A Point Mutation of Integrin β_1 Subunit Blocks Binding of $\alpha_5\beta_1$ to Fibronectin and Invasin but not Recruitment to Adhesion Plaques

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Abstract. A point mutation in a highly conserved region of the β_1 subunit, Asp^{130} to Ala (D130A) substitution, abrogates the Arg-Gly-Asp (RGD)-dependent binding of $\alpha_5\beta_1$ to fibronectin (FN) without disrupting gross structure or heterodimer assembly. The D130A mutation also interferes with binding to invasin, a ligand that lacks RGD sequence. In spite of the lack of detectable FN binding by $\alpha_5\beta_1$(D130A), it was recruited to adhesion plaques formed on FN by endogenous hamster receptors. Thus, intact ligand binding function is not required for recruitment of $\alpha_5\beta_1$ to adhesion plaques. Overexpression of β_1(D130A) partially interfered with endogenous $\alpha_5\beta_1$ function, thus defining a dominant negative β_1 integrin mutation.

THE integrins comprise a family of at least six heterodimers of cell surface receptors which share common β_1 subunits but have distinct α subunits. They recognize multiple ligands (for review see Hemler, 1990) including fibronectin (FN)[1], laminin, collagen, epiligrin (Carter et al., 1991), invasin (Isberg and Leong, 1990), and vascular cell adhesion molecule-1 (Elices et al., 1990), and mediate cell–cell and cell–extracellular matrix interactions. The identification of the ligand binding domain of β_1 integrins is important for understanding their functions. Chemical cross-linking studies of Arg-Gly-Asp (RGD)-containing peptides to the RGD-dependent β_3 integrins, platelet $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) and $\alpha_v\beta_3$ (vitronectin receptor), have shown that RGD-containing peptide cross-links to residues 109–171 of β_3 of $\alpha_{IIb}\beta_3$ (D'Souza et al., 1988), and to an overlapping region (residues 61–203) of the β_3 of $\alpha_v\beta_3$ (Smith and Cheresh, 1988). In addition, β_3 of $\alpha_{IIb}\beta_3$ from a thrombasthenic patient has a point substitution at Asp^{119} to Tyr, which inactivates $\alpha_{IIb}\beta_3$ binding to fibrinogen (Loftus et al., 1990), underscoring the importance of this region in ligand–β_3 integrin interaction.

Based on the high similarity of this region (residues 109–171 of β_3) among integrin β subunits, especially the smaller region consisting of residues 108–127 (76 and 81%, respectively), compared to overall similarity (~45%), we hypothesized that the corresponding region in β_1 is also involved in the ligand–β_1 integrin interaction. To address this hypothesis, we examined the effects of the substitution of Asp^{130} of β_1 (which corresponds to the Asp^{119} in β_3) to Ala (D130A substitution) on $\alpha_5\beta_1$ functions. $\alpha_5\beta_1$ binds to the 110K cell binding domain of FN in an RGD-dependent manner (Pytela et al., 1985), as well as to invasin, a protein involved in the entry of the bacterial cells into eukaryotic cells (Isberg and Leong, 1990). The affinity of $\alpha_5\beta_1$ for invasin is two orders of magnitude higher than for FN (Isberg, 1991; Nhieu and Isberg, 1991). In addition, the COOH-terminal 192 amino acid residues of invasin, which contain the integrin binding region, do not contain RGD sequence.

We report here that the D130A substitution in the β_1 subunit blocks the binding of $\alpha_5\beta_1$ both to the FN 110K cell binding fragment and to invasin. Overexpression of β_1(D130A) interfered with the endogenous $\alpha_5\beta_1$ function. Furthermore, ligand-binding defective β_1(D130A) was assembled into the adhesion plaques formed in transfected cells cultured on Fn, suggesting that intact ligand binding is not essential for the recruitment of this receptor to existing adhesion plaques.

Materials and Methods

Materials

FN was purified from fresh human plasma by using gelatin Sepharose (Engval and Ruoslahti, 1977; Plow et al., 1979). FN 110K fragment was obtained by chymotryptic digestion of FN and subsequent gelatin-Sepharose and Sephacryl S-200 gel filtration chromatographies (Pierschbacher et al., 1982). MBP-Inv479 was prepared by using pJL309 plasmid in *Escherichia coli* MC1000 which was provided by R. Isberg (Tufts University, Boston, MA) (Leong et al., 1990). FN 110K fragment and MBP-Inv479 was coupled to CNBr-activated Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) at 2.4 and 2.0 mg/ml packed volume, respectively, according to the manufacturer's instructions. PMSF and octyl-β-D-glucopyranoside were purchased from Calbiochem Corp. (La Jolla, CA). *N*-Ethylmaleimide was purchased from Sigma Chem. Co. (St. Louis, MO), and $Na^{125}I$ from Amersham Corp. (Arlington Heights, IL).

mAb 7E2 (to hamster β_1) and PB1 (to hamster α_5) (Brown and Juliano, 1985, 1988) were from R. L. Juliano (University of North Carolina, Chapel Hill, NC). mAbs to human β_1 were obtained from the following sources: A1A5 from M. E. Hemler (Dana-Farber Cancer Institute, Boston, MA); mAb13 (Akiyama et al., 1989) from K. Yamada (NIH, Washington, DC); LM534 (Cheresh et al., 1989) from D. Cheresh (The Scripps Research In-

1. *Abbreviations used in this paper*: DHFR, dihydrofolate reductase; FN, fibronectin; MBP-Inv479, maltose binding protein-invasin fusion protein.

stitute), and QE.2E5 (Faull, 1991) from R. Faull (The Scripps Research Institute). 102DF5 was prepared as described (Ylänne and Virtanen, 1989). Goat anti-human β_1 antiserum (antibody 172) was prepared by immunizing a goat with purified human $\alpha_4\beta_1$ preparation, and recognizes human but not hamster β_1. Anti-denatured β_1 antiserum (cross-reactive to both human and hamster β_1) was prepared by immunizing rabbits with the SDS-denatured $\alpha_1\beta_1$ preparation purified from human placenta (Takada et al., 1987). The serum recognizes human α_1 and β_1, and mouse and hamster β_1. Anti-α_3 cytoplasmic peptide antibody (reactive to human, mouse, and chicken α_3 [Solowska et al., 1989; Takada et al., 1991]) and anti-α_5 cytoplasmic peptide antibody were obtained from E. Marcantonio (Massachusetts Institute of Technology, Cambridge, MA). The anti-denatured human α_4 antibody (reactive to human and mouse α_4 [Holzman et al., 1989]), was prepared as described (Hemler et al., 1987; Takada et al., 1989). The anti-α_2 cytoplasmic peptide antibody was prepared as described (Takada and Hemler, 1989).

