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A novel role for α3β1 integrins in extracellular matrix assembly

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SUMMARY

To study the biological role of α3β1 integrins in cell adhesion, migration, and in the deposition of extracellular matrix, we stably expressed the human α3 integrin subunit in the α4, α5 integrin deficient CHO cell line B2. The expression of α3β1 integrins enhanced cell adhesion on entactin (also known as nidogen), but not on fibronectin. Using recombinant GST-fusion proteins that span the entire length of the entactin molecule, we located cell adhesive activity to the G2 domain of entactin. These results suggest that the α3β1 integrin functions as an adhesion receptor interacting with the G2 domain of entactin. On the other hand, the expression of α3β1 integrins did not confer the ability to migrate on entactin. Strikingly, the expression of α3β1 dramatically increased the deposition of entactin and fibronectin into the pericellular matrix. This was accompanied by increased binding activity of the 29 kDa amino-terminal domain of fibronectin. Thus, similar to α5β1 integrins, α3β1 integrins can play an important role in modulating the assembly of pericellular matrices. However, unlike fibronectin deposition supported by α5β1, α3β1 supported fibronectin deposition into pericellular matrix was not inhibited by antibodies binding to the RGD containing cell adhesion domain of fibronectin, demonstrating that the two processes are mechanistically distinct. The role of α3β1 in pericellular matrix assembly potentially implicates this receptor in the assembly and/or recognition of entactin-containing pericellular matrices, an observation consistent with its apparent role in the renal glomerulus of the mammalian kidney.

Key words: entactin, fibronectin, integrin, matrix assembly

INTRODUCTION

Extracellular matrices play a major role in cell adhesion, migration, growth and differentiation as well as in the creation and maintenance of tissue architecture. During embryogenesis and other biological processes, such as the repair of tissue, the assembly of complex, polymeric matrices is both temporally and spatially regulated. Many extracellular matrix components (e.g. collagen and laminin) self-assemble into homo- and heteromeric polymers and networks in vitro (Yurchenco, 1994). However, cells also play a critical role in modulating the assembly of pericellular matrices (McDonald, 1988; Mosher et al., 1992).

Previous studies have demonstrated that α5β1 integrin functions in an early and essential step in the assembly of fibrillar fibronectin matrices by a number of different cultured cells. Fibronectin fragments containing the α5β1 binding site or antibodies to the α5β1 binding site in fibronectin inhibited fibronectin matrix assembly in cultured fibroblasts and developing amphibian embryos (McDonald et al., 1987; Darribere et al., 1992). In addition, antibodies to β1 integrin, and antibodies to α5 integrin to a lesser degree, reduced the deposition of fibronectin into extracellular matrix (Akiyama et al., 1989; Roman et al., 1989; Fogerty et al., 1990). When binding of both sites to the cell surface is inhibited, fibronectin matrix assembly by cultured fibroblasts is essentially abolished (Roman et al., 1989). The participation of α5β1 integrins in fibronectin matrix assembly has been extensively studied in Chinese hamster ovary (CHO) cells. Overexpressing α5β1 in CHO cells with endogenous α5β1 increased fibronectin deposition in extracellular matrix (Giancotti and Ruoslahti, 1990). The CHO B2 line is a particularly useful model, as it does not express α5β1 integrin, and attaches and migrates poorly on fibronectin. CHO B2 cells cannot assemble plasma fibronectin into the extracellular matrix (Wu et al., 1993). Reconstituting α5β1 integrin expression by transfecting the cells with a full length cDNA encoding the human α5 chain completely restored fibrillar fibronectin matrix assembly (Wu et al., 1993).

Although compelling evidence links α5β1 integrin with fibronectin matrix assembly in cultured cells, mechanisms for assembling a fibronectin matrix in the absence of α5β1 clearly exist. In particular, fibroblastic cells derived from α5 integrin null mutant embryos assembled a fibronectin matrix in vitro (Yang et al., 1993). Moreover, recombinant fibronectins mutated to lack the α5β1 binding site are still incorporated into a pre-existing fibronectin-containing matrix (Schwarzbauer, 1991). Although it is not known how cells lacking α5β1 assemble a fibronectin-containing extracellular matrix, it is likely that other cell surface molecules bind fibronectin thereby bypassing the requirement for α5β1 integrin in fibronectin matrix assembly.

In addition to α5β1, fibronectin interacts with several other

integrins including α4β1, αvβ1, and αvβ6. However, neither α4β1 nor αvβ1 support fibronectin matrix assembly by CHO B2 cells. Their expression in α5 null CHO B2 cells enabled the cells to adhere on fibronectin, but not to assemble a fibronectin matrix (Zhang et al., 1993; Wu et al., 1995). The β1 integrin α3β1 (VLA-3) has also been implicated in mediating cell adhesion on fibronectin (Carter et al., 1990; Hemler et al., 1990; Elices et al., 1991). This integrin is expressed in many tissues including the renal glomeruli, basal cells of epidermis, and other epithelia (Fradet et al., 1984; Morhenn et al., 1985; Peltonen et al., 1989; Carter et al., 1990; Choy et al., 1990; Korhonen et al., 1990; Simon and McDonald, 1990; Beaulieu, 1992; Bartolazzi et al., 1993; Bosman, 1993; Patey et al., 1994). However, recent studies suggest that binding of α3β1 integrins to fibronectin is very weak (Sriramarao et al., 1993; Weitzman et al., 1993). Instead, α3β1 integrins appear more likely to function as receptors for basement membrane components including epiligrin/kalinin (Carter et al., 1991; Sonnenberg et al., 1993; Weitzman et al., 1993; Rousselle and Aumailley, 1994) and entactin (Dedhar et al., 1992). Entactin is a ubiquitous basement membrane glycoprotein of M_r 150,000 comprised of several structurally and functionally distinctive domains (Chung and Durkin, 1990; Mayer and Timpl, 1994). A larger (190,000 Da) molecule immunologically related to entactin has also been identified (Carlin et al., 1983). Entactin supports cell adhesion (Durkin et al., 1988; Mann et al., 1989; Chakravarti et al., 1990; Tsao et al., 1990), migration (Yelian et al., 1993) and chemotaxis (Senior et al., 1992) of a number of types of cells. In addition to binding to the cell surface, entactin also interacts with itself (Tsao et al., 1990) as well as with collagen IV (Aumailley et al., 1989; Fox et al., 1991), laminin (Carlin et al., 1983; Dziadek et al., 1985; Paulsson et al., 1987), perlecan (Battaglia et al., 1992), fibrinogen (Wu and Chung, 1991) and fibronectin (Dziadek et al., 1985; Wu et al., 1991; Hsieh et al., 1994). This extensive binding repertoire suggests that entactin may function as a bridging molecule during the assembly of extracellular matrices, and hence is a candidate for a role in fibronectin deposition.

To determine the biological role of α3β1 integrin in cell adhesion, migration, and in the deposition of extracellular matrix, we have stably expressed the human α3 integrin subunit in the α4, α5 deficient CHO cell line B2. We report that overexpressing α3β1 integrins in these cells conferred the ability to attach to entactin substrates, but not to fibronectin, and also dramatically increased the deposition of entactin and fibronectin into the pericellular matrix. Furthermore, we provide evidence that α3β1 integrin mediated deposition of fibronectin into pericellular matrix is mechanistically distinct from that mediated by α5β1 integrins.

MATERIALS AND METHODS

Cells and reagents

CHO B2 cells (Schreiner et al., 1989) and the mouse monoclonal anti-hamster β1 antibody 7E2 (Brown and Juliano, 1988) were generously provided by Dr R. L. Juliano (Department of Pharmacology, University of North Carolina, Chapel Hill, NC). The CHO B2 cells were grown and maintained in α-MEM (Gibco Laboratories, Grand Island, NY) containing 10% FBS (Atlanta Biologicals, Norcross, GA), and 1% antibiotic-antimycotic mixture (Sigma Chemical Co., St Louis, MO) in monolayer culture. Recombinant entactin (Tsao et al., 1990), GST fusion proteins containing G1 (amino acid residues 7 to 301), G2 (amino acid residues 301 to 647), E (amino acid residues 639 to 941) or G3 domain (amino acid residues 941 to 1217) of entactin (Hsieh et al., 1994), entactin mutants in which the RGD sequence (amino acid residues 672 to 674) is deleted or replaced with RGE (Senior et al., 1992), and rabbit anti-entactin polyclonal antibodies (Carlin et al., 1981) were produced as previously described. A Bluescript KSII+ vector containing the entire coding sequence of the human α3 cDNA (Bluescript KSII+/α3) (Takada et al., 1991) was provided by Dr Martin Hemler (Harvard Medical School, Boston, MA). The eukaryotic expression vector pcDNA3 was purchased from InVitrogen (San Diego, CA). Mouse monoclonal anti-α3 antibody J143 was kindly provided by Dr Kenneth O. Lloyd (Memorial Sloan-Kettering cancer center) or prepared from the J143 mouse hybridoma cell line purchased from the American Type Culture Collection (Rockville, MD). A polyclonal rabbit anti-α3 cytoplasmic domain antibody (AB85) was prepared in our laboratory against a synthetic peptide mimicking the carboxyl-terminal sequence of the human α3 cytoplasmic domain (CRIQPSETERLTDDY) (Takada et al., 1991) as described previously (Roman et al., 1989). FITC-conjugated goat anti-rabbit IgG antibodies and CY3-conjugated anti-mouse IgG+IgM antibodies were purchased from Sigma (St Louis, MO), Dako Corp. (Carpinteria, CA) or Jackson ImmunoResearch Lab (West Grove, PA). Biotin-X-NHS (water soluble) was from Calbiochem (San Diego, CA).

