Functional Role of the Cytoplasmic Domain of the Integrin α5 Subunit

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Abstract. The purpose of this study was to explore the functional role of the cytoplasmic domain of the α subunit of the α5/β1 integrin, a fibronectin receptor. Mutant CHO cells that express very low levels of endogenous hamster α5 subunit (CHO clone B2) were transfected with an expression vector containing full-length or truncated human α5 cDNAs to form chimeric human α5/hamster β1 integrins. Three transfectants were examined: B2a27 expresses a full-length human α5 subunit with 27 amino acids in the cytoplasmic domain; B2a10 expresses an α5 with a 17-amino acid cytoplasmic truncation; B2a1 expresses an α5 with a 26-amino acid truncation. Levels of α5/β1 surface expression in B2a27 and B2a10 cells were similar to that in wild type CHO cells. The expression of α5/β1 in B2a1 cells was less, amounting to 15–20% of WT levels, despite message levels that were three to five times greater than those of B2a27. The transfectants were used to examine the role of the α5 cytoplasmic domain in cell adhesion, cell motility, cytoskeletal organization, and integrin-mediated tyrosine phosphorylation.

The adhesion characteristics of B2a27 and B2a10 cells on fibronectin substrata were similar to each other and to wild type CHO cells. B2a1 cells displayed slight reductions in the strength and rate of adhesion to fibronectin. Cell motility in the presence of fibronectin was similar for B2a27, B2a10, and wild type CHO cells, while the B2a1 cells were substantially less motile. Comparable degrees of cell spreading and extensive organization of actin filaments were observed for B2a27, B2a10, and wild type CHO cells on fibronectin substrata. The B2a1 cells spread to a lesser degree, and some organization of actin was observed; the untransfected B2 cells remained round on fibronectin substrata and showed no actin reorganization. Since the reduced motility and cell spreading observed in the B2a1 cells might be due either to reduced surface expression of α5/β1 or to the truncation in the α5 cytoplasmic domain, we used flow cytometric cell sorting to select populations of B2a1 and B2a27 cells expressing similar levels of cell surface α5. The deficits in spreading and motility were present in B2a1 cells expressing high levels of α5. Thus the region of the α5 cytoplasmic domain adjacent to the membrane seems to play an important role in cytoskeletal organization and cell motility. We also examined whether α subunit truncation would affect integrin-mediated tyrosine phosphorylation. When B2a27 cells interacted with fibronectin substrata, increased tyrosine phosphorylation was observed in proteins of ~125 kD. A similar pattern of phosphorylation was observed in wild type CHO, B2a10, and B2a1 cells, but not in B2 cells. Thus, the α5 cytoplasmic domain does not seem to be essential for integrin-mediated tyrosine phosphorylation of intracellular proteins.

CELL interactions with the extracellular matrix are a vital aspect of embryogenesis, angiogenesis, the inflammatory response, tissue differentiation, and hemostasis. These cellular processes are mediated in part by the integrin family of cell surface glycoproteins (Buck and Horwitz, 1987; Hynes, 1987; Juliano, 1987). Integrins are formed by the noncovalent association of an α subunit with a β subunit; to date, at least 14 α and 8 β subunits have been described giving rise to at least 20 distinct integrin receptors (Dedhar, 1990; Rouslahti, 1991; Hynes, 1992). The largest subgrouping of integrin receptors are those that share the β1 subunit, including receptors for fibronectin, vitronectin, laminin, and collagen (Guan and Hynes, 1990; Sonnenberg et al., 1988; Santoro et al., 1988; Takada, 1987; Elices et al., 1991). Both the β1 subunit and its α partners have large extracellular domains, transmembrane-spanning regions, and short cytoplasmic domains. The extracellular domains of integrin α and β subunits cooperate to bind matrix proteins, while the cytoplasmic domains articulate with a number of cytoskeletal elements, including talin, vinculin, and α actinin (Burridge et al., 1988; Otey et al., 1990).

Much of the research on integrins to date has focused on the extracellular interactions of integrins with their ligands, while the role of cytoplasmic domains of integrins has only been explored to a limited degree. In an early study, Horwitz et al. (1986) demonstrated the association of the cytoplasmic

domain of the β1 subunit with talin, while more recently an interaction between the β1 subunit and α actinin has been found (Otey et al., 1990). Using murine cells expressing avian β1 constructs, Hayashi et al. (1990) and Marcantonio et al. (1990), independently demonstrated that truncating the cytoplasmic domain by five or more amino acids resulted in partial or complete loss of β1 integrin localization in focal contacts, while Solowska et al. (1989) showed that truncation of the β1 cytoplasmic domain does not prevent the in vitro binding between fibronectin and chimeric fibronectin receptor. Hibbs et al. (1991) expressed various constructs of the LFA-1 receptor (a member of the β2 subclass of integrin receptors) in COS cells, and demonstrated that truncation of the carboxy terminus of the β subunit diminished phorbol ester stimulation of LFA-1 binding to ICAM. The importance of the cytoplasmic domain of integrin β subunits has also been demonstrated in vivo; antibodies against the cytoplasmic domain of the β1 subunit injected intracellularly into developing amphibian embryos prevented extracellular fibronectin fibril formation and delayed embryo development (Darribére et al., 1990).

It is apparent from the studies cited above that the cytoplasmic domain of the β subunit contributes significantly to integrin function. Less information, however, is available on the participation of the cytoplasmic domain of the α subunit. Hibbs et al. (1991) demonstrated that partially truncating the cytoplasmic domain of the α subunit of LFA-1 had no effect on receptor function. Truncating the entire cytoplasmic domain of the α subunit, however, did inhibit expression of the receptor on the cell surface. O'Toole et al. (1991) produced various constructs of the platelet glycoprotein αII/β3, an integrin receptor that requires activation before binding; these were expressed in CHO cells. Interestingly, truncating the entire cytoplasmic domain of the α subunit of the platelet glycoprotein αII/β3 yielded a functional receptor that bound fibrinogen without activation. Recently, Chan et al. (1992) have explored the role of the α subunit cytoplasmic domain by preparing a series of chimeras having the α2 external and transmembrane domains coupled to the cytoplasmic domains of α2, α4, or α5. They found that the presence of the α4 cytoplasmic domain contributed to cell motility while the α2 or α5 cytoplasmic domains permitted collagen gel contraction.

It seems clear that cytoplasmic domains play an important role in integrin interactions with cytoskeletal proteins and in cellular activation of integrins. Recently, however, another potential role has been identified. Studies of human carcinoma cells (Kornberg et al., 1991), rodent fibroblasts (Guan and Hynes, 1991), and platelets (Golden et al., 1991) all suggest that integrins can mediate a signal transduction process resulting in phosphorylation of tyrosine residues on intracellular proteins. Integrin-mediated tyrosine phosphorylation seems to involve a novel 125-kD cytoplasmic tyrosine kinase (Kanner et al., 1990; Shaller et al., 1992; Hanks et al., 1992). Early evidence suggests that integrin β subunit cytoplasmic domains may be required in this process (Guan and Hynes, 1991). Whether integrin-mediated tyrosine phosphorylation relates to other aspects of adhesion-related signal transduction such as de novo gene expression (Haskell et al., 1988; Werb et al., 1989) remains to be determined.

To address the role of the α5 cytoplasmic domain in fibronectin receptor (FnR)[1] function, we have used an existing human α5 cDNA expression vector (Giancotti and Ruoslahti, 1990), to generate two constructs that give rise to α5 subunits with truncated cytoplasmic domains. One construct yields a protein with 17 amino acids deleted from the α5 cytoplasmic domain; the other essentially eliminates the entire cytoplasmic domain. These cDNAs were transfected into CHO cell α5/β1 FnR variants developed previously in our laboratory that fail to express significant levels of endogenous hamster α5 subunit (Schreiner et al., 1989). These FnR-deficient variants provide an elegant system to explore the contributions of normal or mutated transfected α5 subunits to α5/β1 FnR function.

Materials and Methods

Cell Culture

Wild type CHO cells, CHO 2% FnR variants (B2), and CHO 20% FnR variants (1-23), as well as the transfectants, were grown and maintained as previously described (Schreiner et al., 1989; Bauer et al., 1992). Cells were either grown as monolayers in α-MEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS (Irvine Scientific, Santa Ana, CA) and 1% antibiotic-antimycotic mixture (GIBCO BRL) or in suspension culture where the FBS was reduced to 5%. Cells grown in monolayer were routinely passaged with trypsin-EDTA (GIBCO BRL) and cell number was determined with an ElectroZone celloscope (Particle Data, Inc., Elmhurst, IL).

Extracellular Matrix Proteins

Fibronectin was prepared from human plasma as described (Schwarz et al., 1984). Vitronectin was also prepared from human plasma using a modification (Danilov and Juliano, 1989) of a previously published procedure (Ruoslahti et al., 1987).

Vector Construction

A eukaryotic expression vector containing a full-length human cDNA insert (pECE-α5) was a gift from Dr. E. Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA) (Giancotti and Ruoslahti, 1990). The pECE-α5/10 construct was generated by first digesting the original vector with restriction enzymes NdeI and XbaI, filling in the ends with the Klenow fragment of DNA polymerase 1, and religating the shortened vector. The pECE-α5/1 construct was generated by excising the section of DNA in the α5 insert within the HindIII and XbaI restriction sites. The pECE expression vector, however, also contained a HindIII site. Therefore, pECE-α5 was first partially digested with HindIII, filled in with the Klenow fragment of DNA polymerase I, and blunt-end ligated (clones containing only one HindIII site within the α5 sequence were identified by restriction screening). The modified vector was then further digested with HindIII and XbaI. The ends of the shortened vector were filled in with the Klenow fragment of DNA polymerase 1 and religated. Both constructs were ~0.3-kb shorter than the intact construct (3.6 kb). Each construct was verified by dideoxy sequencing using a 15-base primer 35 bases upstream of the truncated region. The pECE-α5/10 and pECE-α5/1 constructs gave rise to truncated α5 subunits expressing either a 10- or 1-amino acid cytoplasmic domain, respectively.