Construction of β_1 cDNA Expression Vector and Mutagenesis

The human β_1 cDNA clone (B-3) was cloned from human endothelial λgt11 library (obtained from T. Collins, Brigham and Women's Hospital, Boston, MA) with partial β_1 cDNA (obtained from E. Ruoslahti, La Jolla Cancer Research Foundation, La Jolla, CA) as probe. The clone B-3 (~3.55 kb) includes a 2,334-bp complete coding region, and 58-bp 5′-noncoding and 1,100-bp 3′-noncoding regions. The amino acid sequence deduced from the nucleotide sequence of the clone B-3 was the same as the published sequence (Argraves et al., 1987) except that His^{92} was Thr and Thr^{195} was Ser in clone B-3. Oligonucleotide-directed mutagenesis of β_1 cDNA clone B-3 in pBluescript KS+ was carried out using a combination of the primer extension method (Zoller and Smith, 1982), and the method of Kunkel et al. (1987) with synthetic oligonucleotide, 5′-ACTACCTTATGGCCCTGTCTTATTC-3′ (YLMALSYS) based on the published β_1 sequence 5′-ACTACCTTATGGACCTGTCTTATTC-3′ (YLMDLSYS) as primer. The whole coding region of the mutagenized cDNA was sequenced and the absence of other mutations was confirmed. The wild-type β_1 or mutagenized β_1 (D130A) cDNA in pBluescript was digested with XbaI (XbaI site is in the vector) and NheI (NheI site is in a 3′ noncoding region in cDNA). After gel purification, the 2.9-kb XbaI/NheI fragment containing whole coding region was ligated to XbaI-digested/calf intestinal phosphatase-treated pCDneo vector (Aruffo and Seed, 1987).

Transfection and Amplification of the Transfected Gene

DG44 cells, a dihydrofolate reductase (DHFR)-deficient mutant of CHO cell line (Urlaub and Chasin, 1980) (obtained from L. A. Chasin, Columbia University, NY), were maintained in the α-modified minimal essential medium (α-minus MEM, Sigma Chem. Co.) supplemented with 10% heat-inactivated FCS, hypoxanthine, and thymidine in a 5% CO_2 incubator at 37°C. β_1 or β_1(D130A) cDNA in pCDneo vector (10 μg) and 1 μg of pLTRdhfr26 plasmid (Subramani et al., 1981) (obtained from Amer. Type Culture Collection, Rockville, MD) were cotransfected into DG44 cells (10^7 cells) by electroporation. Transfected cells were maintained for 3 d in the above medium, and then transferred to α-minus MEM supplemented with 10% dialyzed FCS and 700 μg/ml G418 (Gibco Laboratories, Grand Island, NY). After 10–14 d, resulting colonies were harvested and cells expressing human β_1 were collected by sorting with mAb A1A5 in FACStar (Becton Dickinson Immunocytometry Sys., Mountain View, CA). mAb A1A5 reacts with both human β_1 and β_1(D130A) (Fig. 1, *e* and *f*). The transfected genes were then amplified in α-minus MEM supplemented with 10% dialyzed FCS, 700 μg/ml G418, and the increasing concentration (up to 100 nM) of the DHFR inhibitor methotrexate (Kaufman and Sharp, 1982).

FN 110K Fragment–Sepharose Affinity Chromatography

Cells were harvested by washing the attached cells with PBS (0.15 M NaCl, 10 mM potassium phosphate, pH 7.4) twice and incubating in 3.5 mM EDTA in PBS at room temperature for 3 min, and washed twice with PBS. Cells (~5 × 10^6) were then surface labeled with ^{125}I by using iodogen (Pierce Chem., Rockford, IL) (Braciale et al., 1986), washed three times with PBS, and solubilized in 0.4 ml of 100 mM octyl-β-D-glucopyranoside in 10 mM Tris-HCl/0.15 M NaCl (TBS), 1 mM $MnCl_2$, 1 mM PMSF, 1 mM *N*-ethylmaleimide, pH 7.4, at 4°C for 1 h. The insoluble materials were removed by centrifugation at 15,000 *g* for 10 min. The supernatant was then incubated with a small amount of underivatized Sepharose 4B at 4°C for 1 h to remove nonspecific binding material. The supernatant was incubated with 1 ml of packed FN 110K fragment-Sepharose at 4°C overnight, which had been equilibrated with TBS containing 1 mM $MnCl_2$, 1 mM PMSF, 1 mM *N*-ethylmaleimide, 25 mM octyl-β-D-glucopyranoside, pH 7.4 (washing buffer). The unbound materials were washed with 20 ml of washing buffer and the bound materials were eluted with 20 mM EDTA instead of 1 mM $MnCl_2$ in washing buffer, and 2.5-ml fractions were collected. 0.25-ml aliquots of the peak fraction (2.5 ml) of the eluted radioactivity were used for immunoprecipitation. The samples were precleared by incubating with protein G–Sepharose and immunoprecipitated as described below.

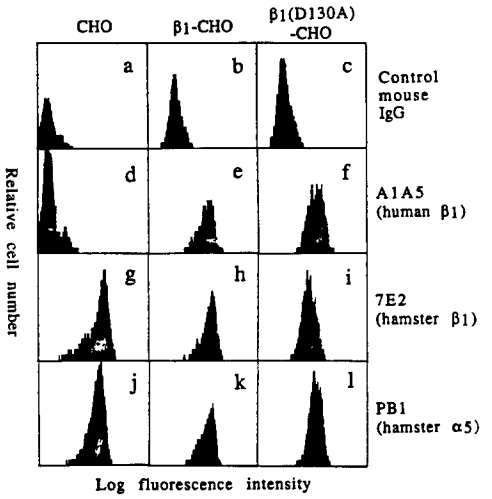

Figure 1. The expression of human β_1 or β_1(D130A) in CHO cells. The β_1-, β_1(D130A)-, or parent CHO cells were harvested and incubated with the first antibodies (*a–c*, control purified mouse IgG [10 μg/ml]; *d–f*, A1A5 [anti-human β_1]; *g–i*, 7E2 [anti-hamster β_1]; or *j–l*, PB1 [anti-hamster α_5]), and then with the FITC-labeled goat anti-mouse IgG. Ascites A1A5, 7E2, and PB1 were diluted 500-fold and used. The stained cells were analyzed by FACStar (Becton Dickinson Immunocytometry Sys.) The mean fluorescent intensities were (*a*) 4.1, (*b*) 14.2, (*c*) 15.1, (*d*) 5.2, (*e*) 168, (*f*) 174, (*g*) 221, (*h*) 210, (*i*) 98.4, (*j*) 170, (*k*) 174, and (*l*) 168.

Maltose Binding Protein–Invasin Fusion Protein (MBP-Inv479)-Sepharose Affinity Chromatography

The MBP-Inv479-Sepharose affinity chromatography was carried out as described above for FN 110K–Sepharose chromatography except that (*a*) 50 μl packed volume of MBP-Inv479-Sepharose was used, (*b*) unbound materials were removed by washing three times in 1 ml of washing buffer, and (*c*) 500 μl of 20 mM EDTA in washing buffer was used for elution.