Construction of the α3 expression vector and transfection

A plasmid containing the sequence encoding the human α3 integrin subunit (Bluescript KSII+/α3 (Takada et al., 1991)) was digested with *Xba*I and the 3.47 kb fragment consisting of nucleotides 1-3470 encoding human α3 integrin was ligated into pcDNA3 expression vector cut with *Xba*I. The correct orientation of the resulting expression vector (pcDNA3/α3) was determined by restriction mapping with *Eco*RI and the sequence confirmed by DNA sequencing using a Sequenase^R version 2.0 DNA sequencing kit (UAB, Cleveland, OH).

CHO B2 cells (4×10^6) were suspended in 0.4 ml of RPMI 1640 medium containing 25 μg of pcDNA3/α3 linearized with *Bgl*II and 100 μg/ml of Salmon sperm DNA (Sigma, St Louis, MO). Cells were electroporated with a BTX 600 electroporator at 360 V and 600 μF. To create vector transfected control cells, CHO B2 cells were electroporated with 25 μg of pcDNA3 vector alone using identical conditions. Twenty four hours after transfection, the cells were placed in α-MEM supplemented with 10% BCS, 2 mM L-glutamine and 1 mg active geneticin/ml (G-418 sulfate, Gibco/BRL). Geneticin-resistant cells were cloned by limited dilution and the clones expressing human α3 integrin identified by immunofluorescent staining of the cells with monoclonal anti-human α3 antibody J143 as previously described (Wu et al., 1993).

Immunoprecipitation

Cell surface proteins of cells in one confluent, 100 mm tissue culture dish were biotinylated with biotin-X-NHS (0.2 mg/ml) and extracted with 0.25 ml of a solution containing 1% (v/v) Triton X-100, 10 mg/ml bovine hemoglobin, 140 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ in 50 mM HEPES (pH 7.36) containing 2 mM PMSF and 1× general protease inhibitor cocktail (Calbiochem, San Diego, CA). Aliquots (75 μl) of the cell extracts were incubated with monoclonal anti-α3 antibody J143 (5 μl of 0.625 mg/ml IgG), followed by precipitation with an anti-mouse κ chain antibody coupled to Sepharose 4B (20 μl of 50% suspension, Zymed, San Francisco, CA). The precipitated proteins were separated by non-reducing SDS-PAGE and transferred onto an Immobilon-p^R membrane (Millipore, Bedford, MA). The biotinylated cell surface proteins were detected using an avidin-biotinylated horseradish peroxidase complex (Vector Laboratories,

Burlingame, CA) and the ECL[R] detection system (Amersham, England) following the manufacturer's protocols.

Immunoblotting

CH0 B2 and B3 cells were lysed in lysis buffer (1% Triton X-100, 140 mM NaCl, 50 mM Tris-HCl, pH 8.0, 2 mM PMSF, 5 mM EDTA, 0.2 mM AEBSF, 10 mM leupeptin and 1 mM pepstatin). The cellular proteins were separated by non-reducing SDS-PAGE, transferred onto an Immobilon-p[R] membrane, and the α3 subunit was detected by immunoblotting with the Ab85 rabbit anti-α3 cytoplasmic domain antibody (20 μg IgG/ml), followed by horseradish peroxidase labeled anti-rabbit IgG (Amersham, England, 1:1,000 dilution). Immunolabeled polypeptides were detected using the ECL[R] detection system (Amersham, England) following the manufacturer's protocols.

Integrin analysis by flow cytometry

The expression of cell surface β1 integrins on CHO cells was estimated by indirect immunofluorescence using flow cytometric techniques similar to those described previously (Schreiner et al., 1989). The CHO cells were detached with 0.3 mM EDTA in 2.7 mM KCl, 137 mM NaCl, 1 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4 (PBS). After three washes with α-MEM, the cells were stained with mouse monoclonal anti-hamster β1 antibody 7E2 (160 μg/ml IgG in 10 mg/ml BSA in PBS) followed by staining with a FITC-conjugated rabbit anti-mouse IgG (Dako Corp., Carpinteria, CA). The cells were fixed with 1% paraformaldehyde in PBS and analyzed with a FACS Vantage Fluorescent Activated Cell Sorter (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Cell adhesion assay

Cell adhesion was performed in 96-well ELISA plates (Corning). The wells were coated with 40 μg/ml recombinant entactin or 10 μg/ml human plasma fibronectin in PBS at 37°C for at least one hour. Then, each well was incubated with 200 μl of 10 mg/ml heat treated BSA in PBS at 37°C for one hour or longer and rinsed with α-MEM (Gibco, Grand Island, NY). The cells were harvested with 0.3 mM EDTA in PBS, rinsed three times with α-MEM, and suspended to a final density of 3×10^5 cells/ml in α-MEM or α-MEM supplemented with 0.5 mM $MnCl_2$ as specified in each experiment. Cells (3×10^{4}) were added to each well of the 96-well ELISA plate and incubated for 90 minutes in a 37°C incubator under a 5% CO_2-95% air atmosphere. Under these experimental conditions, the maximal number of cells adhered is 3×10^4 cells/well or 933 cells/mm^2. The wells were rinsed twice with PBS, and the number of adherent cells determined by measuring endogenous *N*-acetyl-β-D-hexosaminidase activity as described previously (Landegren, 1984). The cell number was shown in initial experiments to be directly proportional to the absorbance in this assay.

To determine cell adhesion on GST-entactin fusion proteins and entactin mutants in which the RGD sequence is mutated, wells of the 96-well ELISA plates were coated with the same concentration (50 μg/ml) of mouse recombinant entactin, GST fusion proteins containing the E, G1, G2 or G3 domain of entactin in PBS at 4°C overnight, or the entactin RGD mutants (deletion or RGD→RGE mutation). Then, each well was incubated with 200 μl of 10 mg/ml heat treated BSA in PBS at 37°C for 24 hour, followed by rinsing with α-MEM (Gibco, Grand Island, NY). The cells were harvested with Trypsin-EDTA solution (Sigma, St Louis, MO), followed by rinsing once with α-MEM supplemented 10% FBS and three times with α-MEM. The cells were suspended to a final density of 3×10^5 cells/ml in α-MEM supplemented with 0.2 mM $MnCl_2$ and added to each well of the 96-well ELISA plate which was placed in a 37°C incubator under a 5% CO_2-95% air atmosphere for 120 minutes. The number of the cells adhered was quantified as described above.

Cell motility assay

Cell motility assays were performed as previously described (Bauer et al., 1992; Wu et al., 1995). The undersurfaces of 8 μm pore diameter Transwell[R] motility chambers (Costar, Cambridge, MA) were coated with recombinant entactin (5, 20 or 40 μg/ml) or human plasma fibronectin (10 μg/ml) in PBS. Cells (2×10^5 cells in 0.1 ml medium) were suspended in 1% BSA in α-MEM, added to the upper chamber of the Transwell, and incubated in a 37°C incubator under a 5% CO_2-95% air atmosphere for 18 hours. Following incubation, the cells on the upper surface of the membrane were removed using a cotton-tipped applicator. The membranes were fixed with 2% formalin in PBS and the cells on the undersurface were stained with Gill's III hematoxylin. To ensure that we did not count cells present in the pores of the membranes as migrated, we wiped both upper and under surfaces of the filters. This abolished all cell staining, indicating that there was negligible contribution from cells present in the pores. The cells from ten randomly selected microscopic fields were counted and cell motility was expressed as the number of the cells/mm^2 of the microscopic field ± s.d.

Extracellular matrix assembly assays

The deposition of entactin into pericellular matrix by CHO cells was determined by immunofluorescent staining of cell monolayers. Cells were seeded in Lab-Tek 8 chamber slides (Nunc, Inc., Naperville, IL) in α-MEM supplemented with 10% fibronectin depleted fetal bovine serum, 2 mM glutamine and 0.5 mg active geneticin/ml (B3 cells only) at cell densities yielding a confluent monolayer after 3 days. Three days after seeding, the cell monolayers were fixed with 4% paraformaldehyde, followed by staining with a rabbit anti-entactin antiserum (1:100). After washing, the bound antibodies were detected with a FITC-conjugated goat anti-rabbit IgG antibody (10 μg/ml).