Transfection of FnR Deficient Variants

A FnR variant clone (B2) was chosen for expressing the α5 constructs; this clone expresses <2% of wild type levels of endogenous hamster α5/β1 (Schreiner et al., 1989). Cells were transfected with pECE-α5, pECE-α5/10, or pECE-α5/1. Cells were cotransfected with a neo-resistance plasmid (pSVNeo) for selection purposes. B2 cells were grown in monolayer, harvested with trypsin-EDTA and washed twice in ice cold electroporation media (PBS). The cells were then resuspended at a density of 1×10^7 cells/ml. Cells were then added to 0.4-cm electroporation Gene Pulser cu-

1. *Abbreviations used in this paper*: FnR, fibronectin receptor; RCF, relative centrifugal forces.

vettes (Bio-Rad, Richmond, CA) and 20 μg of pECE-α5 construct vectors and 2 μg of pSVNeo were added to the cuvettes and incubated with the cells on ice for 10 min before electroporation. All plasmids were linearized by digestion with PvuI restriction enzyme before addition to the cells to facilitate insertion of the plasmids into host chromosomal DNA. Cells were pulsed using voltages between 200 and 800 V with a constant capacitance of 25 μF. Cells were selected with G418 (1,000 μg/ml) and isolated using cloning rings. After cloning, transfectants were maintained in normally supplemented α-MEM containing 200 μg/ml G418. Cells were screened for expression of the chimeric human α5/hamster β1 FnR by ELISA and by flow cytometry using the BIE5 anti-human α5 antibody (generous gift of Dr. C. Damsky, University of California, San Francisco, San Francisco, CA) (Werb et al., 1989).

Integrin Analysis by Flow Cytometry

The expression of α5/β1 FnR, α5 subunit, VnR, and the integrin β1 subunit was evaluated by indirect immunofluorescence using flow cytometric techniques similar to those previously (Schreiner et al., 1989). PB1 (a mouse monoclonal anti-FnR antibody specific for the intact CHO α5/β1 FnR), 7E2 (a mouse mAb specific for the CHO β1 integrin subunit), BIE5 (a rat monoclonal anti-human α5 antibody), and rabbit polyclonal anti-VnR antibody (Suzuki et al., 1986) were used as primary antibodies, and FITC-conjugated IgG (anti-mouse, -rat, or -rabbit) was used as a secondary reagent. The PB1 and 7E2 monoclonals have been fully described elsewhere (Brown and Juliano, 1985, 1988). Background staining was assessed by omitting the primary antibody. The FnR-deficient cells were also screened by an ELISA technique as described (Schreiner et al., 1989) to confirm the phenotype. The FnR-deficient variants were periodically screened thereafter by ELISA using PB1 and BIE5 to ensure stability of the variant and mutant phenotypes.

Adhesion Assays

Adhesion assays using radiolabeled cells were conducted in tissue culture plates (48 well; Costar Corp., Cambridge, MA) coated with various amounts of fibronectin and subsequently blocked with bovine albumin. The assay procedure has been described in detail previously (Bauer et al., 1992; Schreiner et al., 1989). Adhesion assays were also conducted by reverse centrifugation to gauge the strength of adhesion of the modified receptors. These experiments were performed essentially as described by Lotz et al. (1989). Briefly, 96-well microtiter plates (3912 Microtiter Test III; Falcon Plastics, Cocheysville, MD) were coated as described above with 1 μg/ml Fn. Wells coated only with 3% BSA were used to determine background adhesion. Metabolically labeled cells (4×10^4) were added to the plates and the cells were then centrifuged onto the immobilized Fn substratum using a Beckman GP centrifuge at 17 *g* for 10 min at 4°C. The plates then were incubated at 37°C for 30 min while the cells were allowed to attach. The plates were then sealed with Costar plate sealers, inverted, and centrifuged at varying speeds corresponding to relative centrifugal forces (RCF) of 10, 50, 100, 500, and 1,000 *g* for 10 min. After centrifugation, the inverted plates were quick-frozen at −70°C. The bottoms of the plate wells were clipped and the radioactivity of the remaining cells was quantitated. Background adhesion of cells to BSA was subtracted from specific adhesion of cells to Fn. Assays were performed in quadruplicate.

Motility Assays

Cell motility experiments were performed essentially as described by Bauer et al. (1992) using Transwell (Costar Corp.) motility chambers. Various concentrations of fibronectin were coated on to the lower side of the Transwell filter insert before the start of the assay. In one case a "wounding" type motility assay was used. Cell layers in 24-well tissue culture plates were scraped with a sharp plastic blade, thus demarcating a line where cells were removed. The migration of cells across the line and into the denuded area was observed as a function of incubation time at 37°C using an inverted phase microscope with a gridded eyepiece.

Immunofluorescent Staining

Cultured cells were harvested by adding 0.05% trypsin and 1 mM EDTA for 5 min at room temperature. The trypsin was neutralized by washing the harvested cells three times in serum-free α-MEM containing 1% BSA and 1% penicillin/streptomycin. The cells were resuspended in this same medium and counted. Cells (1×10^3) were seeded onto 22 mm^2 microscope coverslips that were pre-coated with 100 μg/ml Fn. Cells were allowed to attach for 4 h at 37°C, and then washed in universal buffer (UB; 150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 0.1% NaN_3) and fixed in 3.7% formaldehyde. The cells were further washed in UB and permeabilized with 0.4% Triton X-100. The cells were stained with rhodamine-labeled phalloidin (specific for F-actin; Barak et al., 1980) (Molecular Probes, Inc., Junction City, OR) for 45 min at 37°C. Cells were again washed with UB, mounted onto slides with Gelvatol® and visualized using a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Immunoprecipitation

Equal numbers (1×10^7) of cells were surface labeled in 1 ml PBS by addition of lactoperoxidase (100 μg), 1 mCi $Na^{125}I$, and 10-μl aliquots of 0.03% H_2O_2 added each 3 min for 20 min at room temperature. Cells were washed 4× in PBS and lysed in 1 ml of 2% NP-40, 150 mM NaCl, 50 mM Tris, 1 mM $MgCl_2$, 0.01% aprotinin, 0.01% soybean trypsin inhibitor (pH 8.0) for 30 min on ice. Insoluble material was removed by centrifugation and lysates were stored at −80°C. 50 μl of each lysate was pre-cleared with 30 μl of a 50% suspension of protein A–conjugated Sepharose beads and 10 μl goat anti-mouse or goat anti-rat IgG for 2 h on ice. Aliquots (10 μl) of either PB1 (monoclonal mouse anti-hamster FnR antibody) or of BIE5 (monoclonal rat anti-human α5 subunit antibody) were added to the precleared lysates and allowed to incubate overnight on ice. The immune complexes were precipitated by addition of either 10 μl goat anti-mouse or 10 μl goat anti-rat IgG and 30 μl of 50% protein A–conjugated Sepharose beads for 2 h on ice. The precipitates were washed 3× in a buffer containing 500 mM NaCl, 50 mM Tris, pH 8.0, 10 mM EDTA, 1 mg/ml BSA, 0.025% NaN_3, and 0.5% NP-40. The precipitates were then washed 2× in a buffer containing 150 mM NaCl, 50 mM Tris, pH 8.0, 10 mM EDTA, 1 mg/ml BSA, 0.025% NaN_3, and 0.5% NP-40. Finally, the precipitates were transferred to new microfuge tubes and washed 1× in a buffer containing 150 mM NaCl, 50 mM Tris, pH 8.0, 10 mM EDTA, 0.025% NaN_3, and 0.5% NP-40. The precipitates were eluted by boiling 3 min in SDS sample buffer and analyzed by PAGE under nonreducing conditions. Immunoprecipitates were visualized by autoradiography using X-Omat AR film (Eastman Kodak Co., Rochester, NY). Comparisons of band intensities were made by densitometry; molecular weights of α5 subunits were determined by comparing the relative migrations of the α5 bands on the gel to the relative migrations of the molecular weight markers. Immunoprecipitations were also performed using a rabbit polyclonal anti-human α5 cytoplasmic domain (antibody 161, graciously provided by Dr. R. O. Hynes, Massachusetts Institute of Technology, Cambridge, MA) (Hynes et al., 1989). Antibody 161 cross-reacts with mouse, rat, chicken, and hamster α5 cytoplasmic domains.

Analysis of Protein Tyrosine Phosphorylation

Changes in the levels of phosphotyrosyl residues subsequent to interactions of α5/β1 with its ligand were assessed by using Western blots probed with anti-phosphotyrosine antibody (Kornberg et al., 1991). CHO cells were dispersed using trypsin-EDTA and washed extensively with 1% BSA in α-MEM; cells were maintained in suspension and allowed to recover from trypsinization for 2 h before plating. Cells were then plated on 60-mm tissue culture plates previously coated with 10 μg/ml fibronectin; the cells were incubated for 90 min at 37°C. Control cells were maintained in suspension throughout this period. After the incubation, the cells were washed in PBS and lysed in ice-cold RIPA buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 0.1% aprotinin, 1 mM PMSF, 100 μM Na_3VO_4). The extracts were incubated with 20-μl packed protein A–Sepharose and then centrifuged at 10,000 *g* for 10 min. Total protein in the supernatant was measured using the BCA assay (Pierce Chemical Co., Rockford, IL). Equal amounts of protein were electrophoresed under reducing conditions on 8% denaturing polyacrylamide gels (Laemmli, 1970). The resolved proteins were transferred to nitrocellulose as described (McCune and Earp, 1989) and the blots were pre-blocked by overnight incubation in 3% BSA. Phosphotyrosyl-containing proteins were detected using the anti-phosphotyrosine antibody PT66 (Sigma Immunochemicals, St. Louis, MO). Blots were incubated for 3–4 h in the presence of a 1:2,000 dilution of PT66 and then washed; the blots were then incubated for 1 h in the presence of 2 μg/ml rabbit anti-mouse IgG and then rewashed. Finally, the blots were incubated with 2 μCi/10 ml ^{125}I-protein A (New England Nuclear, Boston, MA) and washed extensively. Radioactive bands were detected by autoradiography; film exposures using Kodak

X-OMat film were generally 1–4 d. The specificity of the anti-phosphotyrosyl antibody for P-tyrosine was confirmed by adding 1 mg/ml phenyl phosphate or phosphoserine during the incubation with primary antibody; the phenyl phosphate abolished the labeling pattern while the phosphoserine had no effect.