Immunoprecipitation of Cell Extracts

Cells were ^{125}I surface-labeled, and washed as described above and solubilized in 1 ml of 20 mM Tris, 0.15 M NaCl, 1% Triton X-100, 0.05% Tween 20, pH 7.4, containing 2 mM PMSF, 10 mM benzamidine HCl, 5 U/ml trasylol, and 0.02% NaN_3 (IPB), and incubated on ice for 1 h and centrifuged at 15,000 *g* for 10 min. The supernatant was precleared by incubating with protein G–Sepharose (Pharmacia LKB Biotechnology Inc.) for 30 min at 4°C and incubated with antibody for 1 h at room temperature. The immune complex was recovered by incubating with protein G–Sepharose for 30 min at 4°C, washed three times with 1 ml IPB, boiled for 5 min in 50 μl of SDS-PAGE sample buffer, and analyzed by SDS-PAGE (7% gel) (Laemmli, 1970) under nonreducing conditions.

Adhesion of Cells to FN-coated Plates

Wells of the Immulon 2 removable 96-well plates (Dynatech Labs., Inc.,

Chantilly, VA) were incubated with 100 μl of PBS containing different concentrations of FN overnight at 4°C. The other protein binding sites were blocked by incubating with 100 μl of 1% BSA for 30 min at room temperature, and wells were washed twice with PBS. Cells were harvested with TPCK trypsin (Worthington Biochem. Corp., Freehold, NJ), 3.5 mM EDTA in PBS, washed once with 0.5 mg/ml soybean trypsin inhibitor (Sigma Chem. Co.), and washed twice with incubation buffer (137.5 mM NaCl, 12 mM $NaHCO_3$, 2.6 mM KCl, 5 mM Hepes, 5 mM glucose, 2 mM $CaCl_2$, 0.1% BSA). 3×10^6 cells in 200 μl incubation buffer were incubated with 50 μCi $Na^{51}Cr$ (12.5–25 Ci/mmol, Amersham Corp.) for 1 h at 37°C, and washed three times with incubation buffer. 4×10^4 cells (100 μl) were added to each well and incubated for 1 h at 37°C. The wells were washed three times with incubation buffer and then bound radioactivity was counted in a γ counter.

Indirect Immunofluorescence Microscopy

Cells were harvested with 0.05% trypsin, 0.5 mM EDTA (Irvine Scientific, Santa Ana, CA); washed once with α-minus MEM supplemented with 10% FCS, hypoxanthine, and thymidine; and then twice with the medium minus serum. Round glass coverslips were coated overnight at 4°C with 20 μg/ml of FN in PBS; then the other protein binding sites were blocked by incubating with 1% BSA for 30 min at room temperature. The harvested cells were plated onto the coverslips in the medium minus serum, incubated for 4 h at 37°C, and then fixed with methanol for 10 min in −20°C. The number of spreading cells were counted from three high power microscopic fields (28–144 cells were counted in each field). The fixed cells were incubated with the primary antibodies, and then with FITC-coupled goat anti-mouse IgG (Tago, Inc., Burlingame, CA), for 30 min at room temperature. The stained cells were observed in an Axiophot fluorescent microscope (Carl Zeiss, Inc., Oberkochen, Germany).

Results

Expression and Characterization of Human β_1 or β_1(D130A) in CHO Cells

The β_1 or β_1(D130A) cDNA was transfected into CHO cells. After selection by resistance to G418 and $DHFR^+$ phenotype, human β_1 or β_1(D130A) expression was further amplified by using increasing methotrexate, a DHFR inhibitor, concentration in the medium. Human β_1 or β_1(D130A) was expressed at levels approximately equal to that of endogenous hamster β_1 in CHO cells (Fig. 1). Both β_1 and β_1(D130A) were recognized by four other anti-human β_1 mAbs, in addition to A1A5–mAb13, LM534, QE.2E5, and 102DF5–but none of these mAbs recognized the endogenous hamster β_1 (data not shown).

Immunoprecipitations of the ^{125}I surface-labeled cell extracts (Fig. 2) showed that the human β_1 or β_1(D130A) (M_r