To evaluate fibronectin deposition by CHO cells, we suspended them in α-MEM containing 10% fibronectin-depleted fetal bovine serum, or α-MEM containing 10% fibronectin-depleted fetal bovine serum supplemented with 50 μg/ml human plasma fibronectin at a density of 2.2×10^5 cells/ml in Lab-Tek 8 chamber slides (0.4 ml/well). The cells reached 100% confluence after one day and were allowed to grow for two more days. Three days after seeding, the cell monolayers were fixed with 4% paraformaldehyde, stained with mouse monoclonal anti-fibronectin antibody N294 IgG (23 μg/ml), followed by a CY3-conjugated anti-mouse IgG+IgM antibody (10 μg/ml). In double staining experiments, the cells were incubated with a mixture of a rabbit anti-mouse entactin antiserum (1:100 dilution) and a mouse anti-fibronectin antibody (N294, 23 μg/ml). After washing, the bound rabbit and mouse antibodies were detected with FITC-conjugated goat anti-rabbit IgG and CY3-conjugated anti-mouse IgG+IgM antibodies, respectively. The fluorescent images of entactin and fibronectin were then observed using a epifluorescence microscope with a FITC filter set (entactin) or a CY3 filter set (fibronectin) and photographed.

For fibronectin matrix assembly inhibition studies, CHO B3 cells were seeded in Lab-Tek slides in α-MEM, 10% fibronectin depleted fetal bovine serum supplemented with 22.5 μg/ml (45 nM) human plasma fibronectin alone, 22.5 μg/ml human plasma fibronectin mixed with 2.3 μM N294 anti-fibronectin IgG or 22.5 μg/ml human plasma fibronectin mixed with 0.67 μM J143 anti-human α3 IgG as specified in each experiment. The cells were cultured for 41 hours and then fixed with 4% paraformaldehyde. Fibronectin was detected by staining the cells with the N294 anti-fibronectin antibody and a CY3-conjugated anti-mouse IgG+IgM antibody. For the cells cultured with mouse J143 IgG, fibronectin was detected with a rabbit polyclonal anti-fibronectin antibody and FITC-conjugated anti-rabbit IgG antibodies. The fluorescent images were observed using an epifluorescence microscope (Nikon Microphot-FXA) and photographed under identical exposure conditions.

The deposition of entactin into the extracellular matrix fraction resisting solubilization in 2% deoxycholate was determined by immunoblotting (Wu et al., 1993). The CHO B2 and B3 cells were cultured in 100 mm tissue culture plates in α-MEM supplemented with 10% heat-inactivated, fibronectin-depleted fetal bovine serum, 2 mM glutamine, 0.5 mg active geneticin/ml (B3 cells only) for three

days. The confluent cells were rinsed with PBS and extracted with 3% Triton X-100 and 2% deoxycholate sequentially to isolate the detergent insoluble extracellular matrix fraction (Wu et al., 1993). The fraction insoluble in 2% deoxycholate was solubilized in SDS and separated by SDS-PAGE under reducing conditions. Entactin was detected using a rabbit anti-entactin antiserum (1:1,000) and alkaline phosphatase-conjugated goat anti-rabbit IgG after transfer to a PVDF membrane (Immobilon-P^{R}, Millipore Corp.). Each lane was loaded with the matrix fraction corresponding to 40 μg of the 3% Triton X-100 soluble cellular proteins as determined by BCA protein assay using BSA as standard (Pierce, Rockford, IL).

Binding of the 29 kDa amino-terminal fragment of fibronectin to CHO cells

CHO cells (2.5×10^5 cells/ml) were suspended in α-MEM supplemented with 2 mM glutamine and 10% heat-inactivated fetal bovine serum depleted of fibronectin, or supplemented with human plasma fibronectin (final concentration, 50 μg/ml) as specified in each experiment. The cells (0.25 ml) were seeded into each well of a 96-well tissue culture plate (ViewPlate-96^{TM}, Packard Instrument Co., Meriden, CT). The cells reached confluence after one day and were cultured for two more days. The binding of the amino-terminal 29 kDa fragment of fibronectin to the confluent cell monolayers was measured as previously described with slight modifications (Quade and McDonald, 1988). The cells were rinsed three times with 2 mg/ml BSA in α-MEM, followed by incubation with binding medium (100 μl/well) containing 7.2 μg/ml (0.25 μM) ^{125}I-labeled 29 kDa amino-terminal domain (specific activity = 4.5×10^5 cpm/μg) and 2 mg/ml BSA in α-MEM at 37°C for 1 hour. Following incubation, the wells were rinsed four times with 2 mg/ml BSA in α-MEM and the ^{125}I-labeled 29 kDa domain bound to the cells was quantified using a TopCount microplate scintillation counter (Packard Instrument Co., Meriden, CT). Nonspecific binding was determined by adding a 100-fold excess of unlabeled 29 kDa domain, and ranged between 0.1 to 0.25 ng/well. Specific binding was calculated by subtracting nonspecific binding from total binding.

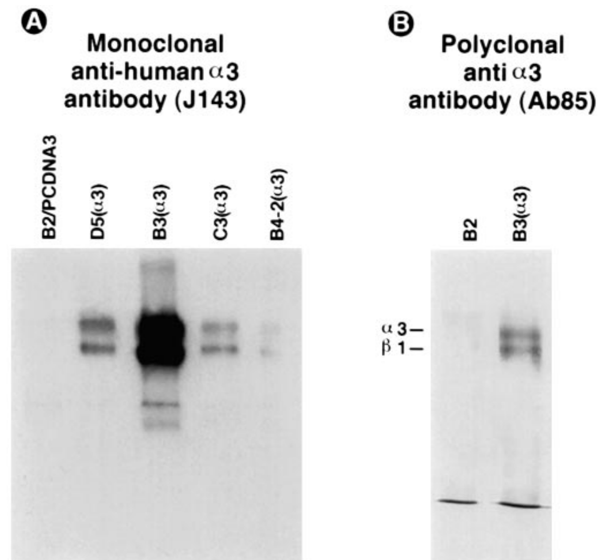


Fig. 1. Cell surface expression of α3β1 integrin in CHO B3, D5, C3 and B4-2 cells. CHO B2, B2/pcDNA3, B3, D5, C3 and B4-2 cells were surface biotinylated with biotin-X-NHS and lysed as described in Materials and Methods. (A) α3β1 integrin was immunoprecipitated from B2/PcDNA3, B3, D5, C3 and B4-2 cell lysates with monoclonal anti-human α3 antibody J143 and an anti-mouse κ chain antibody coupled to Sepharose 4B. (B) α3β1 integrin was immunoprecipitated from B2 and B3 lysates with polyclonal anti-α3 antibody Ab85 and Protein A coupled to Sepharose 4B. The precipitated proteins obtained from equal amounts of 1% Triton X-100 soluble proteins were separated on non-reducing SDS-PAGE gels and transferred onto Immobilon^{TM}. The biotinylated proteins were detected by avidin-biotinylated horseradish peroxidase complex and chemiluminescence using the ECL^{R} detection system.

RESULTS

Expression of human α3 integrin in α4, α5 deficient CHO B2 cells

The CHO B2 cell line expresses neither α4 (Wu et al., 1995) nor α5 integrins (Schreiner et al., 1989), and very low levels of endogenous α3 (see below). To increase α3 expression in the B2 cells, we transfected the cells with the expression vector pCDNA3/α3 encoding full length human α3, or as a control, pCDNA3 that does not contain the α3 insert. The resulting geneticin-resistant cells were cloned by limited dilution and screened for expression of human α3 by immunofluorescent staining with the J143 monoclonal antibody specific for human α3. A number of cell lines (designated CHO B3, D5, C3 and B4-2) that stably expressed human α3 were isolated. The human α3 subunit expressed by these cells was associated with the hamster β1 subunit on the cell surface, as shown by immunoprecipitation of the integrin complex with J143 anti-human α3 antibody from lysates of surface biotinylated cells (Fig. 1A). Two major biotinylated bands of 150 and 110 kDa were detected in the immunoprecipitates from all four α3 transfectants but not in the immunoprecipitates from B2/pcDNA3 cells, which were obtained by transfecting B2 cells with vector alone (Fig. 1A). The 150 kDa band represents the human α3 subunit and the 110 kDa band the associated hamster β1 subunit, as they were recognized by a polyclonal rabbit anti-α3 antibody (Ab85) and a polyclonal rabbit anti-β1 cytoplasmic domain antibody, respectively, in immunoblots (data not shown). The B3 cells expressed approximately three and four times more human α3/hamster β1 than D5 and C3 cells, respectively (Table 1). The α3β1 complex was also precipitated from B3 cell lysates using the polyclonal rabbit anti-α3 antibody Ab85 that recognizes both human and hamster α3 (Fig. 1B). Both immunoprecipitation (Fig. 1B) and immunoblotting (Fig. 2) of α3β1 integrins from B2 and B3 cell lysates with Ab85 indicates that B2 cells express much less α3β1 than B3 cells. The identity of the slower migrating band detected by anti-cytoplasmic domain antibody Ab85 in immunoblot is unknown, but may represent intracellular, unprocessed pro-α3 subunit. We concluded from these results that the human α3 subunit was associated with hamster β1, and was expressed on the cell surface of CHO B3, D5, C3 and B4-2 cells. The resulting α3 cell lines varied in their relative expression of α3, but all expressed a much higher level of α3β1 integrins than the parental B2 cells.