Evaluation of Cells Expressing Similar Levels of Normal or Truncated α5

B2a1 cells or B2a27 cells were labeled with B1E5 monoclonal followed by fluorescein anti-rat polyclonal, as described above. The B2a27 population was analyzed for α5 expression by flow cytometry, and gates were chosen to include the main peak of fluorescence intensity. The labeled B2a1 cells were sorted using the gates set from the B2a27 population. A small population of high α5 expressors was selected from the B2a1 population (this population is termed B2a1H); the residual B2a1 cells comprised a low expressing population (termed B2a1L). Cell populations were collected in α-MEM plus 1% BSA and were maintained at 4°C. Light scatter characteristics were used to discriminate dead cells and to remove them from the sort. After the sorting runs, which lasted ~3 h, the level of α5 expression in the positively sorted B2a1 population (B2a1H) was analyzed and compared to the B2a27 population. The labeled, sorted, B2a27 and B2a1 populations were briefly treated with trypsin-EDTA, rinsed in serum-containing medium, and then placed in suspension culture for 1 h at 37°C to regenerate cell surface integrins (Sczekan and Juliano, 1990). Thereafter, the cells were suspended in α-MEM plus 1% bovine albumin and plated on to 24-well tissue culture plates that had previously been coated with 10 μg/ml of fibronectin. Cell morphology was observed and photographed using a phase contrast photomicroscope after 2 or 24 h of incubation at 37°C. Alternatively, cell motility was evaluated using a cell layer "wounding" assay, as described above.

Results

Preparation of Integrin α5 Subunits with Truncated Cytoplasmic Domains

Vectors capable of expressing truncated α5 subunits were constructed by deleting segments of DNA that corresponded to specific regions of the cytoplasmic domain from an α5 containing expression vector (pECE-α5). The new cDNA constructs were sequenced through the altered regions by Sanger's dideoxy chain termination technique (data not shown). Translation of the truncated α5 constructs was terminated by stop codons present within the vector. The intact α5 contains a 27 amino acid cytoplasmic domain. Two constructs were generated; pECE-α5/10, which encodes an α5 subunit with the cytoplasmic domain truncated by 17 amino acids; pECE-α5/1, which encodes an α5 subunit that has 26 of the 27 amino acids of the cytoplasmic domain deleted (Fig. 1). Both pECE-α5 and the mutated α5 vector constructs were transfected into a CHO FnR variant (the B2 clonal cell line; originally described by Schreiner et al., 1989) that expresses very low levels of the endogenous hamster α5 subunit. The transfection yielded chimeric fibronectin receptors; i.e., human α5 associating with hamster β1.

Expression of the Human α5/Hamster β1 Constructs

Several cell lines that expressed either pECE-α5 or one of the truncated α5 constructs were screened by ELISA using the B1E5 anti-α5 subunit antibody (data not shown). The cell lines that expressed the greatest amounts of α5 subunit were chosen for study. The selected cell lines were B2/a27, B2/a10, and B2/a1, corresponding to cells expressing pECE-α5, pECE-α5/10, or pECE-α5/1, respectively. ELISA analysis showed that both B2/a27 and B2/a10 cells expressed similar levels of α5 subunit on the surface, while less α5 subunit was expressed by the B2/a1 cells (data not shown). Expression of chimeric α5/β1 FnR on the surface of the transfectants was also evaluated by flow cytometry (Fig. 2). Analysis of cells stained with PB1, an antibody which recognizes the intact α5/β1 FnR (Brown and Juliano, 1985), showed that wild type, B2/a27, and B2/a10 expressed similar levels of FnR. B2/a1 expressed substantially less α5/β1 FnR than either the other transfectants or wild type cells. B2 cells exhibited levels of hamster α5/β1 FnR expression only marginally above background. When cells were analyzed using B1E5, an antibody to the α5 subunit, wild type, B2/a27, and B2/a10 were shown to express similar levels of the subunit. In agreement with the PB1 antibody data, B2/a1 expressed substantially less α5 subunit, ~15–20% of wild type cells. B2 cells were devoid of measurable α5 subunit expression. Expression of β1 subunit was tested using an anti-hamster β1 mAb (7E2). As reported previously, β1 subunit expression in B2 cells was substantially reduced due to lack of α5 (Schreiner et al., 1989); β1 expression in B2/a27 and B2/a10 was restored to wild type levels, while β1 subunit expression was only marginally increased in B2/a1 cells. Finally, αv/β3 vitronectin receptor expression was tested using a polyclonal anti-receptor antibody; all cell lines were shown to exhibit similar levels of vitronectin receptor expression. Thus levels

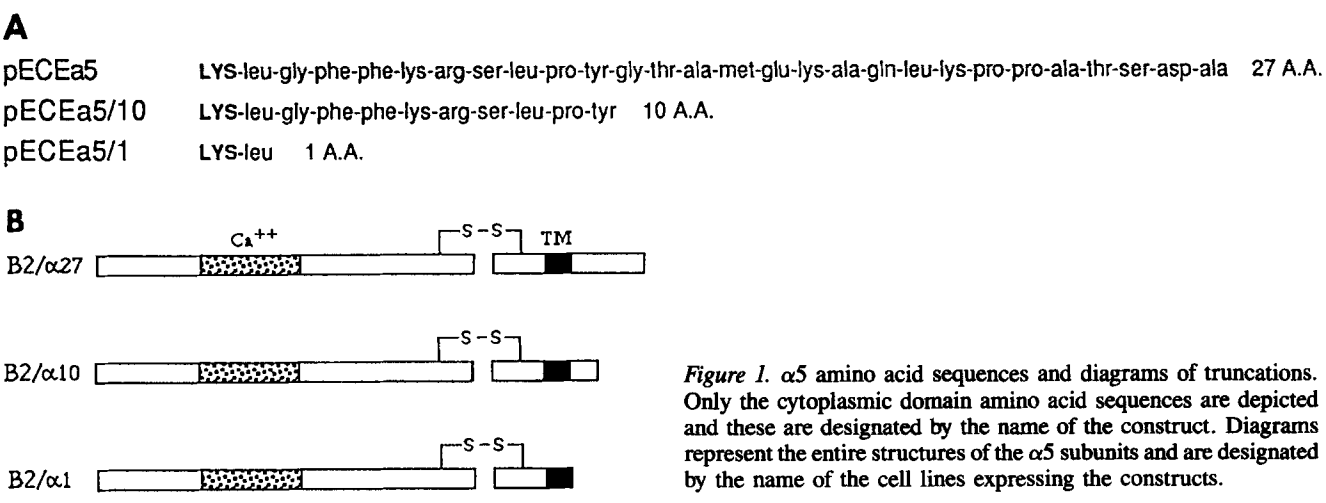

Figure 1. α5 amino acid sequences and diagrams of truncations. Only the cytoplasmic domain amino acid sequences are depicted and these are designated by the name of the construct. Diagrams represent the entire structures of the α5 subunits and are designated by the name of the cell lines expressing the constructs.

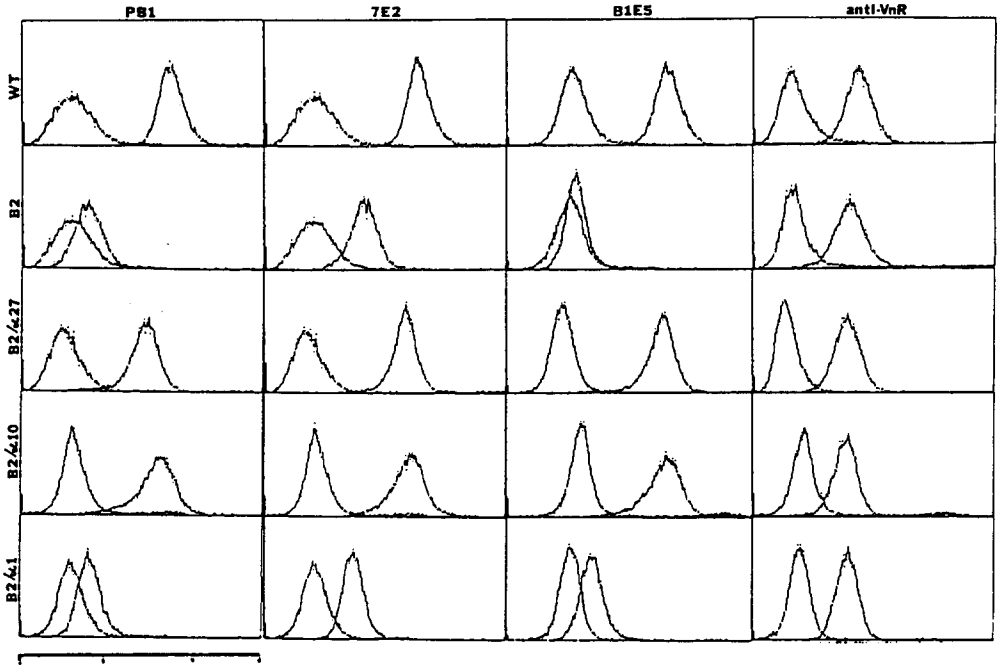

Figure 2. FACS Histograms of FnR, β1, α5, and VnR expression on CHO wild type, B2, B2/a27, B2/al and B2/al0. Equal numbers of each cell type were washed with cold α-MEM with 1% BSA and stained with primary antibodies PB1 (anti-hamster α5/β1), 7E2(anti-hamster β1), B1E5 (anti-human α5), or an anti-VnR antibody (right side histogram in each panel). Cells were then stained with appropriate secondary antibodies, either fluorescein-conjugated sheep anti-mouse IgG (PB1, 7E2), fluorescein-conjugated goat anti-rat IgG (B1E5), or fluorescein-conjugated goat anti-rabbit IgG (antiVnR). Cells not treated with primary antibody and stained with fluorescein-conjugated secondary antibody were used as negative controls (left histogram in each panel). The ordinate is the number of cells per channel of fluorescence intensity with the full height of the ordinate being 512. The abscissa is the log of the fluorescence intensity in arbitrary units. A three-log scale of fluorescence intensity is indicated under the lowest panel on the left and is applicable to all the panels.