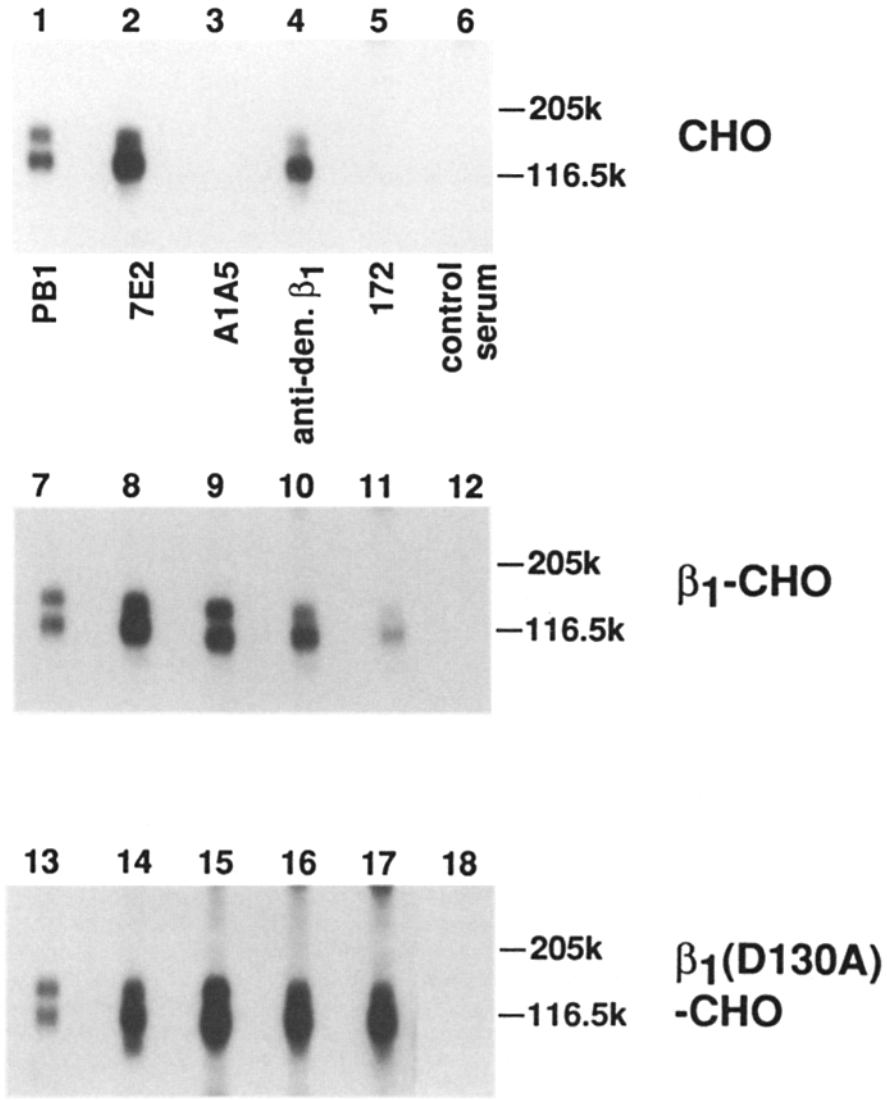

Figure 2. Immunoprecipitation of human β_1- or β_1(D130A)-CHO cells. The detergent extracts of the ^{125}I surface-labeled CHO cells (lanes *1–6*), β_1- (lanes *7–12*), or β_1(D130A)- (lanes *13–18*) CHO cells were immunoprecipitated with mAb PB1 (anti-hamster α_5) (lanes *1, 7, 13*), mAb 7E2 (anti-hamster β_1 (lanes *2, 8, 14*), mAb A1A5 (lanes *3, 9, 15*), anti-denatured β_1 (nonspecies specific (lanes *4, 10, 16*), antibody 172 (anti-human β_1) (lanes *5, 11, 17*), or rabbit preimmune serum (lanes *6, 12, 18*). The immunoprecipitated materials were analyzed by SDS-PAGE (7% gel) under nonreducing conditions.

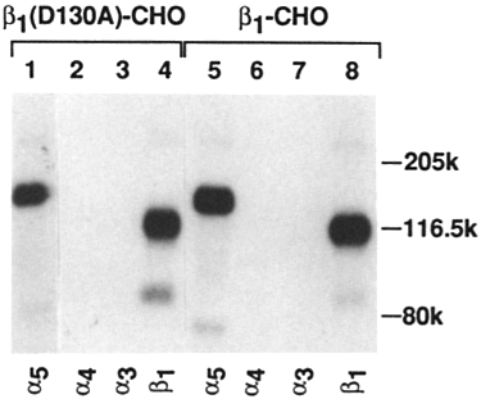

Figure 3. Human β_1 or β_1(D130A) is associated with endogenous α_5 in CHO cells. A detergent extract of ^{125}I surface-labeled β_1- or β_1(D130A)-CHO cells was first immunopurified with human β_1-specific A1A5-Sepharose overnight at 4°C. After washing the A1A5-Sepharose, the bound materials were recovered by boiling in SDS-PAGE sample buffer containing 1% (wt/vol) SDS for 5 min. The recovered material was diluted fivehold with IPB (see Materials and Methods), and again immunoprecipitated with anti-α_5 cytoplasmic peptide antibody (lanes *1* and *5*), anti-denatured α_4 polyclonal antibody (lanes *2* and *6*), anti-α_3 cytoplasmic antibody (lanes *3* and *7*), or anti-denatured β_1 polyclonal antibody (lanes *4* and *8*). The immunoprecipitated materials were analyzed as described in the legend of Fig. 1.

110K, nonreduced) associated with endogenous hamster α subunits (M_r 150K, nonreduced) in both β_1-CHO (lanes *9* and *11*) and β_1(D130A)-CHO (lanes *15* and *17*). Anti-denatured β_1 recognized both hamster β_1 and human β_1/β_1(D130A) (lanes *4*, *10*, and *16*). Antibody 172 recognized human β_1/β_1(D130A) but not hamster β_1 (lanes *5*, *11*, and *17*).

We undertook studies to identify the α subunit associated with the recombinant human β_1/β_1(D130A). CHO cells contain $\alpha_5\beta_1$ as the major β_1 integrin species with a small amount of $\alpha_3\beta_1$ (Giancotti and Ruoslahti, 1990). α_5-deficient CHO cells lose ~80% of β_1 integrin expression (Schreiner et al., 1989), and CHO cells express similar quantities of α_5 and β_1 (Fig. 1). Consistent with these, α_1 or α_2 (which are larger in size than other α subunits) was not detected in CHO cells by immunoprecipitation with anti-β_1 (Fig. 2) or anti-α_2 cytoplasmic peptide antibody (Takada and Hemler, 1989) (Fig. 4, lanes *15* and *21*). To determine whether the transfected human β_1 or β_1(D130A) is associated with α_5 and/or α_3, extracts of surface ^{125}I-labeled transfected cells were immunopurified with human β_1-specific mAb A1A5 immobilized to Sepharose. The recovered recombinant β_1 integrins were solubilized and then reimmunoprecipitated with antibodies that react with multiple species α subunits. The anti-α_5 cytoplasmic domain antibody precipitated the α subunit associated with human β_1