α3β1 integrins support CHO cell adhesion on entactin but not on fibronectin

The adhesion of B2 cells to fibronectin was restored when any one of several integrin receptors for fibronectin, including α5β1 (Bauer et al., 1993), α4β1 (Wu et al., 1995) or αvβ1 (Zhang et al., 1993), were expressed. A previous report using

Table 1. Relative cell surface α3β1 integrin expression levels of CHO clones

Clone	Expression relative to CHO B3 (α3)
B3 (α3)	100
D5 (α3)	35
C3 (α3)	23

The CHO cells were surface biotinylated and immunoprecipitated with monoclonal anti-α3 antibody J143 as described in Materials and Methods. The precipitated proteins obtained from an equal amount of 1% Triton X-100 soluble proteins were separated on non-reducing SDS-PAGE and transferred onto Immobilon™. The biotinylated proteins were detected by avidin-biotinylated horseradish peroxidase complex and chemiluminescence using the ECL^R detection system and quantified using The Discovery Series™ image analysis system (PDI, Huntington Station, NY). Results were normalized using the level of α3β1 integrin expression on CHO B3 cells as 100.

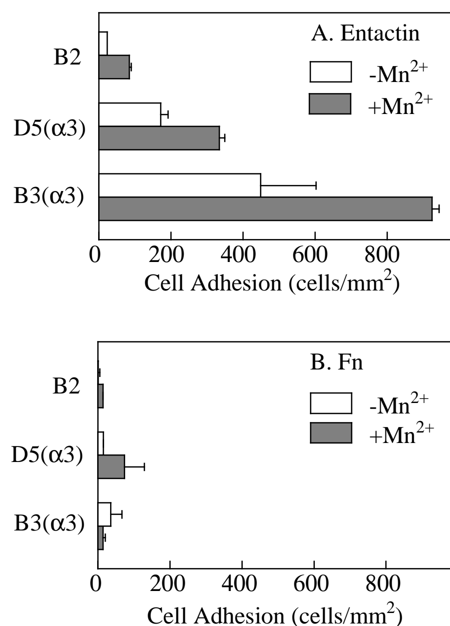


Fig. 3. Adhesion of CHO cells to entactin and fibronectin. CHO B2, D5 and B3 cells were suspended in α-MEM ($-Mn^{2+}$) or α-MEM supplemented with Mn^{2+} ($+Mn^{2+}$) and seeded into wells coated with 40 µg/ml entactin (A) or 10 µg/ml human plasma fibronectin (B). Cell adhesion was quantified as described in Materials and Methods. The data represent means ± s.d. from duplicate wells.

affinity chromatography and anti-α3 antibody suggested that α3β1 integrins mediate the adhesion of prostate carcinoma cells to entactin (Dedhar et al., 1992). To determine if the human α3/hamster β1 complex functions as an entactin and/or fibronectin receptor mediating cell adhesion, we studied the adhesion of D5, B3 cells and B2 cells to entactin and fibronectin. B2 cells, which expressed a very low level of endogenous α3β1 (Fig. 2), adhered poorly to entactin, whereas the B3 cells, which expressed a much higher level of α3β1 (Figs 1 and 2), adhered readily (Fig. 3A). The D5 cells expressed approximately one-third the level of α3β1 integrin of B3 cells (Table 1). In accordance with a role of α3β1 integrin in mediating cell adhesion on entactin, the adhesion of D5 cells on entactin is weaker than that of B3 cells, although stronger than that of B2 cells (Fig. 3A). D5 and B3 cells adhered to fibronectin no better than the parental B2 cells (Fig. 3B). This is in contrast to the α5β1 expressing CHO 1-23 cells, which adhered to fibronectin much better than the B2 cells (Schreiner et al., 1989). To ensure that the level of α3β1 integrin expression by the B3 cells was equivalent to the expression of α5β1 integrins and thus adequate to support cell adhesion, we used flow cytometric analysis to compare their expression. The CHO B3 cells expressed a higher level of α3β1 than the level of α5β1 expressed by the 1-23 cells (data not shown). Thus, in contrast to α5β1, α3β1 did not mediate cell adhesion to fibronectin. Cell adhesion on entactin, but not on fibronectin, was significantly enhanced by Mn^{2+} (Fig. 3). Taken together, these results demonstrated that the human α3/hamster β1 complex mediated cell adhesion on entactin and this activity is stimulated by Mn^{2+}.

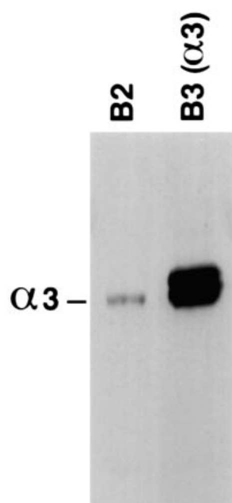


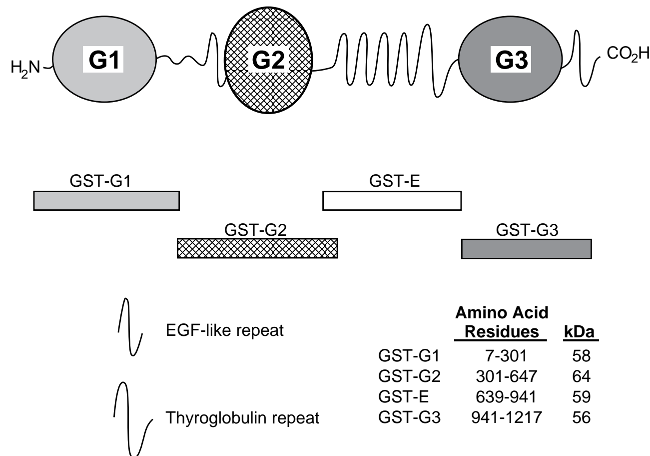
Fig. 2. Immunoblot of α3 from CHO B2 and B3 cells. CHO B2 or B3 cells from one confluent 100-mm tissue culture dish were lysed with 0.2 ml of lysis buffer (1% Triton X-100, 140 mM NaCl, 50 mM Tris-HCl, pH 8, 2 mM PMSF, 5 mM EDTA, 0.2 mM AEBSF, 10 mM leupeptin and 1 mM pepstatin) and equal amounts of soluble cellular proteins were separated on non-reducing SDS-PAGE. The α3 subunit was recognized by Ab85 rabbit polyclonal anti-α3 cytoplasmic domain antibody. The antibody bound to the α3 subunit was detected with horseradish peroxidase-conjugated anti-rabbit IgG and the ECL^R detection system.

The G2 domain supports CHO cell adhesion to entactin

Entactin contains an RGD sequence in the first EGF-like repeat of domain E that was implicated in cell adhesion (Chung and Durkin, 1990; Yelian et al., 1993; Mayer and Timpl, 1994). To determine if the RGD sequence is involved in the α3β1 integrin mediated CHO cell adhesion to entactin, we studied entactin mutants in which the RGD sequence is deleted or changed to RGE. Both mutants supported the adhesion of CHO B3 cells expressing a high level of α3β1 integrin (Fig. 4), whereas lower levels of α3β1 expression significantly reduced adhesion. Thus, the RGD sequence is not required for α3β1 integrin mediated cell adhesion to entactin. To identify the domain of entactin supporting cell adhesion, we tested GST-fusion proteins containing the G1, G2, E or G3 domain of entactin. CHO B3 cells preferentially adhered to the G2 domain (Fig. 4), whereas a much lower level of adhesion was observed with CHO B2 cells. These results suggest that the α3β1 integrin mediates cell adhesion to entactin via interaction with the G2 domain.