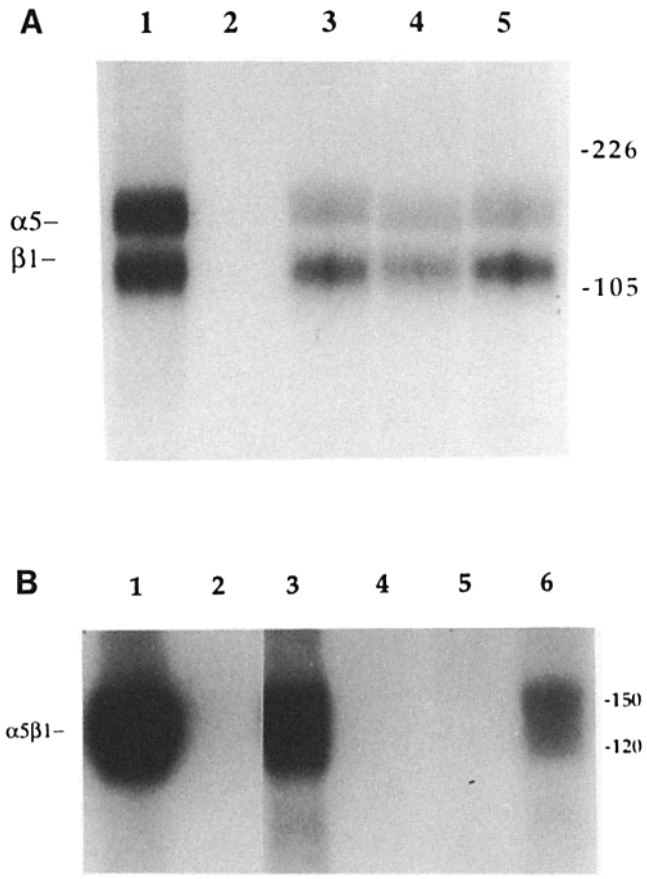

Figure 3. Immunoprecipitation of FnR from the α5 truncated transfectants. Cells were surface labeled with $Na^{125}I$ and detergent lysates were prepared as described in the Materials and Methods section. (*A*) FnR was immunoprecipitated from detergent lysates with either PB1 (wild type, B2) or B1E5 (α5 transfectants) and resolved under nonreducing conditions on a 4.5–8.6% gradient polyacrylamide gel. Equal cpm were applied to each gel lane: lane *1*, wild type; lane *2*, B2; lane *3*, B2/a27; lane *4*, B2/al; and lane *5*, B2/al0. (*B*) FnR was immunoprecipitated from detergent lysates with anti-α5 cytoplasmic domain polyclonal antibody (161). Proteins were resolved on a 7.5% polyacrylamide gel. Lane *1*, wild type; lane *2*, B2; lanes *3* and *6*, B2/a27 (only half as much protein was loaded in lane 6); lane *4*, B2/al; lane *5*, B2/al0.

of expression of chimeric α5/β1 in B2/a27 and B2/a10 cells are similar to levels of expression of native α5/β1 in wild type cells, while the B2/al cells have a reduced level of α5/β1 FnR expressed at the cell surface.

Immunoprecipitation of Expressed α5 Constructs and Evaluation of mRNA Levels

Immunoprecipitations of ^{125}I-labeled cell-surface proteins were performed on detergent lysates prepared from equal numbers of wild type CHO cells, B2 cells, and the α5 transfectants. PB1 was used to precipitate α5/B1 FnR from wild type and B2 cells, while chimeric FnR from transfected cells was immunoprecipitated with B1E5. Different antibodies were used to ensure the highest efficiency immunoprecipitation. The precipitated FnR was analyzed on a non-reducing 4.5–8.5% gradient polyacrylamide gel to discriminate size differences between the various α5 constructs (Fig. 3 *A*). Immunoprecipitation of native α5/β1 FnR or chimeric FnR with either PB1 or B1E5, respectively, yielded two bands of equal intensity; the 140-kD band is the α5 subunit while the 120-kD band is the β1 subunit. Apparent molecular weights of the precipitated α and β subunits were calculated from their relative migration factors (n = 2). α5 subunits from

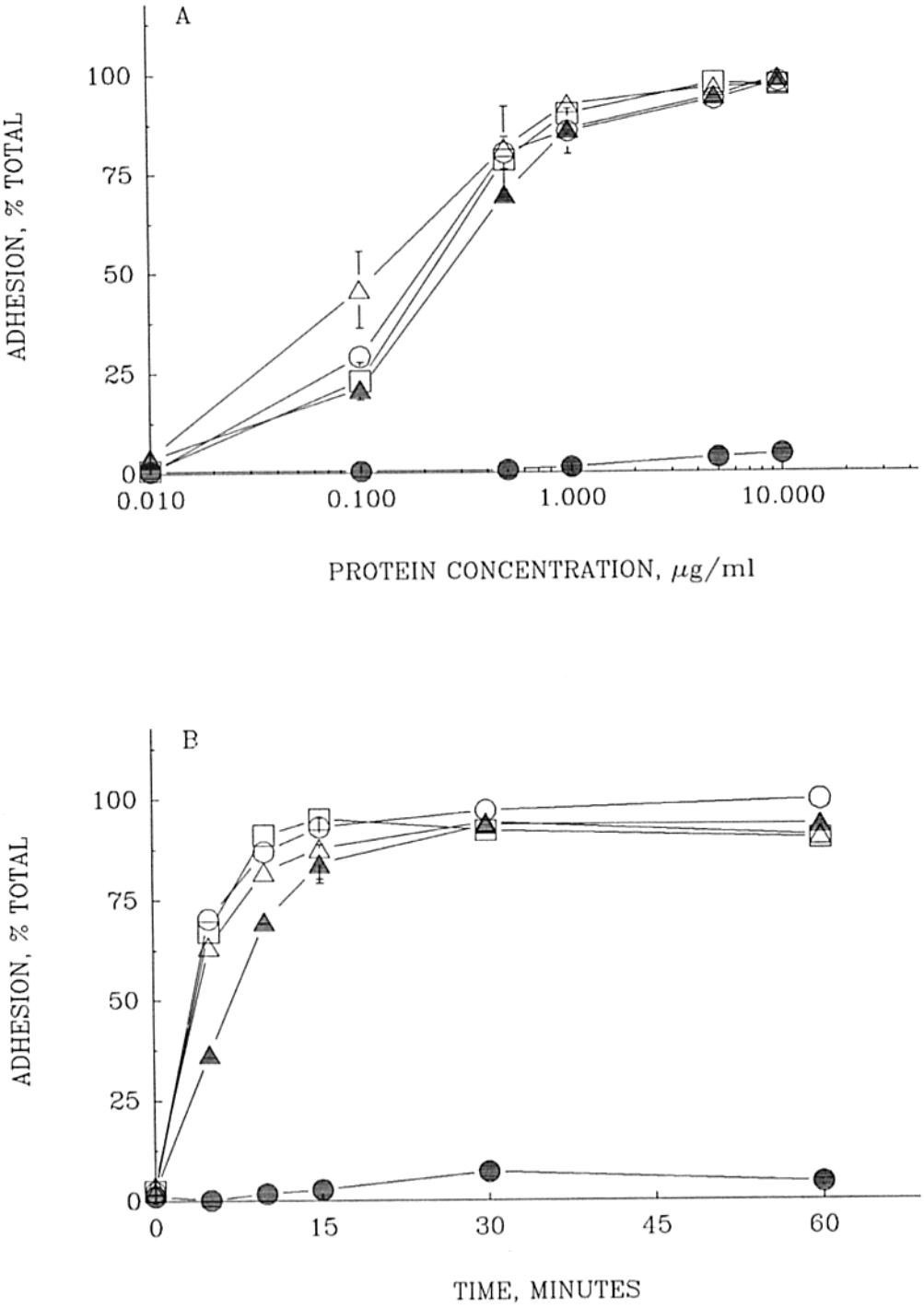

Figure 4. Adhesion of α5 transfectants to Fn-coated substrata. (*A*) Efficiency of adhesion to Fn-coated substrata. 48-well tissue culture plates were coated with various concentrations of Fn for 2 h at 37°C in PBS, and then blocked with 3% BSA as described in Materials and Methods. Cells that were metabolically labeled overnight with ^{35}S-Trans label were washed and resuspended at 5 × 10^5 cells/ml in adhesion buffer. Cells were added to the plates and allowed to adhere to the substrata at 37°C for 60 min. The adherent cells were recovered and analyzed for radioactivity as described in Materials and Methods. Data is presented as a percentage of the total cells added to each well. (○—○), wild type; (●—●), B2; (△—△), B2/a27; (▲—▲), B2/a1; (□—□), B2/a10. (*B*) Kinetics of adhesion. 48-well tissue culture plates were coated with non-limiting concentrations of fibronectin (1 μg/ml) and then blocked with 3% BSA. Metabolically labeled cells were prepared as described in Fig. 5 *A*, added to the Fn-coated plates and allowed to attach at 37°C for varying times up to 60 min. The adherent cells were recovered and analyzed for radioactivity as described in Materials and Methods. Data is presented as a percentage of the total cells added to each well. (○—○), wild type; (●—●), B2; (△—△), B2/a27; (▲—▲), B2/a1; (□—□), B2/a10.

wild type cells (Fig. 3 *A*, lane *1*) or B2/a27 cells (Fig. 3 *A*, lane *3*) exhibited similar mobilities on gradient gels, with apparent subunit molecular weights of 147 and 149 kD, respectively. In comparison, the truncated α5 subunits from B2/a1 cells (Fig. 3 *A*, lane *4*) or B2/a10 cells (Fig. 3 *A*, lane *5*) migrated on the gradient gel slightly faster than intact human α5 subunit, exhibiting apparent molecular weight values of 143 and 147 kD, respectively. The β1 subunit for all cell types tested, excluding B2, exhibited an apparent molecular weight of 117 kD; α5/β1 was absent in immunoprecipitates from B2 cells (Fig. 3 *A*, lane *2*).

