If both the α and β subunits are missing their transmembrane domain, they form heterodimers with one another and are shed as such from the cell. If, however, only the β subunit is missing its transmembrane domain, it is unable to combine with membrane associated α subunits and is instead shed from the cell (Fig. 6). Thus, there appears to be a separation of membrane-associated subunits from nonmembrane-associated subunits during the biosynthetic process making it impossible for those receptors not inserted in the membrane to pair with those that will eventually appear at the cell surface as mature, functional heterodimers.

Finally, the fact that individual β subunits can be constructed in a soluble form that is shed from the cell and the fact that such constructs can participate in heterodimer formation (Dana et al., 1991) suggests that it may be possible to quantitatively evaluate subunit association in vitro using readily available α and β subunits that are shed from the cell. It may also be possible to use such soluble subunits, either alone or in combination, to interfere with specific adhesive events for both experimental and therapeutic purposes. In this respect, it will be interesting to determine if α subunits can be shed from the cell in the absence of an accompanying β subunit.

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