α3β1 integrins do not support cell motility on entactin

Cell migration along solid substrata requires cell surface



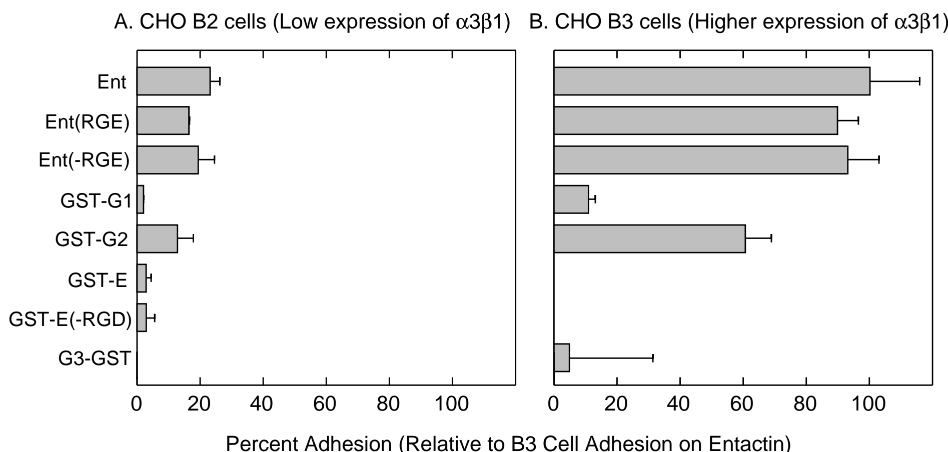


Fig. 4. The G2 domain of entactin supports CHO cell adhesion. Cell adhesion to entactin, entactin mutants and GST-entactin domain fusion proteins was performed as described in Materials and Methods. The data is presented as percentages of the B3 cell adhesion to mouse recombinant entactin. Ent: mouse recombinant entactin; Ent(RGE): mouse recombinant entactin in which the Asp at amino acid 674 of the molecule is replaced with a Glu, Ent(–RGD): mouse recombinant entactin in which the RGD sequence is deleted; GST-G1, GST-G2, GST-E, GST-G3: GST fusion proteins containing the G1, G2, E or G3 domain of entactin; GST-E(–RGD): GST-E fusion protein in which the RGD sequence is deleted.

receptors interacting with both the substrata and cytoskeleton. The B2 cells, which lack fibronectin adhesion receptors, do not migrate on fibronectin. Expression of either α5β1 (Bauer et al., 1992) or α4β1 (Wu et al., 1995), but not αvβ1 (Zhang et al., 1993), in B2 cells enables them to migrate on fibronectin, indicating they are capable of migration on matrices. Previous studies showed that entactin promoted migration of mouse primary trophoblast cells (Yelian et al., 1993). To determine if α3β1 integrin supported cell migration, we tested the motility of B2, D5 and B3 cells on entactin and fibronectin, respectively, in a Transwell motility assay. Both D5 and B3 cells, like the parental B2 cells, migrated poorly on either entactin or fibronectin (Fig. 5). This is in sharp contrast to the α5β1 expressing CHO B2B4 cells, which migrated well on fibronectin (Fig. 5B). Thus, although α3β1 integrin mediated cell adhesion on entactin, it did not support cell migration.

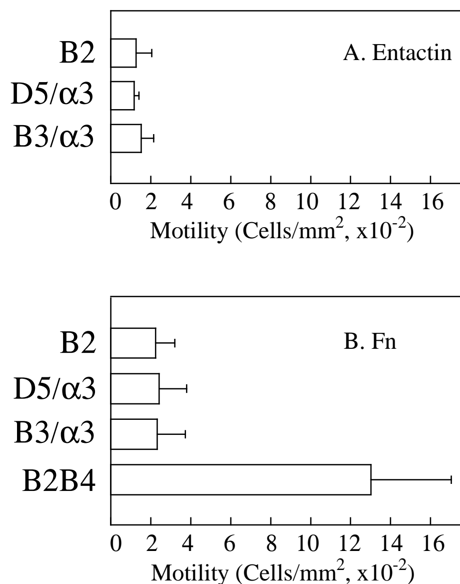


Fig. 5. Motility of CHO cells on entactin and fibronectin. CHO B2 and B3 cells were suspended in 1% BSA-α-MEM and added to the upper chamber of Transwell inserts in which the undersurface of membranes was coated with 40 μg/ml of entactin (A) or 10 μg/ml human plasma fibronectin (B). The motility assay was performed as described in Materials and Methods. The cells from ten randomly selected microscopic fields were counted and the cell motility was expressed as the number of cells/mm^2 of the membrane undersurface (error bars = s.d.).

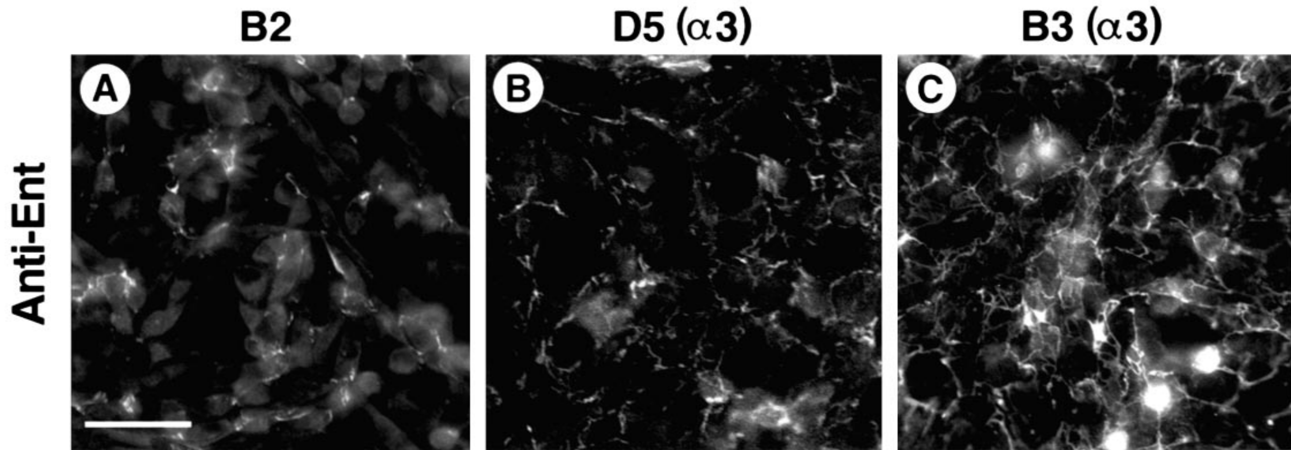


Fig. 6. Modulation of entactin deposition into pericellular matrix by α3β1 integrin. CHO B2 (A), D5(B) and B3 cells (C) were seeded in Lab-Tek 8 chamber slides in α-MEM supplemented with 10% fibronectin depleted fetal bovine serum, 2 mM glutamine and 0.5 mg active geneticin/ml (D5 and B3) at densities yielding confluent monolayers after 3 days. The cell monolayers were fixed and stained with a rabbit anti-entactin antiserum and a FITC-conjugated goat anti-rabbit IgG antibody as described in Materials and Methods. Bar in A equals 50 μm and applies to all panels.

α3β1 integrin promotes deposition of entactin and fibronectin into pericellular matrix

Because α3β1 is a receptor for entactin (this work; Dedhar et al., 1992), and in certain circumstances possibly for fibronectin (Wayner and Carter, 1987; Gehlsen et al., 1988; Gehlsen et al., 1989; Hemler et al., 1990; Elices et al., 1991), we studied its role in the deposition of entactin and fibronectin into pericellular matrix. Immunofluorescent staining of cells with an anti-entactin antiserum showed that B2 cells deposited small amounts of entactin into the pericellular matrix (Fig. 6A). In contrast, D5 and B3 cell lines expressing higher levels of α3β1 deposited much more entactin into the matrices (Fig. 6B and C). This result was confirmed by immunoblot analysis of entactin in the extracellular matrix fraction resisting deoxycholate solubilization (Fig. 7). In addition to the 150 kDa entactin polypeptide, a 190 kDa protein in the extracellular matrix fraction was also recognized by the anti-entactin antibodies. A similar size, entactin-related protein was previously detected in mouse embryonal carcinoma F9 cells (Carlin et al., 1983). The deposition of both the 150 kDa entactin and the 190 kDa entactin-related protein was dramatically increased in the B3 cell line (Fig. 7). This increase was specific for α3β1 as expression of α5β1 (Wu et al., 1993) or α4β1 (data not shown) in the B2 cells did not significantly increase entactin deposition into the extracellular matrix.

We next examined the effect of overexpressing α3β1 on the deposition of fibronectin into pericellular matrix. The α5β1 deficient B2 cells did not accumulate significant amounts of fibronectin in the pericellular matrix before or shortly after they reached confluence, as shown previously (Wu et al., 1993). However, if the B2 cells were allowed to grow in the presence of exogenously supplied plasma fibronectin for several days after they reached confluence, they began to accumulate small amounts of fibronectin in the pericellular matrix (Fig. 8A). Overexpressing α3β1 integrin in the B2 cells significantly increased the accumulation of fibronectin (Fig. 8C and E). On the other hand, culturing the same cells in the presence of an inhibitory anti-α3 antibody J143 resulted in less fibronectin deposition (compare Fig. 9A and C). Thus, α3β1 integrin supports the deposition of fibronectin into pericellular matrix, even though it is not a functional fibronectin receptor in the CHO cells. Perusal of the antibody staining patterns revealed that the fibronectin was mostly deposited at the cell periphery in short stitches. In contrast, α5β1 expressing CHO B2B4 cells typically deposit an extensive matrix with long fibrils between cells (Fig. 8G; Wu et al., 1993). Double staining of B3 cells with anti-entactin and anti-fibronectin antibodies revealed that entactin and fibronectin were co-localized in the pericellular matrix (Fig. 10).