Native and chimeric α5/β1 FnR were also immunoprecipitated with a rabbit polyclonal antibody (No. 161) directed against the cytoplasmic domain of the human α5 subunit and were resolved on a 7.5% polyacrylamide gel (Fig. 3 *B*). Antibody 161 shows cross-reactivity with α5 subunits originating from other species due to interspecies sequence homologies within the α5 cytoplasmic domain. Antibody 161 precipitated native α5/β1FnR from wild type cells (Fig. 3 *B*, lane *1*). Chimeric α5/β1 was also precipitated from B2/a27 (Fig. 3 *B*, lanes *3* and *6*). In contrast, the truncated α5 subunits arising from B2/a1 cells (Fig. 3 *B*, lane *5*) and

B2/a10 cells (Fig. 3 *B*, lane *6*) were not precipitated by the 161 antibody, confirming that the α5 subunit is truncated in these cells. No α5/β1 was detected in B2 cells with antibody 161.

Equal amounts of total cytoplasmic RNAs derived from wild type, B2, B2/a27, B2/a10, and B2/a1 cells were evaluated by Northern blot hybridization (Chomczynski and Sacci, 1987; Sambrook et al., 1989) for the presence of human α5 subunit mRNA. Northern analysis showed a single band at ~4.2 kb for cells transfected with pECE-α5, pECE-α5/1, and pECE-α5/10 (data not shown), in agreement with the sizes predicted from the cDNA constructs taking into account polyadenylation of the transcribed mRNA sequence. Slot blot hybridizations were performed to compare relative amounts of α5 mRNA present in all three transfectants. Densitometric analysis of the slot blot hybridization indicated that five times more α5 message was present in B2/a10, and three times more message was present in B2/a1 when compared to α5 message within B2/a27; thus, data from Northern and slot blot analysis, compared with surface expression of the mutated α5 subunits, suggests that partial or complete deletion of the cytoplasmic domain impedes effective translation or surface expression.

Adhesion of Cells to Fibronectin

Although the truncated α5 subunits were expressed and could be precipitated using the BlE5 antibody, it remained to be seen whether the modified receptors were functional. To explore this, cells were tested for their ability to adhere to purified Fn immobilized on tissue culture plastic. These experiments show that B2/a27 and B2/a10 adhered to Fn to a similar extent as wild type CHO cells (Fig. 4 *A*). Despite lower α5/β1 FnR expression, B2/a1 cells also attached to Fn in a manner similar to wild type cells. Initial binding was evident at coating concentrations of 0.1 μg/ml Fn, while maximum adhesion of cells to Fn was demonstrated at coating concentrations of 1 μg/ml. The cells not expressing α5/β1 (B2 cells) showed very limited adhesion to Fn even at coating concentrations of 10 μg/ml. To determine whether the truncations within the α5 subunit could affect the rapidity of binding to immobilized Fn, time courses of adhesion assays were performed. Kinetic experiments were done using non-limiting concentrations of Fn (1 μg/ml). Both B2/a27 and B2/a10 cells displayed kinetics of adhesion to Fn similar to wild type cells (Fig. 4 *B*), adhering maximally in 15 min. B2/a1 cells showed a slower rate of adhesion to Fn than wild type cells during the initial 10 min, but approached wild type levels of adhesion at 15 min. This slower rate of adhesion may be a reflection of the decreased surface expression of the chimeric α5/β1 FnR. This latter result is in agreement with results reported previously in our laboratory which showed that CHO FnR variants that exhibit reduced (20% of wild type) α5/β1 FnR expression, adhered to immobilized Fn substrata at a slightly slower rate than wild type cells (Schreiner et al., 1989).

Centrifugal force was used to gauge the strength of adhesion of cells to non-limiting concentrations of immobilized Fn (1 μg/ml) (Fig. 5). Wild type cells and B2/a27 cells were completely adherent at relative centrifugal forces (RCFs) up to 100 *g*. Beyond this, adhesive resistance to centrifugal force decreased for both cell lines. At the maximal centrifugal force used (RCF = 1,000 *g*), 80 and 75% of wild type cells and B2/a27 cells, respectively, remain attached to the Fn-coated substrata. B2/a1 and B2/a10 cells showed slight decreases in adhesion at 100 *g*. The decrease in adhesion was more pronounced at 500 *g*, falling to 50 and 75% for B2/a1 and B2/a10, respectively. At 1,000 *g* both B2/a1 and B2/a10 displayed ~50% adhesion. To distinguish between the effects of reduced α5/β1 FnR expression, and effects caused by the cytoplasmic domain truncation, a variant that expresses 20% of wild type hamster α5/β1 FnR (clone 1-23; previously described by Schreiner et al., 1989), was used as a control. At all centrifugal forces tested, the adhesion of B2/a1 to Fn paralleled that of the 20% FnR variant. The 2% FnR variant B2 showed only background adhesion to Fn

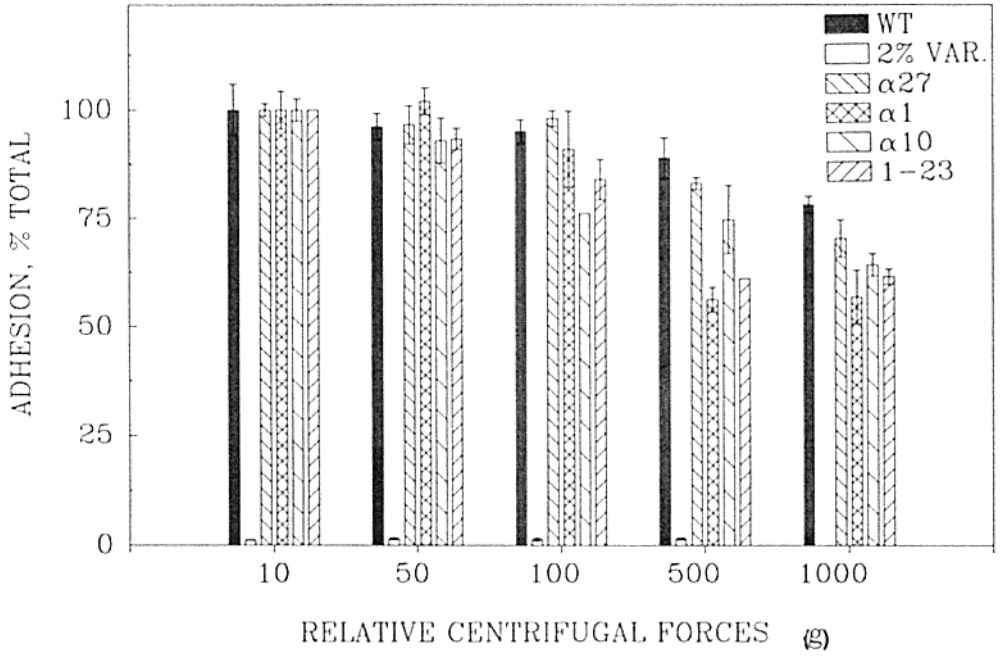

Figure 5. Strength of adhesion of α5 transfectants to Fn. 96-well microtiter plates were coated with non-limiting concentrations of Fn (1 μg/ml) and then blocked with 3% BSA. Metabolically labeled (as described in Materials and Methods) cells were added to the plates and then were spun onto the substrata at 17 *g* for 10 min at 4°C. The plates were then incubated at 37°C for 30 min. After incubation, plates were sealed, inverted, and centrifuged at 4°C for 10 min at varying speeds to dislodge non- or weakly adherent cells. Adhesion was quantitated by counting the radioactivity of the attached cells. Wild type, ■; B2, □; B2/a27, ▨; B2/a1, ▩; B2/a10, ▧; 1-23, ▨.

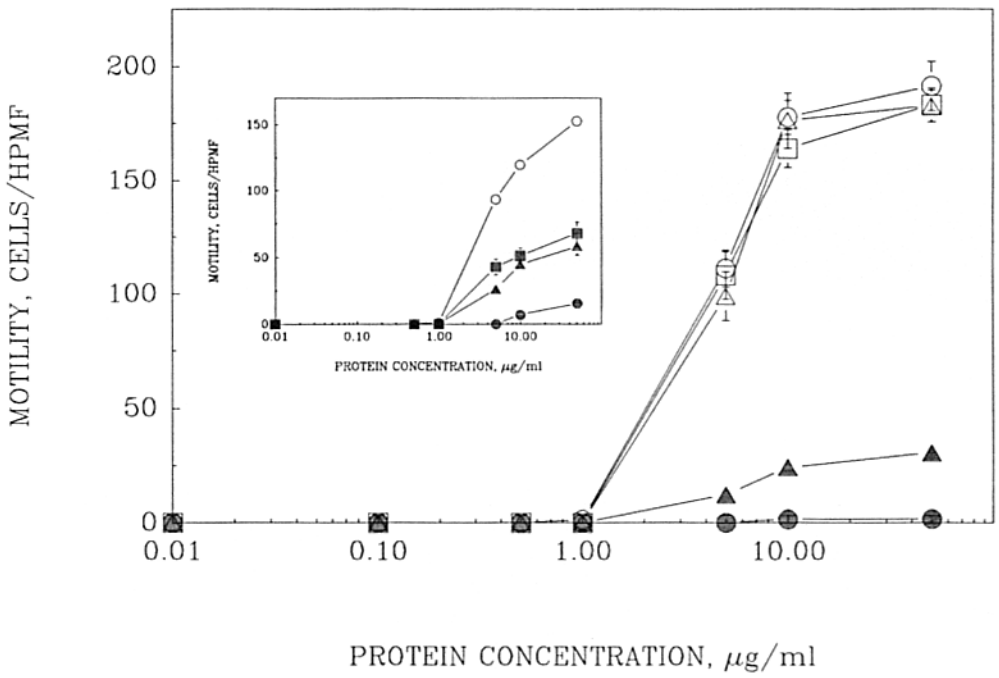

Figure 6. Motilities of CHO α5 transfectants on Fn. The undersides of Transwell motility chamber inserts were coated with various concentrations of Fn in PBs. Cells were washed and resuspended in α-MEM containing 1% BSA. 1 × 10^5 cells were added to the upper chamber and the motility chambers were incubated at 37°C for 8 h. Motility was quantitated by counting the number of cells which migrated to the undersides of the membranes. The results are averages of at least 10 random high powered microscopic fields. (○—○), wild type; (●—●), B2; (△—△), B2/a27; (▲—▲), B2/a1; (□—□), B2/a10. (*Inset*) Comparison of B2/a1 and 1-23 motilities Fn. Motility assay was performed as described above. (○—○), wild type; (●—●), B2; (▲—▲), B2/a1; (■—■), 1-23.

even at low centrifugal forces (RCF = 10 *g*). Overall, the adhesive behavior of B2/a10 and B2/a1 cells was fairly similar to wild type or B2/a27 cells. The modest reduction in adherence of the B2/a1 cells seemed to be primarily attributable to the lower expression of α5, rather than being directly due to the cytoplasmic domain truncation.