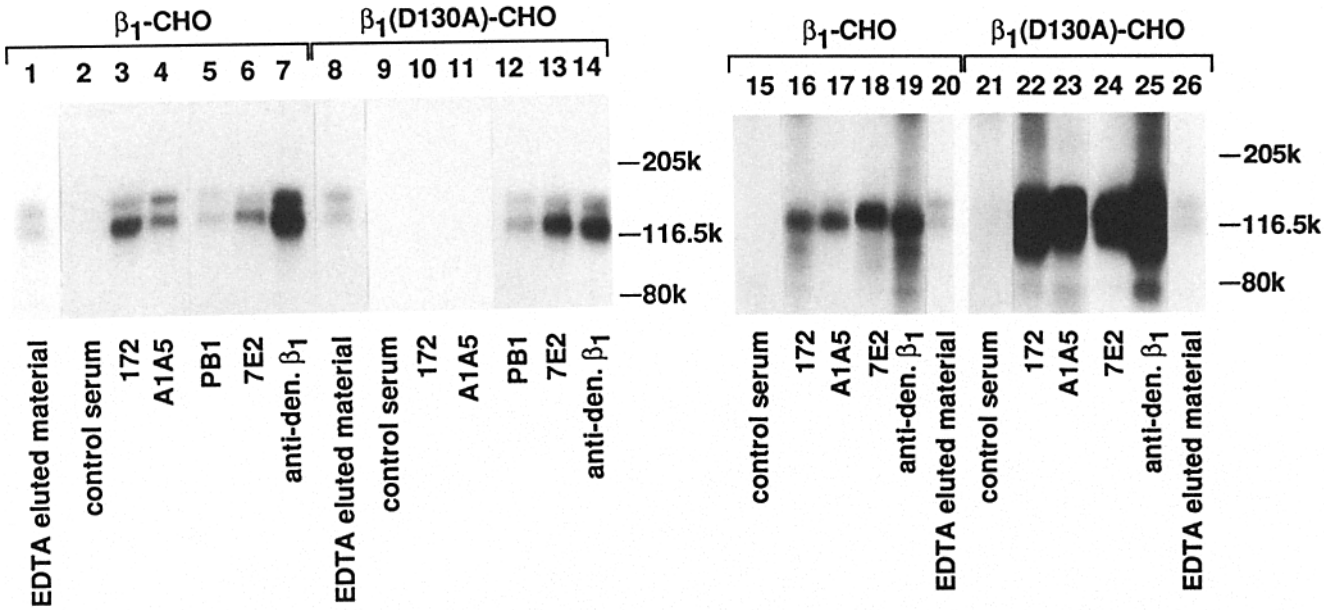

Figure 4. $\alpha_5\beta_1$ but not $\alpha_5\beta_1$(D130A) binds to cell-binding domain of FN. Detergent extracts of ^{125}I surface-labeled CHO cells bearing $\alpha_5\beta_1$ or $\alpha_5\beta_1$(D130A) were passed through immobilized FN 110K (central cell-binding domain) affinity columns as described in Materials and Methods. 250-μl aliquots of the peak EDTA-eluted fraction (2.5 ml) from the FN 110K Sepharose column with β_1 (lanes *1–7*) or β_1(D130A) (lanes *8–14*) CHO extract, or the peak pass-through fraction (2.5 ml) with β_1 (lanes *15–20*) or β_1(D130A) (lanes *21–26*) CHO extract were analyzed by immunoprecipitation with anti-human β_1 polyclonal (*172*) (lanes *3*, *10*, *16* and *22*) or monoclonal A1A5 (lanes *4*, *11*, *17* and *23*) antibodies; anti-hamster α_5 monoclonal (PB1) (lanes *5* and *12*); anti-hamster β_1 monoclonal (*7E2*) (lanes *6*, *13*, *18* and *24*); anti-denatured β_1 (nonspecies-specific) polyclonal (lanes *7*, *14*, *19* and *25*); or irrelevant polyclonal antibody (antiintegrin α_2 cytoplasmic domain) (lanes *2*, *9*, *15* and *21*). The EDTA-eluted material (20 μl each) from the FN 110K-Sepharose with β_1- or β_1(D130A)-CHO extract were also analyzed without immunoprecipitation (lanes *1* and *20*, and *8* and *26*, respectively). The samples were analyzed by SDS-PAGE (7% gel) under nonreducing conditions. (Lanes *1–14*) The EDTA eluate from β_1-CHO contained hamster α_5 and β_1, and human β_1. The eluate from the β_1(D130A)-CHO contained hamster α_5 and β_1, but no human β_1(D130A). (Lanes *15–26*) The human β_1 and β_1(D130A) were present in the unbound fractions.

while the anti-α_3 and anti-α_4 antibodies did not produce detectable immunoprecipitates (Fig. 3). Thus, the recombinant human β_1 or β_1(D130A) was associated with endogenous hamster α_5.

$\alpha_5\beta_1$(D130A) Does Not Bind to FN 110K Fragment

The previous experiments established that both human β_1 and β_1(D130A) were expressed as heterodimers with hamster α_5. Since FN is a major ligand for $\alpha_5\beta_1$, we assessed the binding of these recombinants to the immobilized cell binding domain of FN. When extracts of surface ^{125}I-labeled CHO cells bearing recombinant β_1 integrins were subjected to FN 110K affinity chromatography, the bound fraction (EDTA eluate) contained only two ^{125}I-labeled protein bands corresponding to α_5 and β_1 subunits in size (M_r 150K and 110K, nonreduced) (Fig. 4, lanes *1* and *8*, for β_1-CHO and β_1(D130A)-CHO cells, respectively). Immunoprecipitation of the EDTA eluate (Fig. 4) showed that the eluate from β_1-CHO contained hamster α_5 (lane *5*) and hamster (lane *6*) and human (lanes *3* and *4*) β_1. The eluate from the β_1(D130A)-CHO contained hamster α_5 (lane *12*) and β_1 (lane *13*), but no human β_1(D130A) (lanes *10* and *11*). The human β_1 and β_1(D130A) were detected in the unbound fractions by immunoprecipitation (Fig. 4, lanes *16* and *17*; *22* and *23*, respectively). Thus, $\alpha_5\beta_1$(D130A) had no detectable affinity for the FN 110K fragment.

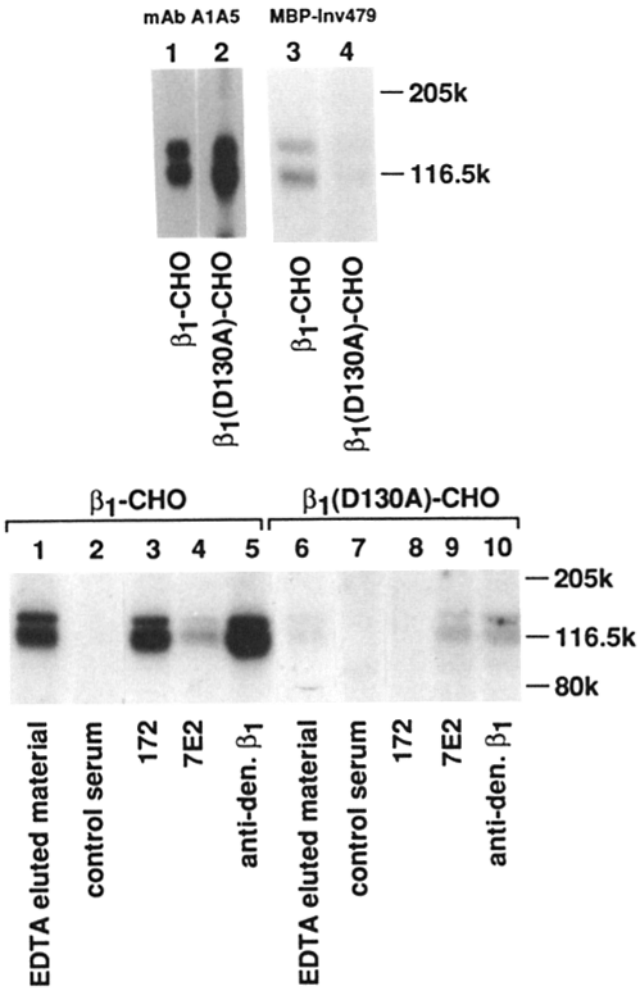

Figure 5. $\alpha_5\beta_1$ but not $\alpha_5\beta_1$(D130A) binds to MBP-Inv479-Sepharose. (*Top*) Immunoprecipitation of 200 μl of the same detergent extract of β_1- (lane *1*) or β_1(D130A)-CHO (lane *2*) cells as in Fig. 2 by A1A5 (human-specific anti-β_1). The detergent extracts were incubated with 50 μl of MBP-Inv479-Sepharose in the presence of 1 mM Mn^{2+}, and washed; and the bound material was eluted with 20 mM EDTA in the column buffer (lanes *3* and *4*). Samples were analyzed by SDS-PAGE (7% gel) under nonreducing conditions. The density of two bands of M_r ~110 kD and 150 kD was lower with β_1(D130A)-CHO cells in four separate experiments (lane *4*). (*Bottom*) The bound fraction (Fig. 5, *top*) from β_1 (lanes *1–5*) or β_1(D130A) (lanes *6–10*) immunoprecipitated with preimmune rabbit serum (lanes *2* and *7*); antibody 172 (lanes *3* and *8*); mAb 7E2 (lanes *4* and *9*); and anti-denatured β_1 (lanes *5* and *10*). 20 μl each of the EDTA fraction from β_1- or β_1(D130A)-CHO cells was analyzed without immunoprecipitation in lanes *1* and *6*, respectively. Samples were analyzed by SDS-PAGE (7% gel) under nonreducing conditions.