The deposition of fibronectin into pericellular matrix supported by α3β1 integrin is mechanistically distinct from that mediated by α5β1 integrin

The failure of α3β1 integrin to support cell adhesion to fibronectin (Fig. 3B) and the marked difference in morphology of the fibronectin matrix deposited by the B3 and B2B4 cells suggested that α3β1 integrin modulated fibronectin deposition via a mechanism distinct from that of α5β1 integrins. To

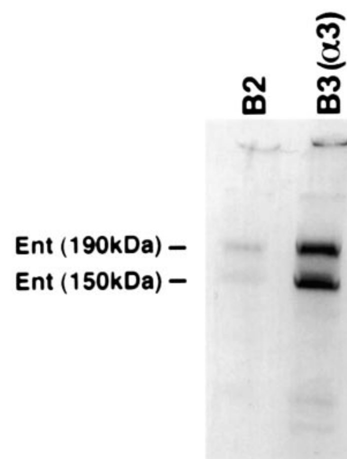


Fig. 7. Deposition of entactin into an extracellular matrix resisting 2% deoxycholate solubilization by CHO cells. Entactin in the extracellular matrix fraction resisting 2% deoxycholate solubilization was detected using a rabbit anti-entactin antiserum and alkaline phosphatase-conjugated goat anti-rabbit IgG as described in Materials and Methods. Each lane was loaded with the matrix fraction corresponding to 40 μg of the 3% Triton X-100 soluble cellular proteins as determined by BCA protein assay using BSA as standard.

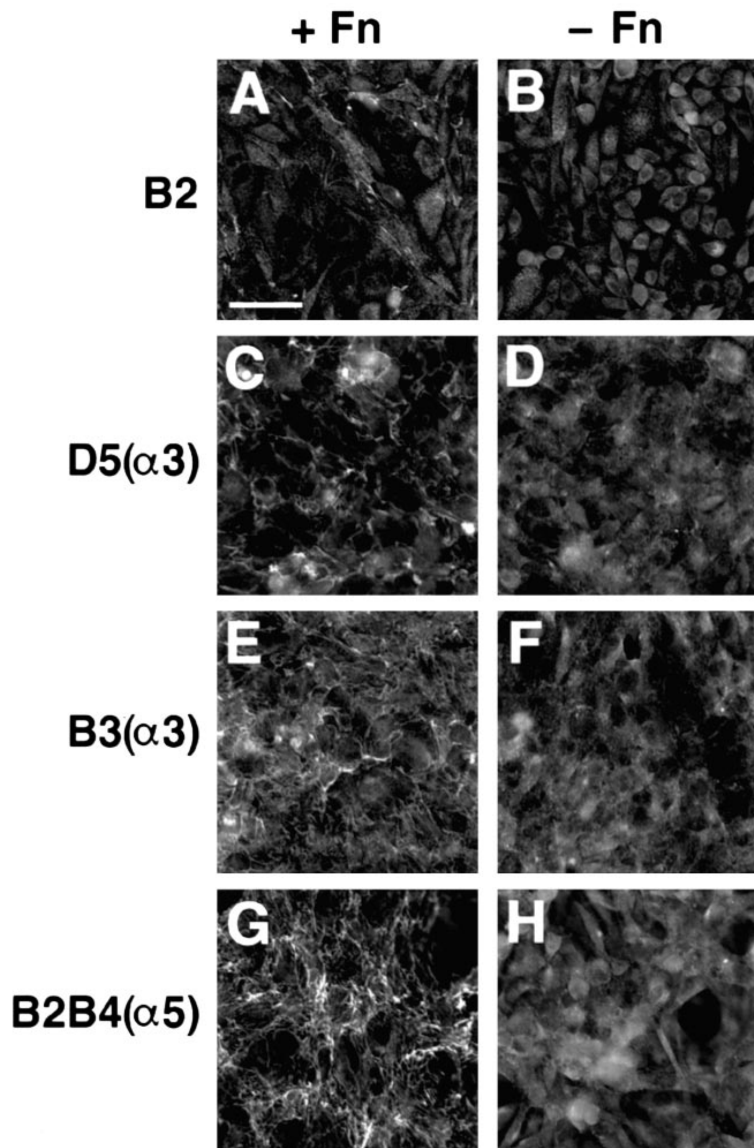


Fig. 8. Modulation of fibronectin deposition into pericellular matrix by α3β1 integrin. CHO B2 (A and B), D5 (C and D), B3 (E and F) and B2B4 (G and H) cells were seeded at a density of 2.2×10^5 cells/ml in Lab-Tek 8 chamber slides (0.4 ml/well) in α-MEM containing 10% fibronectin depleted fetal bovine serum (B,D,F and H), or α-MEM containing 10% fibronectin depleted fetal bovine serum supplemented with 50 μg/ml of human plasma fibronectin (A,C,E, and G). The cells reached 100% confluence after one day and were allowed to grow for two more days. The cells were fixed and stained with a mouse monoclonal anti-fibronectin antibody as described in Materials and Methods. Bar in A equals 50 μm and applies to all panels.

further test this hypothesis, we cultured CHO B3 and B2B4 cells in the presence of monoclonal antibody N294, which binds to the RGD containing cell adhesion domain of fibronectin and inhibits α5β1 mediated fibronectin assembly (McDonald et al., 1987). Indeed, fibronectin matrix assembly by α5β1 expressing B2B4 cells was markedly inhibited by N294 (Fig. 9, compare D and E). However, the deposition of fibronectin into extracellular matrix by CHO B3 cells overexpressing α3β1 was not inhibited (Fig. 9, compare A and B). Thus, the RGD containing cell adhesion domain of fibronectin is not involved in the α3β1 mediated fibronectin deposition into pericellular matrix, supporting the notion that α3β1 participates in fibronectin matrix assembly through a mechanism distinct from that of α5β1.

Overexpressing α3β1 integrin in CHO cells increases their binding activity for the 29 kDa amino-terminal fragment of fibronectin

Two principal domains in fibronectin have been implicated in its assembly mediated by α5β1 integrins, the RGD-containing α5β1 integrin binding domain, and the 29 kDa amino-terminal domain, which binds to the surface of mesenchymal cells with high avidity. The 29 kDa domain also binds to the III-1 domain in fibronectin (Hocking et al., 1994) and to a 70 kDa candidate for the matrix assembly receptor on myoblasts (Moon et al., 1994). In CHO B2 cells, expressing α5β1 integrin significantly increased their ability to bind the 29 kDa domain, but only when they were cultured in the presence of exogenously supplied fibronectin and had formed a fibronectin-containing matrix (Wu et al., 1993). To determine if α3β1 integrin played a role in binding of the 29 kDa domain to cells, we quantified binding by B2, D5 and B3 cells. Fig. 11 shows that all three α3β1 expressing cell lines exhibited similar, low levels of 29 kDa domain binding when cultured in the absence of exogenously supplied fibronectin. Under these culture conditions, no fibronectin was detected in the pericellular matrix (Fig. 8B,D and F). Binding of the 29 kDa domain was significantly increased when confluent cell monolayers were cultured with exogenous fibronectin and a fibronectin-containing matrix formed (Figs 8 and 11). The increase in binding activity for the