Motility of Cells on Fn

It has been shown that the α5/β1 FnR is involved in the motility of cells on Fn substrata (Bauer et al., 1992). Using a modified Boyden chamber assay, we examined the contributions of the α5 subunit cytoplasmic domain to cellular haptotaxis on Fn. B2/a27 cells migrate on Fn to an extent similar to wild type cells. B2/a10 cells also exhibited wild type levels of motility on Fn, while migration of B2/a1 cells on Fn was substantially reduced (15–35% of wild type) (Fig. 6). The B2 cells failed to show any appreciable motility on Fn. Decreased motility of B2/a1 cells on Fn could be due to either decreased α5/β1 FnR expression or to less efficient receptor function due to the deletion of the α5 cytoplasmic domain. To help evaluate these two possibilities, the motility of the B2/a1 on Fn was compared to that of FnR-deficient clone 1-23 (Fig. 6, *inset*). B2/a1 and 1-23 both exhibited reduced degrees of motility as compared to wild type cells. This comparison suggested that the decreased motility of B2/a1 cells on Fn might be partly due to reduced α5/β1 FnR expression. However, additional experiments (see below) indicate that α5 truncation has a direct effect on motility. Our standard motility assay incubation is eight hours. A kinetic study was performed to determine whether the deletions in the cytoplasmic domain of the α5 might cause changes in the motility of cells on Fn discernable beyond this 8-h incubation time. On non-limiting concentrations of Fn (10 μg/ml) migration of B2/a27 and B2/a10 cells paralleled migration of wild type cells at all time points tested (Fig. 7). B2/a1, however, showed slower rates of motility on Fn initially, and failed to achieve wild type levels of motility even after 24 h of incubation.

Cytoskeletal Organization

Subsequential to integrin binding to ECM proteins, integrins cluster to form adhesion plaques (focal contacts), a process which involves cytoskeletal rearrangement and actin stress fiber development (Burridge et al., 1988). It has already been shown that truncation of the β1 subunit within the cytoplasmic domain significantly impairs the ability of integrins to participate in adhesion plaque formation and cytoskeletal organization (Hayashi et al., 1990; Marcantonio et al., 1990). We investigated whether cytoplasmic truncation of the α5 subunit also affects cytoskeletal organization. Morphology and stress fiber development were evaluated in cells plated on substrata coated with 100 μg/ml Fn. Wild type, B2/a27, and B2/a10 cells spread equally well on Fn exhibiting strong stress fiber formation (Fig. 8, *A*, *C*, and *E*, respectively). CHO cells do not produce well-developed focal contacts; however, in the wild type, B2/a27 and B2/a10 cells, α5/β1 was shown to localize at the edges of the stress fiber (data not shown). B2/a1 cells (Fig. 8, *D*) spread on Fn but differed morphologically from wild type cells, maintaining a less-elongated appearance. This was similar to the appearance previously described for 1–23 cells that express comparable levels of α5/β1 (Schreiner et al., 1989). A poorly spread morphology was also evident in several other independent clones arising from transfection with the fully truncated α5 construct (data not shown). As in the other cells (with the exception of B2), stress fibers were produced in

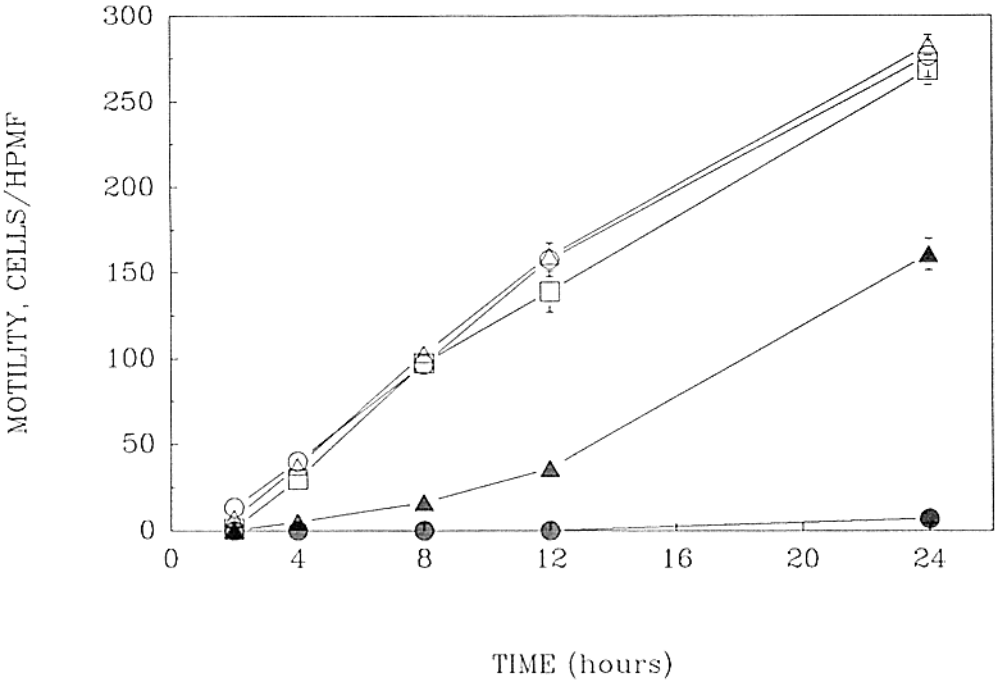

Figure 7. Kinetics of CHO motility on Fn. Transwell motility inserts were coated with 30 μg/ml Fn. 1 × 10^5 cells were added to the upper chamber and incubated for various times at 37°C. Motility was quantitated as before. (○—○), wild type; (●—●), B2; (△—△), B2/a27; (▲—▲), B2/a1; (□—□) B2/a10.

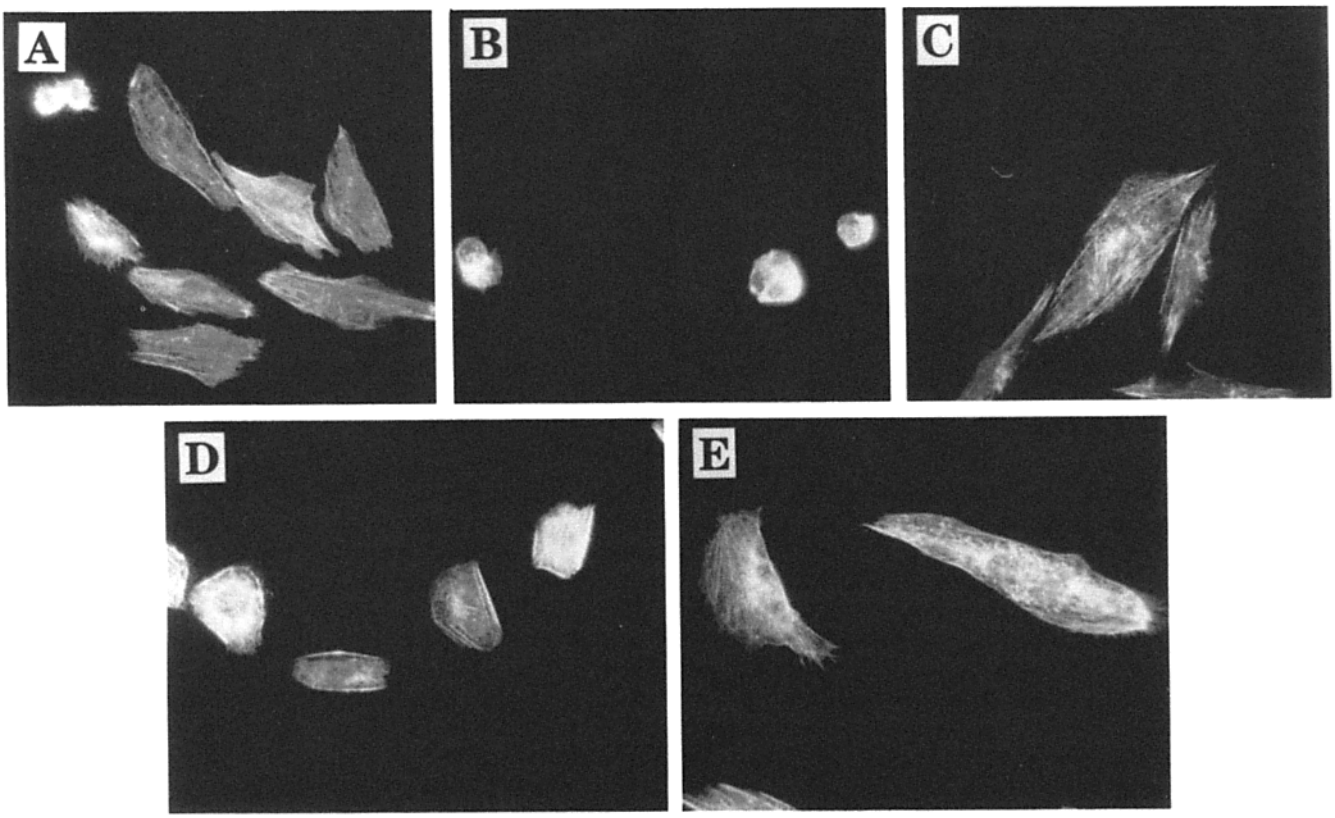

Figure 8. Actin cytoskeletal stress fiber development in α5 transfectants spread on Fn. Trypsinized cells were plated onto fibronectin-coated (50 μg/ml) coverslips and allowed to spread for 4 h. Cells were then fixed in 3.7% formaldehyde, permeabilized in 0.4% Triton X-100, and stained with rhodamine-labeled phalloidin. Cells were observed using a 100× objective. *A*, wild type; *B*, B2; *C*, B2/a27; *D*, B2/a1; and *E*, B2/a10.