$\alpha_5\beta_1$(D130A) Does Not Bind to Invasin

FN binding to $\alpha_5\beta_1$ depends on the RGD sequence in the 10th type III repeat (Pytela et al., 1985; Pierschbacher et al., 1982; Obara et al., 1988). To determine whether the D130A substitution abolished binding to a ligand that lacks RGD, we assessed the binding of $\alpha_5\beta_1$(D130A) to invasin. The ^{125}I surface-labeled cell extracts were incubated with MBP-Inv479-Sepharose in the presence of Mn^{2+}. SDS-PAGE analysis of the EDTA eluted materials detected two bands corresponding to α_5 and β_1 in size (Fig. 5). The EDTA eluates were analyzed by immunoprecipitation (Fig. 5, *bottom*). Both human and hamster β_1 (lanes *3* and *4*, respectively) were detected with wild-type β_1-CHO cells, while hamster β_1 (lane *9*), but no human β_1(D130A), was detected (lane *8*) with the β_1(D130A)-CHO cells. These findings show that D130A substitution of β_1 subunit blocks the binding of $\alpha_5\beta_1$ to invasin.

Overexpression of β_1(D130A) Produces Dominant Negative Effects

β_1(D130A) forms a binding-defective heterodimer with endogenous α_5 and may, therefore, block cell functions dependent on $\alpha_5\beta_1$. To test this possibility, the adhesion of β_1-CHO or β_1(D130A)-CHO cells to FN was examined (Fig. 6). The β_1(D130A)-CHO cells were less adherent to FN than the β_1-CHO or parent CHO cells (the amount of FN required for 50% of cells to adhere was 0.2 μg/well for β_1(D130A)-CHO cells and 0.1 μg/well for the others), reflecting the decrease in functional $\alpha_5\beta_1$ on the surface. At higher FN concentration (2 μg/well), the difference in adhesion among parent, β_1-CHO, and β_1(D130A)-CHO cells is relatively small. However, only 7.4 ± 1.5% of adhering β_1(D130A)-CHO cells spread in contrast to 56 ± 7.5 and 46 ± 8.1% of adherent CHO and β_1-CHO cells, respectively. Fig. 7 shows the striking difference in morphology between human β_1 and β_1(D130A) CHO cells on FN (2 μg/well). Thus, overexpression of β_1(D130A) reduces the function of endogenous $\alpha_5\beta_1$. The ratio of expression of β_1(D130A) mutant to endogenous wild-type β_1 integrin in β_1(D130A)-CHO cells was ~2:1 based on the mean fluorescent intensity (Fig. 1).

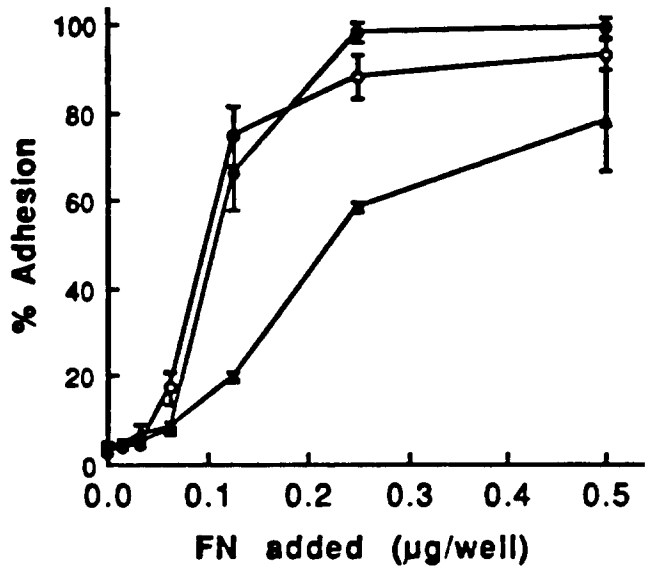

Figure 6. β_1(D130A)-CHO cells were less adherent to FN than CHO or β_1-CHO cells. Wells of the removable 96-well plates were coated with varying concentrations of FN. 4×10^4 ^{51}Cr-labeled cells (100 μl) were added to each well and incubated for 1 h at 37°C. The wells were washed three times with incubation buffer and then bound radioactivity was counted. Data are shown as means of triplicate experiments ± SD. CHO (○), β_1-CHO (●), β_1(D130A)-CHO (▲).

Binding-defective Human β_1(D130A) Is Assembled into Adhesion Plaques on FN

As noted above, a small percentage of β_1(D130A)-CHO cells spread on FN. To determine whether this ligand binding–defective mutant could be recruited to adhesion plaques formed by endogenous hamster $\alpha_5\beta_1$, we localized the human β_1 or β_1(D130A) in the transfectants that spread on the FN-coated glass coverslips (Fig. 8). In β_1-CHO cells, hamster β_1 (mAb 7E2, *b*) and human β_1 (mAbs 102DF5, *e*; QE.2E5, *h*; A1A5, *k*; LM534, data not shown) were localized in the adhesion plaques. In the spreading β_1(D130A)-CHO cells, human β_1(D130A) mutant was present in the adhesion plaques as detected by three antihuman β_1 mAbs (102DF5, *f*; QE.2E5, *i*; LM534, data not shown). This staining was specific for human β_1, since these three antibodies did not stain the hamster β_1-containing adhesion plaques formed by untransfected CHO cells (Fig. 8, *d* and *g*; data not shown for LM534). Surprisingly, the antihuman β_1, A1A5 did not stain the plaques in the β_1(D130A)-CHO cells (Fig. 8 *l*). Essentially, the same results were obtained also with CHO cells transiently expressing human β_1 or β_1(D130A) subunit on the surface after 48 h of transfection (data not shown). These findings indicate that β_1(D130A) mutant can be recruited to adhesion plaques formed by hamster $\alpha_5\beta_1$.

Discussion

This paper establishes that a point mutation in a highly conserved region of the β_1 subunit (Asp^{130} to Ala substitution) abrogates the RGD-dependent binding of $\alpha_5\beta_1$ to FN, and that the substitution also interferes with the recognition of invasin that lacks the RGD sequence, suggesting that the Asp^{130} is critical in multiple ligand–$\alpha_5\beta_1$ interactions. The Asp^{130} to Ala substitution of β_1 does not result in a major structural change in the whole β_1 molecule because (*a*) β_1(D130A) was expressed on the surface of CHO cells in association with endogenous α_5 subunits, and (*b*) five mAbs against human β_1 recognize β_1(D130A). Thus, the D130A mutation in β_1 abrogates $\alpha_5\beta_1$ ligand binding function without disrupting gross structure or heterodimer assembly.

