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The role of $\alpha_4\beta_1$ integrin in cell motility and fibronectin matrix assembly

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SUMMARY

The $\alpha_4\beta_1$ integrin has been suggested to play important roles in embryogenesis and pathogenesis of many diseases which involve both cell adhesion and cell migration. Previous studies using anti-$\alpha_4\beta_1$ antibodies and fibronectin (Fn) fragments have suggested that $\alpha_4\beta_1$ integrins may be involved in cell motility on Fn and vascular cell adhesion molecule-1 (VCAM-1). However, the cells used in these studies also express other Fn integrin receptors including $\alpha_5\beta_1$ integrin, which is known to function in cell motility on Fn. To test whether $\alpha_4\beta_1$ integrins mediate cell motility on Fn and VCAM-1 in the absence of $\alpha_5\beta_1$ integrin, we expressed human α_4 integrin in a Chinese hamster ovary (CHO) cell line that is deficient in $\alpha_5\beta_1$ integrin (CHO B2). The parental α_5 deficient CHO B2 cells were unable to adhere, spread or migrate on Fn, nor could they assemble a fibrillar Fn matrix. Expression of $\alpha_4\beta_1$ integrin in the CHO B2 cells enabled the cells to adhere, spread and migrate on Fn and on VCAM-1 but not to assemble a fibrillar Fn matrix. The cellular processes mediated by the interaction of $\alpha_4\beta_1$ with Fn or VCAM-1 were inhibited by the CS1 peptide derived from the major $\alpha_4\beta_1$ binding site on Fn. These findings demonstrate that $\alpha_4\beta_1$ integrins not only function as cell adhesion receptors but also as cell motility receptors for Fn and VCAM-1 independent of $\alpha_5\beta_1$. Moreover, they reveal important functional differences between Fn binding integrins. The α_4-positive, α_5-negative CHO cells described in this report will be useful tools in studying the mechanism of molecular signalling during integrin mediated cellular processes.

Key words: fibronectin, integrins, VCAM

INTRODUCTION

Cell-extracellular matrix (ECM) and cell-cell interactions play important roles in embryonic development and many physiological and pathological processes including lymphocyte traffic, wound healing, inflammation and metastasis. Many of these interactions are mediated by integrins (Ruoslahti, 1991; Hynes, 1992). A number of β_1 integrins, including $\alpha_5\beta_1$, $\alpha_4\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_1$, have been shown to interact with fibronectin (Fn), a major component of the ECM. Fn is required for embryogenesis (George et al., 1993) and is implicated in many other biological processes. Previous studies have shown that $\alpha_5\beta_1$ integrins participated in many cellular processes involving Fn, including cell adhesion, spreading and migration on Fn substrata (Akiyama et al., 1989; Giancotti and Ruoslahti, 1990; Bauer et al., 1992; Schleimer et al., 1992) and assembly of a Fn-containing extracellular matrix (Akiyama et al., 1989; Roman et al., 1989; Fogerty et al., 1990; Giancotti and Ruoslahti, 1990; Wu et al., 1993). However, it has been shown recently that fibroblastic cells derived from α_5-null mutant embryos are capable of assembling focal contacts on Fn substrata, migrating in response to Fn and assembling a Fn matrix (Yang et al., 1993). Therefore, either integrin Fn receptors other than $\alpha_5\beta_1$ may support these cellular responses to Fn, or non-integrin mechanisms are involved.

One candidate for mediating cell-Fn interactions in the absence of $\alpha_5\beta_1$ integrins is $\alpha_4\beta_1$ integrin, a versatile adhesion receptor involved in both cell-cell and cell-ECM interactions (Hemler et al., 1990). Two ligands have been identified that bind to the $\alpha_4\beta_1$ integrin. One is the vascular cell adhesion molecule-1 (VCAM-1) present on the surface of cytokine-activated endothelial cells (Osborn et al., 1989; Elices et al., 1990). The other is the CS1 or V25 site that is located in the alternatively spliced IIICS or V region of Fn (Wayner et al., 1989; Guan and Hynes, 1990; Mould et al., 1990; Komoriya et al., 1991). It has been shown that $\alpha_4\beta_1$ integrins mediate cell adhesion to VCAM-1 (Elices et al., 1990; Guan and Hynes, 1990; Allavena et al., 1991) and Fn (Wayner et al., 1989; Garcia-Pardo et al., 1990; Guan and Hynes, 1990). This integrin is speculated to play important roles in embryogenesis (Rosen et al., 1992; Yang et al., 1995) and in the pathophysiology of many diseases including asthma, allergy, arthritis, inflammation, and tumor cell metastasis (Rice and Bevilacqua, 1989; Laffon et al., 1991; Kawaguchi et al., 1992; Walsh and Murphy, 1992; Bao et al., 1993; Issekutz, 1993). All of these processes involve extensive cell migration, suggesting that this receptor may also mediate cell migration. Previous studies using Fn fragments and anti-integrin antibodies indicate that the $\alpha_4\beta_1$ integrin may be involved in migration of neural crest cells (Dufour et al., 1988), lymphocytes (Chan and Aruffo, 1993) or lymphocyte progenitors (Miyake et al., 1992) on Fn. However, all three cell types studied previously also expressed other Fn integrin receptors including $\alpha_5\beta_1$ integrins. In fact, $\alpha_5\beta_1$ integrins were also