B2/a1 cells, but cytoskeletal organization was less well defined. $\alpha5/\beta1$ was detectable in adhesion plaques in B2a1 cells despite lower receptor expression (data not shown). The morphology of the B2/a1 cells resembled that previously described for clone 1-23, a line that expresses levels of $\alpha5/\beta1$ similar to the B2/a1 cells (Schreiner et al., 1989). The B2 cells (Fig. 8, *B*) showed no spreading on Fn, maintaining a spherical appearance; stress fiber development in the B2 cells was conspicuously absent.

Protein Tyrosine Phosphorylation

Changes in the pattern of protein tyrosine phosphorylation have been observed subsequent to the clustering of integrins by antibodies (Kornberg et al., 1991), or as a result of adhesion to fibronectin (Guan and Hynes, 1991). In Fig. 9 protein tyrosine phosphorylation patterns subsequent to plating on Fn-coated substrata are illustrated for the FnR-deficient mutant B2, and for $\alpha5$-transfected CHO cells. A substantial increase in tyrosine phosphorylation was seen in a band of ~125 kD in both B2/a27 cells and B2/a1 cells adherent to Fn substrata as compared with non-adherent cells. In other experiments, similar increases in tyrosine phosphorylation were observed in B2a/10 cells and WT CHO cells subsequent to adhesion to fibronectin (data not shown). Exposure to Fn substrata had no effect on the tyrosine phosphorylation pattern of B2 cells; as indicated above, these cells are almost completely nonadherent on Fn substrata. In CHO cells interactions with fibronectin seem to be almost exclusively dependent on the $\alpha5/\beta1$ integrin, since these interactions can be completely abolished by mAbs directed against this integrin (Brown and Juliano, 1985; Bauer et al., 1992). Thus, the results shown in Fig. 9 suggest that the α-cytoplasmic domain is not required for $\alpha5/\beta1$-mediated tyrosine phosphorylation in CHO cells.

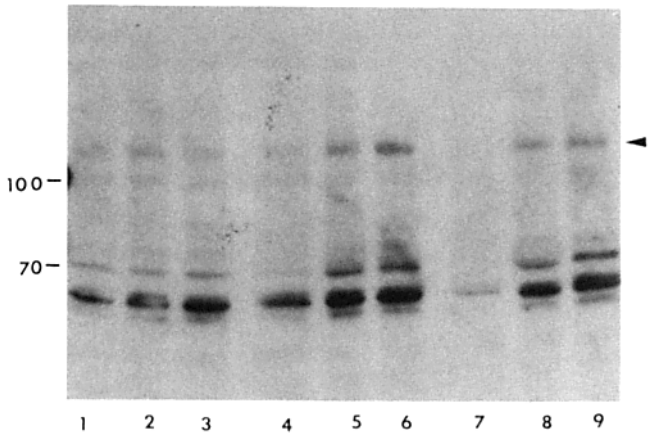

Figure 9. Protein tyrosine phosphorylation subsequent to adhesion on Fn substrata. CHO mutants or transfectants were incubated for 90 min at 37°C on 60-mm dishes coated with 0, 0.5, or 10 μg/ml of fibronectin. The cells were then washed, lysed in 300 μl of RIPA buffer, and then processed for gel electrophoresis and transblotting; gels were run with equal protein loads in each lane. Phosphotyrosyl proteins were detected by Western blotting with an anti-P-tyrosine antibody as described in Materials and Methods. A 24-h autoradiographic exposure is shown. The position of the 125-kD tyrosine phosphorylated protein(s) is indicated by an arrow. Positions of molecular weight markers are indicated at the left of the f gure. (Lanes 1-3) B2 cells on 0, 0.5, or 10 μg/ml of fibronectin; (lanes *4-6*) B2a1 cells on 0, 0.5, or 10 μg/ml of fibronectin; (lanes *7-9*) B2a27 cells on 0, 0.5, or 10 μg/ml fibronectin.

Evaluation of Normal and Truncated $\alpha5$ Function at Similar Levels of Expression

Although a comparison of clones 1-23 and B2a1 (Figs. 6 and 8) suggested that the deficits in cell motility and cell spreading exhibited by B2a1 might be due simply to low levels of $\alpha5$ expression rather than to truncation of the cytoplasmic domain, these results were not unambiguous. Thus we examined cell morphology and motility in cells from clones B2a27 and B2a1 under conditions where the full-length (a27) and truncated (a1) $\alpha5$ subunits were expressed at similar levels. This was done by using a flow cytometer to sort out a population of B2a1 cells that expressed high levels of truncated $\alpha5$, similar to the levels of full-length $\alpha5$ expressed in the B2a27 population (in fact, the average $\alpha5$ expression in the sorted B2a1 cells was slightly higher than the B2a27 cells). The high-expressor B2a1 cells (termed B2a1H) were compared to B2 and B2a27 in terms of ability to spread on Fn substrata, and in terms of cell motility in a "wounding" assay (we were not able to obtain sufficient numbers of B2a1H cells to study cell motility using the Boyden chamber type motility assay). As seen in Fig. 10, during a 2-h incubation at 37°C, B2a27 cells attached and became fully spread on a Fn substratum; in contrast, the B2a1H cells attached, but only a few cells were able to spread. The B2 cells did not attach to the Fn substratum and remained round. The negatively selected, low-expressing population (B2a1L) as well as the unselected B2a1 population attached to the Fn substratum (although some "floaters" were seen in the B2a1L population); these two populations largely remained round after 2 h. After a more extended incubation (24 h) a large fraction of the B2a1H cells and some of the B2a1 cells had partially spread; however, obvious differences with the B2a27 population persisted (data not shown). Table I compiles the number of fully spread, partially spread, or round cells from each population, and confirms the impressions of Fig. 10. Table II illustrates that B2a1H cells migrate less well than B2a27 cells using the wound-type motility assay.

Discussion

Integrins play a vital role in the adhesion and motility of cells on ECM proteins. In doing this, integrins must interact with

Table I. Cell Spreading

Cell type	Percent of total cells per field		
	Fully spread	Partly spread	Round
B2a27	76 ± 7	14 ± 8	9 ± 7
B2a1H	3 ± 3	37 ± 2	59 ± 6
B2a1	0	23 ± 10	77 ± 11
B2a1L	0	8 ± 3	92 ± 4
B2	0	3 ± 3	96 ± 6

Cell populations (1×10^5) in α-MEM + 1% albumin were plated into 24-well tissue culture plates pre-coated with 10 μg/ml fibronectin and incubated for 2 h at 37°C in a CO_2 incubator. The cells were observed with a 40× phase contrast objective and the number of full, partially spread, or round cells per objective field was counted. For each sample fields in three different wells were observed. All cells tested were exposed to primary and secondary antibodies and processed in the same manner. The B2a1H and B2a1L are the sorted populations. Results are given as means and standard errors.