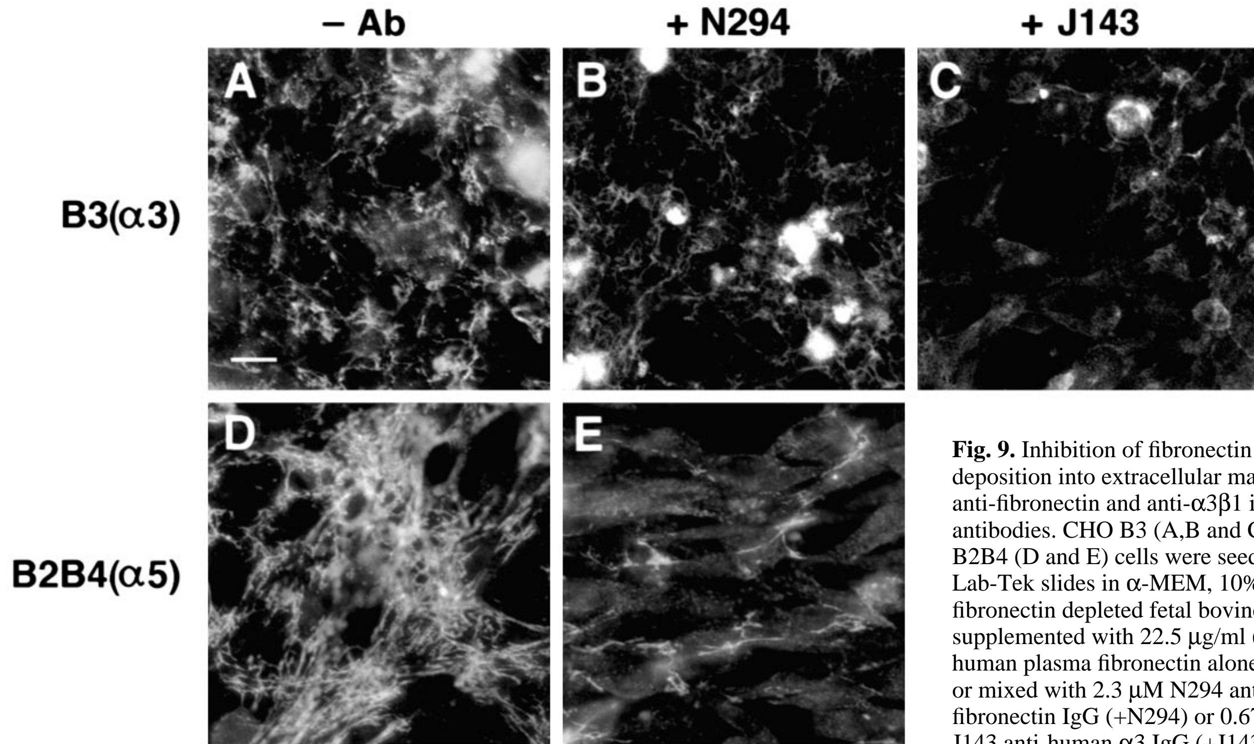


Fig. 9. Inhibition of fibronectin deposition into extracellular matrix by anti-fibronectin and anti-α3β1 integrin antibodies. CHO B3 (A,B and C) and B2B4 (D and E) cells were seeded in Lab-Tek slides in α-MEM, 10% fibronectin depleted fetal bovine serum supplemented with 22.5 μg/ml (45 nM) human plasma fibronectin alone (−Ab) or mixed with 2.3 μM N294 anti-fibronectin IgG (+N294) or 0.67 μM J143 anti-human α3 IgG (+J143). The cells were cultured for 41 hours and then fixed with 4% paraformaldehyde. Fibronectin was detected by staining the cells with a mouse monoclonal anti-fibronectin antibody and CY3-conjugated anti-mouse IgG+IgM antibodies (A,B,D and E). For the cells cultured with mouse J143 IgG (C), fibronectin was detected with a rabbit polyclonal anti-fibronectin antibody and FITC-conjugated anti-rabbit IgG antibodies. The fluorescent images were observed using a epifluorescence microscope with a CY3 filter set (A,B,D and E) or a FITC filter set (C) and photographed under identical exposure conditions. Bar in A equals 15 μm and applies to all panels.

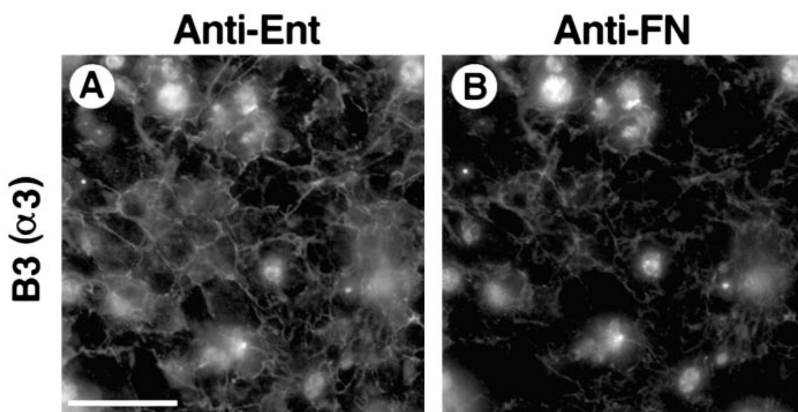


Fig. 10. Co-localization of entactin and fibronectin in pericellular matrix. CHO B3 cells were grown in the presence of 50 μg/ml of human plasma fibronectin as described in Fig. 8. The cells were fixed and doubled stained with a rabbit anti-entactin antiserum and a mouse monoclonal anti-fibronectin antibody as described in Materials and Methods. (A and B) Entactin and fibronectin staining images from the same microscopic field. Bar in A equals 50 μm and applies to both panels.

29 kDa domain was greatest for the B3 cells that also expressed the highest level of α3β1 and least for the B2 cells that expressed the lowest level of α3β1. These results demonstrate that expressing α3β1 integrins in CHO B2 cells supports the formation of a fibronectin-containing matrix, and this in turn increases the binding of the 29 kDa amino-terminal domain of fibronectin.

DISCUSSION

This study demonstrates that α3β1 integrins function as entactin receptors in CHO B2 cells, mediating adhesion but apparently not cell migration, and the deposition of entactin and fibronectin into the pericellular matrix. This latter result is of considerable biological interest, as entactin, like laminin and certain fibrillar collagens, also undergoes rapid homophilic polymerization in vitro under proper conditions (Tsao et al., 1990). The self-assembly of extracellular matrix components is undoubtedly critical for the formation of extracellular suprastructures. However, cells also play an important role in modulating the assembly of matrices. Cells control the biosynthesis, processing, and secretion of various extracellular matrix components and provide specific binding sites for matrix components on their surfaces (Fleischmajer et al., 1983; Kalb and Engel, 1991; Knudson and Knudson, 1993). The modulation

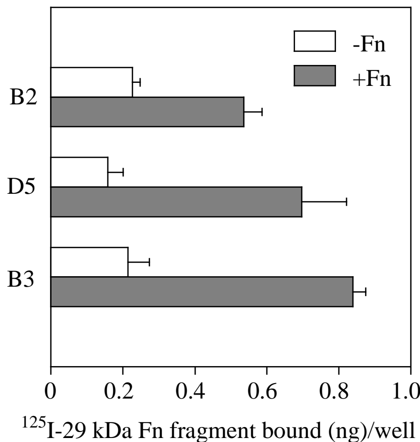


Fig. 11. Modulation of 29 kDa fibronectin fragment binding activity by α3β1 integrins. CHO B2, D5 and B3 were suspended (2.5×10^5 cells/ml) in α-MEM containing 10% fibronectin depleted fetal bovine serum (−Fn), or α-MEM containing 10% fibronectin depleted fetal bovine serum supplemented with 50 μg/ml of human plasma fibronectin (+Fn), and seeded in wells (0.25 ml/well) of 96-well tissue culture plates. The cells reached 100% confluence after one day and were allowed to grow for two more days. Binding of ^{125}I-29 kDa fibronectin fragment to the CHO B2, D5 and B3 monolayers were carried out as described in Materials and Methods. Each bar represents the mean + s.e. of the ^{125}I-29 kDa fragment bound specifically to the cells from duplicate wells.

of fibronectin matrix assembly by cell surface receptors has been studied in cultured cells (McDonald et al., 1987; McDonald, 1988; Akiyama et al., 1989; Roman et al., 1989; Fogerty et al., 1990; Giancotti and Ruoslahti, 1990; Wu et al., 1993). These studies demonstrated that α5β1 integrin plays an important role in the deposition of fibronectin into extracellular matrix. However, it has not been clear if the deposition of other extracellular matrix components can be modulated by integrin receptors. Indeed, it remains formally possible that, by analogy to hemostatic mechanisms, the involvement of α5β1 integrins in fibronectin assembly reflects a specialized adaptation to the presence of high levels of circulating plasma fibronectin and a concomitant need for careful regulation of matrix assembly (McDonald, 1988). Our results demonstrate that increasing the expression of α3β1 integrin in CHO B2 cells dramatically increased entactin deposition into pericellular matrix. This enhancement is α3β1 specific, as expressing α5β1 integrin in the same cells had no detectable effect on entactin deposition (Wu et al., 1993).

The ability of α3β1 integrin to mediate fibronectin deposition into matrix may explain the ability of cells derived from α5-null mutant embryos to assemble a fibronectin-containing matrix (Yang et al., 1993). It is also consistent with the observation that anti-β1 antibodies were more effective than anti-α5 antibodies in inhibiting fibronectin matrix assembly and binding of the amino-terminal domain of fibronectin to cell layers (Fogerty et al., 1990). However, not all fibronectin binding receptors are capable of supporting fibronectin matrix assembly. Expression of α4β1 and αvβ1 integrins in the α5 deficient CHO B2 cells enabled the cells to adhere on fibronectin, but not to assemble a fibronectin matrix (Zhang et al., 1993; Wu et al., 1995). Thus, among the four β1 integrins tested, α3β1 and α5β1 integrins support fibronectin matrix assembly, albeit via distinct mechanisms, whereas α4β1 and αvβ1 do not.