shown to be involved in the migration of these cells on Fn (Dufour et al., 1988; Miyake et al., 1992; Chan and Aruffo, 1993). Therefore, it remains to be established if $\alpha_4\beta_1$ integrins can mediate cell migration on Fn in the absence of $\alpha_5\beta_1$ integrins.

Previous studies have shown that α_5 deficient CHO B2 cells were unable to adhere or migrate on Fn coated surfaces (Schreiner et al., 1989; Giancotti and Ruoslahti, 1990; Bauer et al., 1992) or assemble a Fn-containing extracellular matrix (Wu et al., 1993). Expression of the α_5 integrin in the CHO B2 cells restored all these cellular responses to Fn (Bauer et al., 1992; Wu et al., 1993). In contrast, expression of another integrin Fn receptor, $\alpha_v\beta_1$, in these cells restored cell adhesion and spreading on Fn, but not migration or assembly of Fn matrix (Zhang et al., 1993). To determine whether $\alpha_4\beta_1$ integrins can function in the absence of $\alpha_5\beta_1$ integrins in these responses to Fn, we have expressed human α_4 integrin in CHO B2 cells. We report here that $\alpha_4\beta_1$ integrins mediate cell adhesion and spreading, as well as motility on Fn in the absence of $\alpha_5\beta_1$ integrins. However, $\alpha_4\beta_1$ integrins cannot substitute for the $\alpha_5\beta_1$ integrin in Fn matrix assembly. We also show that the $\alpha_4\beta_1$ integrin promotes cell motility in response to VCAM-1, another ligand, and that this response is partially inhibited by the CS1 peptide mimic of the binding site on Fn.

MATERIALS AND METHODS

Cells and reagents

CHO B2 cells were kindly provided by Dr R. Juliano (Department of Pharmacology, University of North Carolina, Chapel Hill, NC) and were grown and maintained in α-MEM (Gibco Laboratories, Grand Island, NY) containing 10% FBS (Atlanta Biologicals, Norcross, GA), 1% antibiotic-antimycotic mixture (Sigma Chemical Co., St Louis, MO) (Danilov and Juliano, 1989; Schreiner et al., 1989; Zhang et al., 1993). The cells were maintained routinely in monolayer culture. Mouse cellular Fn was purified by gelatin-Sepharose chromatography from mouse 3T6 cell conditioned medium that was prepared by growing the 3T6 cells in Dulbecco's modified essential medium (DMEM) containing 10% Fn-depleted fetal bovine serum (FBS). Recombinant VCAM-1 was kindly provided by Dr Laurelee Osborn (Biogen Inc., Cambridge, MA). A vector containing the entire coding sequence of the human α_4 cDNA (pCDM8/α_4) and a rabbit polyclonal antiserum that was raised against a 21 amino acid synthetic peptide corresponding to the C terminus of human α_4 integrin (N11) were gifts from Dr Yoshikazu Takada (Scripps Research Institute) and Dr Martin Hemler (Harvard Medical School), respectively. Mouse monoclonal anti-α_4 antibody P4C2 was kindly provided by Dr E. A. Wayner (University of Minnesota) or purchased from GIBCO BRL (Gaithersburg, MD). CS1-BSA was a gift from Dr Akira Komoriya (FDA, Bethesda, MD). CS1 peptide (CDELPQLVTLPHPNLHGPEILDVPST) and a scrambled CS1 peptide CS1S (CEHPLNQVLHDLPTLPGPSVDPTLIE) were synthesized and purified by the Peptide Synthesis and Sequencing Core Facility, Mayo Clinic, based on a previously described method (Humphries et al., 1987). Biotin-X-NHS (water soluble) was from Calbiochem (San Diego, CA).