Asp^{130} of β_1 corresponds to Asp^{119} of β_3, a residue reported to be critical in ligand (fibrinogen) binding to $\alpha_{IIb}\beta_3$ (Loftus et al., 1990). Since the protein ligands of $\alpha_{IIb}\beta_3$ contain RGD sequences, it is noteworthy that $\alpha_5\beta_1$(D130A) failed to recognize invasin, a ligand which lacks the RGD sequence (Isberg and Leong, 1990). Nevertheless, the binding of invasin to $\alpha_5\beta_1$ is inhibited by RGD peptide (Nhieu and Isberg, 1991). Taken together, these data are consistent with a common ligand binding mechanism for integrins irrespective of the presence of the RGD sequence in the ligand. The high degree of conservation of the sequence surrounding Asp^{130} is consistent with such a mechanism (Loftus et al., 1990).

Although $\alpha_5\beta_1$(D130A) was ligand binding defective, it was recruited into adhesion plaques on FN. This suggests that integrins can be recruited to adhesion plaques without binding ligands. Clustering of individual receptors and organization thereafter occurred only when cells were exposed to the specific ligand and the clustering preceded the association of vinculin with adhesion plaques and stress fiber formation (Dejana et al., 1988). Chicken β_1 lacking cytoplasmic domain does not enter adhesion plaques; however, it does bind to FN, suggesting that interaction with cytoskeletal components is necessary for the localization of the receptors to adhesion plaques (Solowska et al., 1989). Therefore, one possible sequence of events in the localization of receptor in adhesion plaques is (*a*) the interaction of the receptor with the specific matrix protein ligand, (*b*) clustering of the specific receptor species, and then (*c*) the interaction with cytoskeletal components. One possible explanation for the localization of binding-defective $\alpha_5\beta_1$(D130A) in adhesion plaques is that the initial interaction of intact hamster $\alpha_5\beta_1$ with FN induces clustering of the receptors, including both $\alpha_5\beta_1$ and $\alpha_5\beta_1$(D130A), leading to the incorporation of the β_1(D130A) into adhesion plaques. Consistent with this, LaFlamme et al. (1992) suggested that β_1 cytoplasmic domain has enough information to target $\alpha_5\beta_1$ to existing adhesion plaques, since IL-2 receptor with β_1 cytoplasmic domain was recruited to existing adhesion plaques on FN. However, we could not rule out the possibility that there could be some interaction of the $\alpha_5\beta_1$(D130A) with FN, leading to FN-dependent conformational changes, which allow for the recruitment of β_1(D130A) to focal adhesions. LaFlamme et al. (1992) also suggested that the ligand occupancy regulates the redistribution of the receptor via conformational change that unmasks the β_1 cytoplasmic domain or regions of the cytoplasmic domain that interact with specific cytoskeletal proteins, since RGD peptide promoted the recruitment of $\alpha_5\beta_1$ into adhesion plaques on laminin, an irrelevant ligand. Because ligand binding–defective $\alpha_5\beta_1$-(D130A) was recruited to adhesion plaques, it might have a conformation similar to that of a ligand-occupied form.

β_1(D130A) is detected in adhesion plaques of the transfected CHO cells cultured on FN with four different anti-

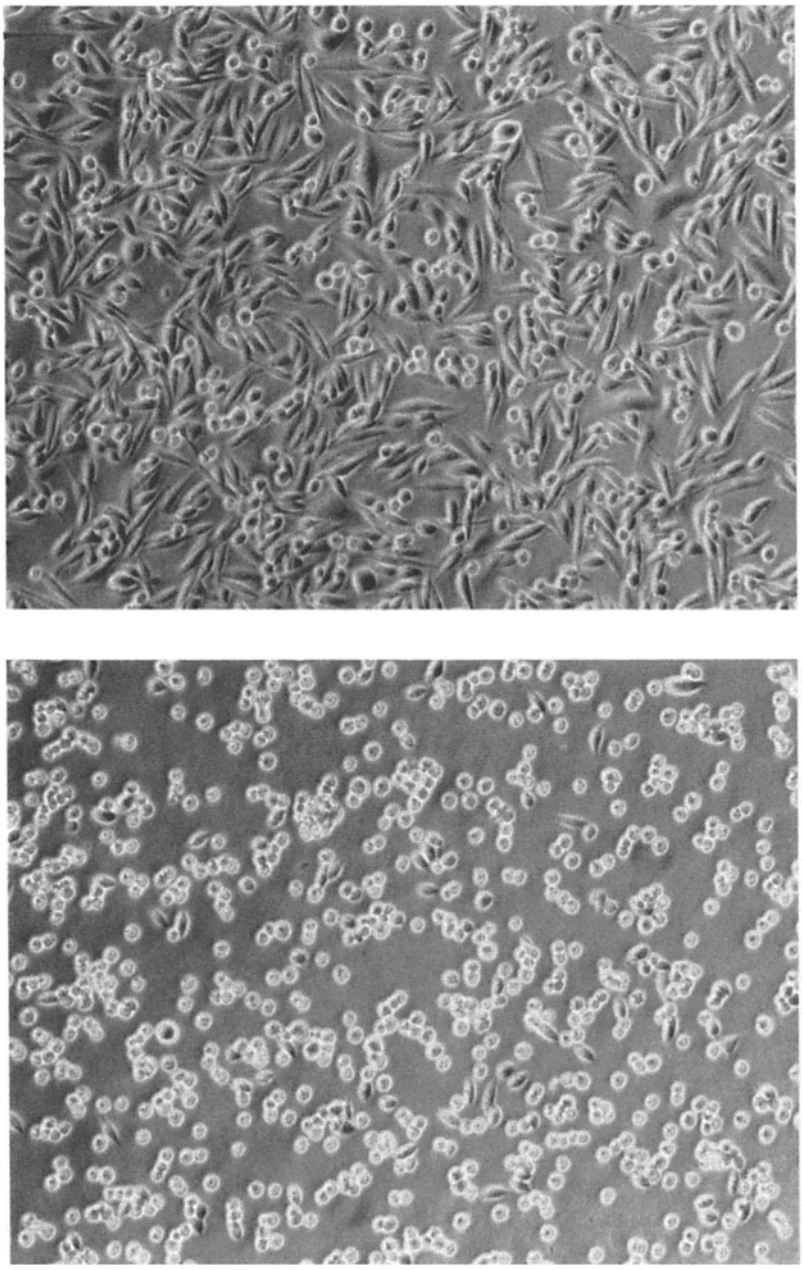

Figure 7. β_1-CHO cells spread on FN but β_1(D130A) CHO cells do not. Wells of the 96-well plates were coated with 100 μl of 20 μg/ml FN in PBS. 4×10^4 unlabeled cells (100 μl) were added to each well and incubated for 1 h at 37°C. Pictures were taken without washing the unbound cells. Details are described in Materials and Methods. (*Top*) Human wild-type β_1-CHO cells. (*Bottom*) Human β_1(D130A) CHO cells.

human β_1 mAbs but, not with mAb A1A5. One possible explanation of this data is that A1A5 epitope in β_1 is related to a site of receptor–receptor interaction during clustering and, therefore, its epitope is not accessible to antibody in adhesion plaques formed in β_1(D130A)-CHO cells because recruitment of β_1(D130A) to adhesion plaques is dependent on interactions that shield the epitope. In contrast, wild-type human β_1 may also be recruited by ligand binding and may express accessible A1A5 epitopes.