Our results strongly suggest that the α3β1 and α5β1 integrins support fibronectin deposition into extracellular matrix via different mechanisms. The α5β1 integrin functions in fibronectin matrix assembly by direct binding to the RGD containing cell adhesion domain of fibronectin (McDonald et al., 1987; Akiyama et al., 1989; Roman et al., 1989), and possibly by modulating a distinct binding event involving the interaction of the 29 kDa amino-terminal matrix assembly domain of fibronectin with the cell surface (Wu et al., 1993). Thus, α5β1 integrin mediated deposition of fibronectin into extracellular matrix was inhibited by antibodies binding to the RGD containing cell adhesion domain of fibronectin, whereas the α3β1 integrin mediated deposition of fibronectin was not (Fig. 9). The interaction between α3β1 integrins and fibronectin is very weak, if it exists at all (Sriramarao et al., 1993; Weitzman et al., 1993). This is confirmed by our observation that overexpressing α3β1 integrins in CHO B2 cells did not enhance cell adhesion on fibronectin (Fig. 3). This, taken with the resistance to N294 inhibition, makes it very unlikely that the enhanced deposition of fibronectin into extracellular matrix is directly mediated by an α3β1 integrin-fibronectin interaction. Despite this, overexpressing either α3β1 (Fig. 11) or α5β1 (Wu et al., 1993) in the B2 cells, together with the presence of a fibronectin-containing matrix, increases the binding of the 29 kDa amino-terminal domain of fibronectin. This supports the hypothesis that the binding of the 29 kDa domain is involved in fibronectin fibril elongation (McDonald, 1988; Wu et al., 1993), although it is equally consistent with 29 kDa domain binding to a component in the fibronectin matrix itself. In either event, it demonstrates that α5β1 is not absolutely required for 29 kDa domain binding.

The mechanism by which α3β1 integrin promotes assembly of entactin into pericellular matrix remains to be elucidated. It seems likely that α3β1 integrins provide cell surface binding sites for entactin, retaining it on the cell surface and effectively increasing the local entactin concentration. This in turn would enhance polymerization and the formation of complexes with other extracellular matrix components including laminin and collagen type IV. Indeed, solubilized α3β1 integrins bind to entactin, and mediate adhesion to entactin (Dedhar et al., 1992; this work). Another possibility, which is not mutually exclusive with the first, is that overexpression of α3β1 integrins enhances cell surface interaction with other putative ligands of the α3β1 integrin such as laminin, which in turn facilitates the deposition of entactin into the pericellular matrix. In support of this, we found that the deposition of laminin into extracellular matrix is also increased in the CHO cells that overexpress α3β1 integrin (C. Wu and J. A. McDonald, unpublished observation). Entactin forms a tight complex with laminin. In fact, in mouse endodermal cells the formation of a laminin-entactin complex precedes secretion (Wu et al., 1988). Thus, anything increasing the deposition of laminin is likely to increase that of entactin into the extracellular matrix and vice versa.

However, entactin also binds to fibronectin with high affinity (Wu et al., 1991; Hsieh et al., 1994) and entactin and fibronectin are co-localized in pericellular matrices (Fig. 10; see also Wu et al., 1991). Accordingly, it may seem paradox-

ical that increasing fibronectin in the pericellular matrix by expressing α5β1 integrins does not also increase entactin deposition. This may be related to the binding site in fibronectin for entactin being localized to the same 29 kDa amino-terminal domain implicated in α5β1-mediated binding (Hsieh et al., 1994). Thus, formation of an initial fibronectin homopolymer mediated by α5β1 may preclude entactin binding. However, because both α3β1 integrin and fibronectin are able to interact with other molecules such as collagen (Wayner and Carter, 1987; Hynes, 1990; Elices et al., 1991), it is possible that in addition to entactin, other molecules are also involved in mediating the deposition of fibronectin into extracellular matrix promoted by α3β1 integrins.

There are now three distinct mechanisms in which fibronectin is involved in matrix deposition. The first, involving α5β1 and possibly other fibronectin-binding integrins involves direct assembly of a fibronectin-based matrix, possibly modulated by other matrix components (Dzamba et al., 1993). This mechanism resembles the direct modulation of hyaluronate deposition mediated by the CD44 mediated binding of hyaluronate (Knudson and Knudson, 1993; Knudson et al., 1993). The second involves the assembly of other matrix components upon a fibronectin scaffold. Fibulin or BM-90 binds to the carboxy-terminal region of fibronectin (Balbona et al., 1992; Pan et al., 1993). We previously demonstrated that its deposition by human lung fibroblasts is dependent upon the presence of a pre-existing fibronectin matrix (Roman and McDonald, 1993). Finally, this work demonstrates that, directly or indirectly, α3β1 integrin functions as a cell surface receptor modulating entactin and fibronectin deposition into extracellular matrix. As α3β1 does not appear to function as a fibronectin receptor in CHO B2 cells, it seems likely that in this case the entactin is serving as a scaffold for fibronectin deposition.

In a recent study, Delwel et al. (1994) found that expression of α3β1 integrin in K562 cells did not induce cell adhesion to entactin. The observed differences in cell adhesion on entactin between the α3 transfectants of K562 (Delwel et al., 1994) and those of the CHO cells (this work) or human prostate carcinoma cells (Dedhar et al., 1992) may relate to the differences of the cell types. A number of studies have indicated that a given integrin may exhibit varying adhesive competence depending on its cellular environment. Such variations in cell adhesion may be due to changes induced by 'post receptor events' (Danilov and Juliano, 1989) or changes in ligand binding affinity of integrin receptors (O'Toole et al., 1994). In the latter study, O'Toole et al. showed that α5β1 integrins expressed in CHO cells bind fibronectin with much higher affinity than those expressed in K562 cells, and that the cytoplasmic domains of α5β1 integrins are involved in this cell type-specific affinity modulation. Thus, it is possible that the entactin binding affinity of α3β1 integrin is modulated in a similar fashion (inside-out signaling) in these cell lines. How cell type-specific modulation of α3β1 integrin is achieved will be an important topic of future studies.

The G2 domain appears to be the major entactin domain involved in cell adhesion mediated by α3β1 integrin (Fig. 4). Thus, α3β1 integrin mediated cell adhesion to entactin is independent of laminin-entactin interaction, which requires the G3 domain of entactin (Mayer and Timpl, 1994). In addition, the RGD sequence, which is located in the E domain of entactin, is not required for the α3β1 integrin mediated cell adhesion. α3β1 integrin expressing CHO cells adhered readily to entactin mutants in which the RGD sequence is deleted or changed to RGE. Because the RGD sequence is involved in adhesion of several other types of cells to entactin (Chung and Durkin, 1990; Mayer and Timpl, 1994), it remains to be elucidated whether these cells adhere to entactin using a different integrin or, alternatively, α3β1 integrin functions in a cell type-specific way so that in certain cellular environments they recognize the RGD sequence.

Typically, α3β1 integrins are expressed in the baso-lateral surfaces of epithelia (see Introduction). Thus, α3β1 integrins may contribute to the organization of basal lamina in vivo. In healing dermal wounds, both the expression of α3β1 integrin and the deposition of extracellular matrix are dramatically increased (Cavani et al., 1993). As keratinocytes synthesize a number of extracellular matrix components (Alitalo et al., 1982; O'Keefe et al., 1984; Carter et al., 1991; Marinkovich et al., 1993), α3β1 integrin expressed on the basal surfaces of keratinocytes may facilitate the formation of extracellular matrix during wound healing. On the other hand, our cell motility assay indicated that α3β1 integrins did not support cell migration. This is consistent with the previous studies demonstrating that blocking α3β1 integrin function enhances rather than inhibits keratinocyte migration on fibronectin and collagen (Kim et al., 1992).

α3β1 integrin is one of the major cell surface receptors expressed in the human renal glomerulus (Simon and McDonald, 1990). Disruption of the mouse α3 integrin gene by homologous recombination resulted in homozygous null animals in disorganization of the basal lamina and altered epithelial cell morphology in the glomerulus, and the α3 null mice died during the first day of life (J. A. Kreidberg and R. Jaenisch, personal communications). Our results are consistent with these observations and suggest that α3β1 integrin participates in the assembly of basal lamina as well as their recognition.

Integrins also play important roles in tumorigenicity (Hynes and Yamada, 1982; Ruoslahti, 1988; Juliano, 1993). Many neoplastic cells show reduced cell adhesion to extracellular matrix substrates and deficiency in deposition of extracellular matrix (Hynes and Yamada, 1982; Ruoslahti, 1988). The loss or down-regulation of α3β1 integrin expression is correlated with increased invasiveness of breast carcinoma (Pignatelli et al., 1991) and prostate carcinoma cells (Dedhar et al., 1993). It is particularly interesting to note that in the latter case, no significant accompanying differences in αvβ3, α2β1 and α5β1 integrin expression were observed between the highly invasive population and the non-invasive population of the prostate carcinoma cells (both expressed very low levels of α5β1 integrins) (Dedhar et al., 1993). Thus, down-regulation of α3β1 integrin expression may be important in tumor progression and metastasis. Our results suggest that this down-regulation of α3β1 integrin expression may also be responsible for the reduction of extracellular matrix deposition associated with oncogenic transformation of certain types of cells.

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