Transfection

CHO B2 cells (4×10^6) were suspended in 0.4 ml of RPMI 1640 medium and electroporated with a BTX 600 electroporator at 360 volts and 600 microfarads with 20 μg of pCDM8/α_4 linearized with *Sac*II, 5 μg of pSV2Neo, and 100 μg/ml of salmon sperm DNA (Sigma, St Louis, MO). To create control cell lines, CHO B2 cells were electroporated with 20 μg of pCDM8/α_4 cleaved in the α_4 coding region with *Xma*I. 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). CHO cells that expressed α_4 on their surface were isolated using anti-human α_4 monoclonal antibody P4C2 (Gibco BRL, Gaithersburg, MD) and DYNABEADS coated with goat anti-mouse IgG (Dynal Inc., Great Neck, NY) following the manufacturer's procedure. The cells stably expressing α_4 were cloned by limited dilution and maintained in α-MEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine and 0.5 mg active geneticin/ml.

Immunoprecipitation

Cells (CHO B2, AA2, AD5 and BA4 cells) from one confluent 100 mm tissue culture dish were surface biotinylated with biotin-X-NHS and extracted with 0.2 ml of 1% (v/v) Triton X-100 containing 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 (50 μl) of the cell extracts were incubated with monoclonal anti-α_4 antibody P4C2 (5 μl of ascites) or as a control, the anti-Fn monoclonal antibody N294, followed by precipitation with an anti-mouse κ chain antibody coupled to Sepharose 4B (Zymed, San Francisco, CA). The precipitated proteins were separated on reducing SDS-PAGE and transferred onto Immobilon-pR (Millipore, Bedford, MA). The biotinylated cell surface proteins were detected by avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) and the ECL^R detection system (Amersham, England) following the manufacturers' protocols.

Immunoblotting of integrins

CHO B2, AA2, AD5, BA4 and human rhabdomyosarcoma (RD) cells were lysed in lysis buffer (1% Triton X-100, 140 mM NaCl, 50 mM Tris-HCl, pH 8, 2 mM PMSF, 5 mM EDTA, 0.2 mM 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF), 10 mM leupeptin and 1 mM pepstatin). The cellular proteins were separated on reducing and nonreducing SDS-PAGE, and the α_4 subunit was detected by immunoblot with N11 rabbit antiserum (1:400 dilution), horseradish peroxidase labeled anti-rabbit IgG (Amersham, England, 1:1000 dilution) and the ECL^R detection system (Amersham, England) following the manufacturers' protocols.

Cell adhesion and spreading assays

Cell adhesion and spreading assays (Humphries et al., 1987; Komoriya et al., 1991) were performed in 96-well ELISA plates (Corning). The wells were coated with 100 μg/ml CS1-BSA, 10 μg/ml mouse cellular Fn, 10 μg/ml or 2.5 μg/ml recombinant VCAM-1 in 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 2.7 mM KCl, 137 mM NaCl, 1 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4 (PBS+Ca^{2+}, Mg^{2+}) as specified in each experiment at 37°C for 1 hour. Then each well was incubated with 200 μl of 10 mg/ml heat treated BSA in PBS at 37°C for 1 hour and rinsed with α-MEM (Gibco, Grand Island, NY). For adhesion, 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 $MnCl_2$, CS1, CS1S peptide or anti-α_4 monoclonal antibody P4C2 (1:50 dilution of the ascites) as specified in each experiment. Cell suspensions containing 3×10^4 cells were added to each well of the 96-well ELISA plates and allowed to attach to the substrata for 90 minutes in a 37°C incubator under a 5% CO_2-95% air atmosphere. Under this experimental condition, the maximum cell adhesion level is 3×10^4 cells/well or 933 cells/mm^2. The wells were then washed twice with PBS, and the numbers of the attached cells in each well were determined by measuring *N*-acetyl-*β*-D-hexosaminidase activity as described previously (Landegren, 1984). The cell numbers (up to 3×10^4) are directly proportional to the absorbance in the hexosaminidase assay.