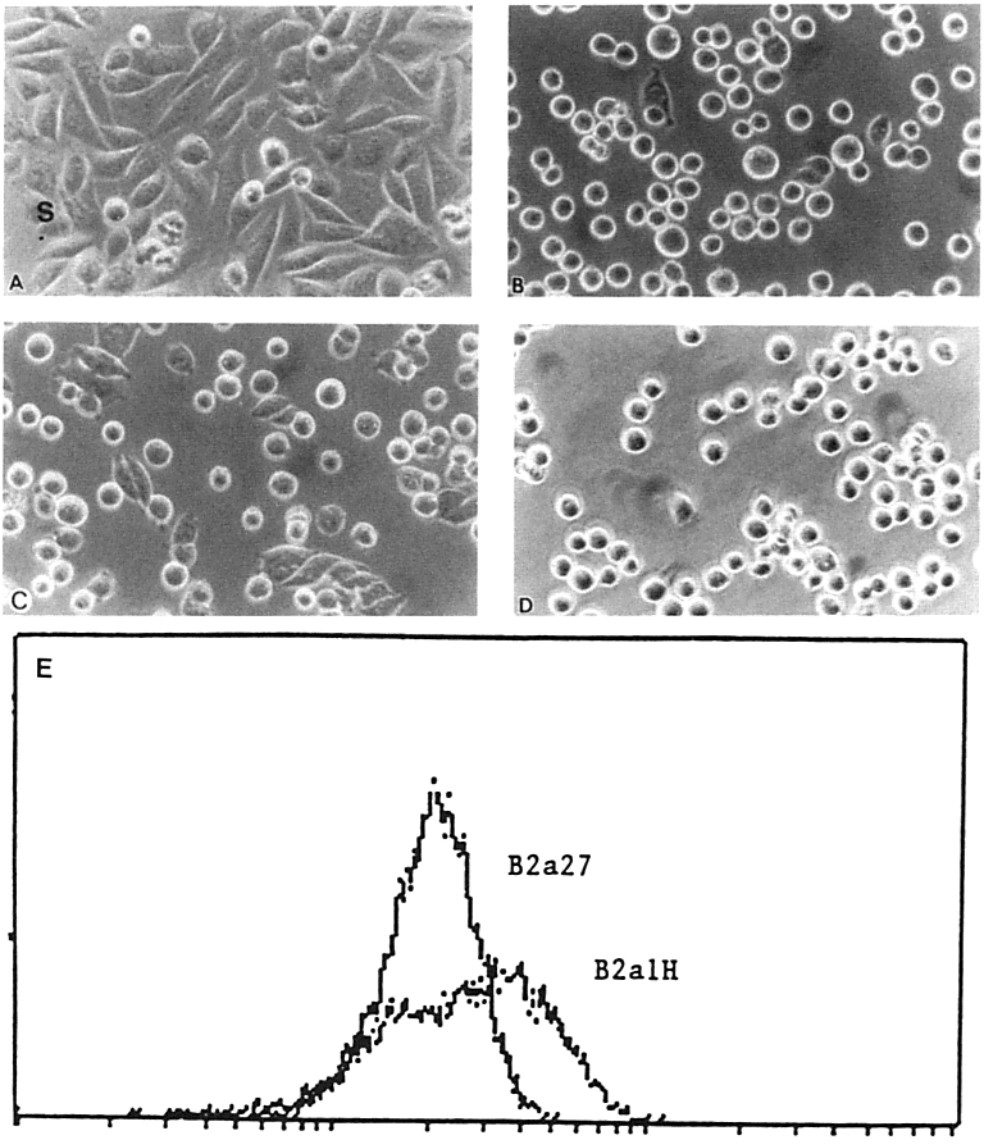

Figure 10. Morphology of cells expressing similar levels of full-length or truncated α5. Using flow cytometry a population of B2a1 cells was obtained (B2a1H) that expresses a level of truncated α5 similar to the expression of full-length α5 in B2a27 cells (see Materials and Methods). A negatively selected low-expressor population was also obtained (B2a1L). Cell populations (B2, B2a1, B2a1L, B2a1H, B2a27) in α-MEM + 1% albumin were placed in 24-well tissue culture plates pre-coated with 10 μg/ml Fn, incubated for 2 h at 37°C, and photographed using a phase contrast photo microscope with a 40× objective. *A*, B2a27; *B*, B2a1; *C*, B2a1H; *D*, B2; *E*, Flow cytometric analysis of α5 expression in B2a27 and B2a1H cells.

the cytoskeletal apparatus in some fashion (Burridge et al., 1988). Recent evidence also suggests that integrins both influence cellular signal transduction mechanisms (Kornberg et al., 1991), and are in turn acted upon by cellular regulatory processes (Van Kooyk et al., 1989). Thus, integrins participate in both "outside in" and "inside out" interactions between the extracellular matrix and the cell interior (Hynes, 1992). It seems reasonable to assume that integrin cytoplasmic domains are essential to these transactions. In this study we have investigated the role of the α5 cytoplasmic domain in several aspects of integrin function including cell adhesion, motility, cytoskeletal organization, and protein tyrosine phosphorylation.

To this end, we engineered two α5 constructs (pECE-α5/1 and pECE-α5/10) that are truncated within the cytoplasmic domain, from an existing full-length human α5 cDNA expression vector (pECE-α5). A CHO cell clone (B2) that produces very low levels of endogenous hamster α5 subunit was chosen as a host system to test the function of these constructs. Transfection of B2 cells with either pECE-α5 or pECE-α5/10 resulted in wild type levels of surface α5/β1 FnR expression. After transfection, pECE-α5/1 was also expressed at the cell surface, albeit at much reduced levels, approximating 15–20% of wild type α5/β1FnR expression. CHO cells transfected with pECE-α5 have previously been shown to give rise to functional chimeric human α5/hamster β1 (Bauer et al., 1992; Giancotti and Ruoslahti, 1990). Our results showed that the truncated α5 constructs can also associate with β1 and that these complexes were expressed on the surface of the B2 cells. Chimeric α5/β1 FnR was immunoprecipitated with the BIE5 anti-human α5 antibody from lysates derived from the three transfectants. Precipitated α5 subunits arising from either B2/a1 or B2/a10 showed altered mobility on gradient polyacrylamide gels, migrating slightly faster than the full-length human α5 subunit. Hamster α5 subunit migrated slightly faster than the intact human α5 presumably due to differences in subunit glycosylation. Additionally, anti-α5 cytoplasmic domain antibody (161) did not precipitate FnR derived from either B2/a1 or B2/a10, thus confirming the truncated nature of the cytoplasmic

Table II. Cell Motility

Cell population	Number of cells crossing demarcation
B2a27	61 ± 8
B2a1H	39 ± 10
B2a1	21 ± 10
B2a1L	10 ± 3

Cell populations (1×10^5) in α-MEM + 1% albumin were placed into 24-well tissue culture plates pre-coated with 10 μg/ml fibronectin and incubated for 2 h at 37°C in a CO_2 incubator. The cell layers were then "wounded" with a sharp plastic blade so as to denude a well-defined region of the well and the displaced cells were washed away. The line separating the residual intact cell layer from the denuded area was marked on the bottom of the plate using a thin-point marker. The samples were incubated for 16 h in a CO_2 incubator. Samples were then observed using a phase contrast microscope with a gridded eyepiece; the grid line was aligned with the demarcation line marked on the bottom of the plate. The number of cells that had moved from the intact cell layer, across the demarcation line, and into the denuded area was counted per unit length of demarcation line using the gridded eyepiece. The data represent triplicate determinations taken from different regions of a single well.

domains, as well as the absence of significant residual hamster α5.

As mentioned above, complete removal of the cytoplasmic domain of the α5 subunit results in lower FnR surface expression. Reduced expression was not due to lower mRNA levels. Thus, B2/a1 cells, which express low levels of cell surface α5/β1, produced three times more α5 message than B2/a27 cells, which express wild type levels of α5/β1, while B2/a10 expressed five times more α5 message than B2/a27, but expressed comparable levels of cell surface α5/β1; both of these observations suggest that truncation of the cytoplasmic domain reduces surface expression. This result is in agreement with the work of Hibbs et al. (1991) who noted reduced surface expression of LFA-1 in COS cells after complete truncation of the cytoplasmic domain of the α subunit. Our pECE α5/1 construct is truncated in the midst of the highly conserved KXGFFR sequence that follows the transmembrane domain in all α chains (Dedhar, 1990); this sequence may be important for efficient cell surface expression of α/β heterodimers (Chan et al., 1992).

Expression of the truncated human α5 constructs in CHO cells gave rise to functional chimeric α5/β1 fibronectin receptor. This conclusion is based on the following observations: (*a*) transfected cells adhered to immobilized Fn substrata; (*b*) the cells exhibited motility on Fn-coated substrata that was consistent with receptor expression; (*c*) cells adhering to Fn underwent cytoskeletal reorganization and focal adhesion plaque development. B2/a27 and B2/a10 cells behaved similarly to wild type cells in terms of adhesion, motility, and cytoskeletal organization. In contrast, B2/a1 cells showed decreased motility on Fn; strength and kinetics of adhesion to Fn were also somewhat attenuated. To understand the behavior of B2/a1 cells, it was essential to dissociate functional effects imposed by the truncation from those due to decreased α5/β1 expression. Thus, we used fluorescence cell sorting to obtain a sub-population of B2a1 cells (B2a1H) that expressed levels of truncated α5 similar to the level of expression of full-length α5 found in B2a27. Despite similar levels of α subunit expression, there were distinct functional differences between the B2a1H cells and B2a27 cells. The latter cells can rapidly spread on fibronectin substrata and develop a fully extended fibroblastic morphology; the B2a1H cells spread to a very limited degree in the short term. After overnight incubation there is considerable spreading of B2a1H, although not as extensive as for B2a27, however, results at these longer times may be partly due to modification of the fibronectin substratum by cell secretion of other adhesion factors. Since both B2a27 cells and B2a10 cells can rapidly spread and develop stress fibers, while B2a1H cannot, it seems that the ability to rapidly reorganize the cytoskeleton and to engage in cell spreading is markedly affected by deletion of the portion of α5 which is proximate to the membrane. This portion of the α5 subunit also seems to be involved in cell migration since the B2a1H cells are less motile than the B2a27 cells. However, the level of expression of α5 is also an important determinant of cell function. This is emphasized by the different behavior of wild type CHO and clone 1-23, which expresses reduced levels of intact hamster α5. It is also apparent from the observation that, although the B2a1H cells spread rather poorly, they do spread more than B2a1L or B2a1 cells that express lower levels of the truncated α5.

Other laboratories have delineated an important role for the β chain cytoplasmic domain in integrin function. For example, Hayashi et al. (1990) found that deleting five or more amino acids from the carboxy terminus of the β1 subunit significantly impaired integrin function, in terms of failure to promote adhesion or to permit integrin co-localization in focal adhesion plaques. Similar results were found by Hibbs et al. (1991) in their experiments truncating the β subunit of LFA-1. Our studies indicate that the situation may be more complex for α chains. Thus, essentially complete truncation of the α5 cytoplasmic domain seems to have little effect on functions such as cell adhesion or integrin-mediated tyrosine phosphorylation. Partial truncation, as in the B2a10 clonal line, also seems to have little effect on the cell's ability to rapidly organize the cytoskeleton or to be motile. However, truncation of the portion of the α5 subunit immediately adjacent to the transmembrane domain, as in the B2a1 clonal line, does seem to produce marked effects on the cell. At minimum, truncation of this region seriously retards the ability of the cell to organize its cytoskeleton and to spread on the substratum; effects on motility are also seen.

Cells seem somewhat more tolerant of modifications of α chain cytoplasmic domains than of modification of corresponding regions in β chains. For example, when Hibbs et al. (1991) truncated the α chain cytoplasmic domain of LFA-1, little effect was noted. This seems consistent with the observations of LaFlamme et al. (1992) who prepared chimeras of the external domain of IL-2 receptor and integrin α or β cytoplasmic domains; these investigators noted that the β subunit chimeras were capable of focal contact localization, while α chimeras were not. Thus our observations, along with those of Hibbs et al. (1991) and LaFlamme et al. (1992), suggest that α chain cytoplasmic domains may not be essential for several important integrin-mediated cellular functions. Our own observations show that partial truncation of the α5 cytoplasmic domain, as in the B2a10 cells, can be tolerated with little recognizable impact on cell function. Truncated of virtually the entire cytoplasmic domain, as in the B2a1 cells, still permits efficient adhesion and presumptive signaling paths mediated by tyrosine phosphorylation; however, these cells are deficient in their ability to rapidly deploy the cytoskeleton.

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