The ability to delete β_1 integrins would be very helpful for defining their roles in adhesion, migration, differentiation, metastasis, transformed phenotype, or signal transduction. Almost all cell lines express β_1 integrins in various combinations (for review see Hemler, 1990). While it is theoretically possible to make such deletions of β_1 in the germline of mice (Capecchi, 1989), deletion of β_1 may be lethal as in *Drosophila* (Mackrell et al., 1989). It is clear that dominant negative receptor mutants can provide insights into receptor function in vivo (Amaya et al., 1991; Ueno et al., 1991). β_1(D130A) is dominant negative since it forms nonfunctional heterodimers with endogenous α_5, leading to suppression of the endogenous β_1 integrin function. In the present study, the partial dominant-negative effects by the mutant β_1(D130A) was seen at the ratio of mutant-to-wild-type β_1 expression of $\sim$2:1 based on the mean fluorescent intensity, suggesting that the level of β_1 integrins expression is just enough to support adhesion and spreading on matrix protein in CHO cells. If the β_1(D130A) is overexpressed in cells with less endogenous β_1 integrins (e.g., lymphocytic cells) instead of fibroblastic CHO cells, higher mutant-to-wild-type ratio and therefore more complete dominant negative effects may be obtained. This suggests that tissue-specific ex-

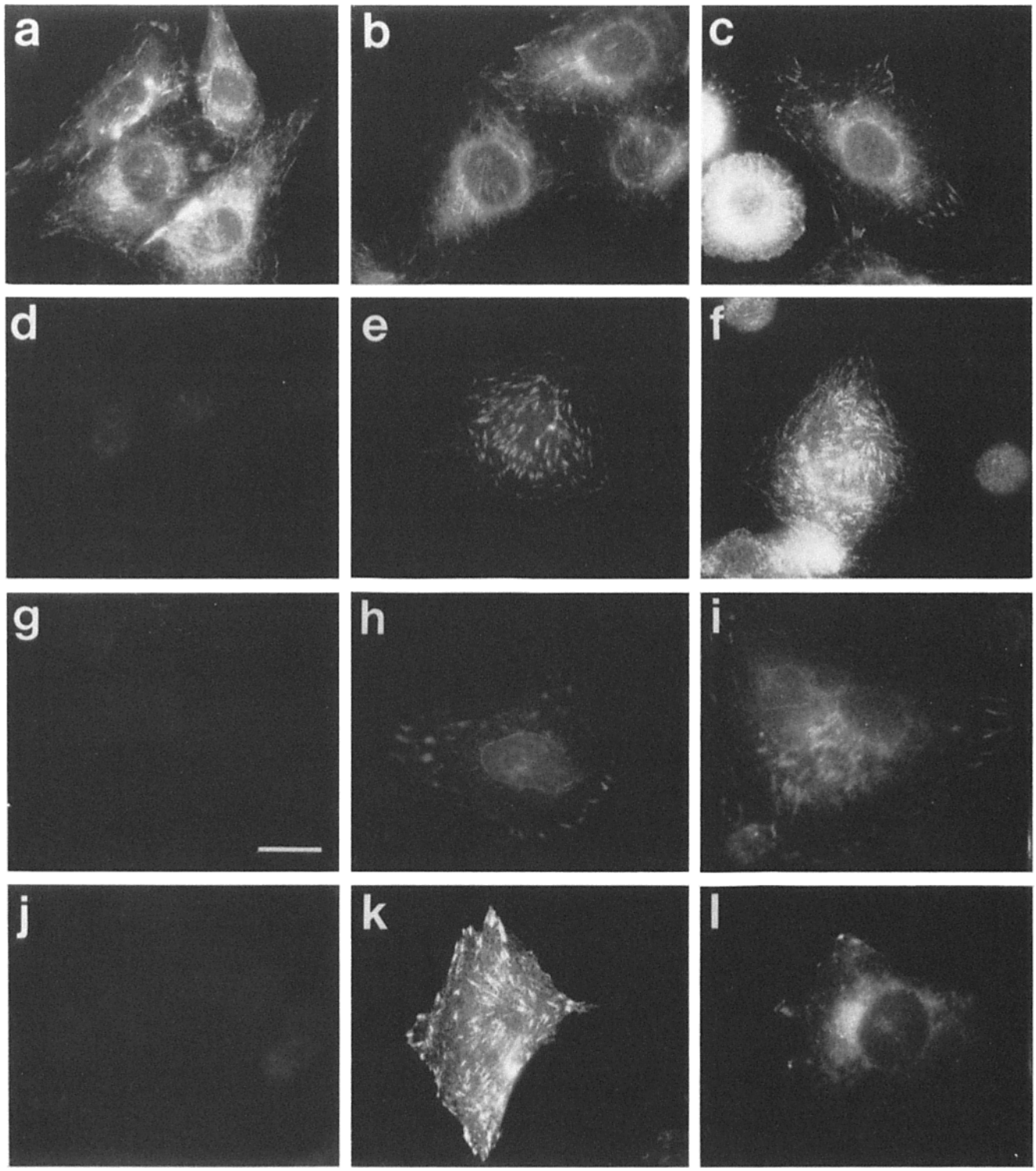

Figure 8. Immunohistochemical localization of β_1 or β_1(D130A) in adhesion plaques on FN. CHO (*a*, *d*, *g*, and *j*), β_1-CHO (*b*, *e*, *h*, and *k*), or β_1(D130A)-CHO (*c*, *f*, *i*, and *l*) cells were plated on FN, incubated for 4 h at 37°C, and then fixed with methanol as described in Materials and Methods. The fixed cells were incubated with primary antibodies (7E2, *a–c*; 102DF5, *d–f*; QE.2E5, *g–i*; A1A5, *j–l*), followed by incubation with FITC-labeled goat anti-mouse IgG, for 30 min at room temperature. Bar, 10 μm.

pression of dominant-negative β_1(D130A) in transgenic animals will provide a powerful means to assess the role of β_1 integrins in the development and function of differentiated tissues.

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