In the cell spreading assay, the cells were seeded and incubated as described above. At the end of incubation, phase-contrast images of

randomly selected fields were recorded with a Sony CCD video camera and a Sony UP-860 video graphic printer. The percentage of cells adopting a well-spread morphology was estimated by counting at least 300 cells (Komoriya et al., 1991).

Cell motility assay

The cell motility assay was performed as previously described (Bauer et al., 1992). The undersurface of Transwell motility chambers (8 μm diameter pore size, Costar, Cambridge, MA) was coated with 10 μg/ml of mouse cellular Fn, 5 μg/ml of VCAM-1 or 100 μg/ml of CS1-IgG. Cells (2×10^5 cells in 0.1 ml medium/chamber) suspended in 1% BSA-α-MEM or 1% BSA-α-MEM containing various peptides as specified in each experiment were 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. At the end of the 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. No cells were observed after both the upper and under surfaces of the filters were wiped with cotton-tipped applicators, indicating the cells counted had indeed migrated through the pores of the membranes. The cells from ten randomly selected microscopic fields were counted and the cell motility was expressed as: the number of the cells/mm^2 of the microscopic field.

Fn matrix assembly assays

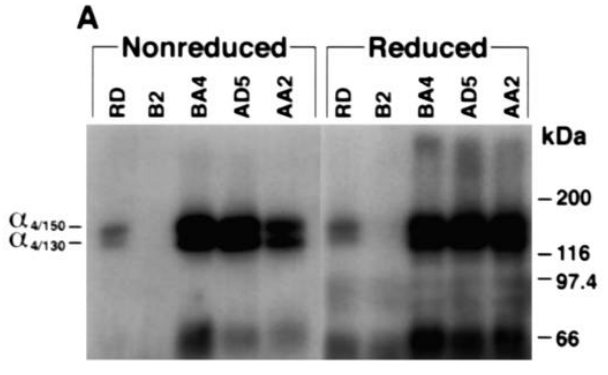
Assembly of a Fn-containing matrix by CHO cells was determined by immunofluorescent staining of the cell monolayers and by immunoblot detection of Fn in the 2% deoxycholate insoluble fractions prepared from the cells. For immunofluorescent staining, CHO Xma, AD5 and B2B4 cells were seeded in Lab-Tek 8 chamber slides in α-MEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 0.5 mg active geneticin/ml at densities yielding confluent monolayers after 3 days. The culture media were supplemented with 50 μg/ml bovine plasma Fn 18 hours after the seeding. Three days after seeding, the cell monolayers were fixed with 4% paraformaldehyde, stained with a rabbit anti-fibronectin antibody and FITC-conjugated goat anti-rabbit IgG antibodies as previously described (Wu et al., 1993). The Fn-containing matrix was observed by epifluorescence microscopy (Nikon FXA) and photographed under identical exposure conditions. For immunoblotting, the CHO cells were grown in 100 mm tissue culture plates under the same culture conditions as described above. Confluent cell layers were washed 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 2% deoxycholate-insoluble material was solubilized in SDS and separated by SDS-PAGE under reducing conditions. Fn was detected using a rabbit anti-Fn antibody and alkaline phosphatase-conjugated goat-anti-rabbit IgG after transfer to a PVDF membrane (Immobilon-PR, Millipore Corp.). Each lane was loaded with the matrix fraction corresponding to 100 μg of the 3% Triton X-100 soluble cellular proteins as determined by BCA protein assay using BSA as standard (Pierce, Rockford, IL).

RESULTS

Expression of α_4 integrin in α_5 deficient CHO B2 cells

The α_4 integrin subunit was introduced into CHO B2 cells by transfection of the cells with an expression vector encoding full-length human α_4 (pCDM8/α_4). The CHO cells that stably expressed α_4 on their surface were isolated and cloned as described in Materials and Methods. The expression of α_4 by the cloned cells was confirmed by both immunoblot and immunoprecipitation. Fig. 1A shows the result of an immunoblot using a polyclonal anti-α_4 antibody raised to a cytoplasmic domain peptide. Two major bands (apparent molecular mass of 150 and 130 kDa, respectively) were recognized by the polyclonal anti-α_4 antibody in all three clones (AA2, AD5 and BA4), as well as in human rhabdomyosarcoma (RD) cells that express $\alpha_4\beta_1$ integrin, whereas no distinct polypeptides were recognized in the parental CHO B2 cells. These two bands could also be precipitated by a monoclonal anti-α_4 antibody (Fig. 1B). The 150 kDa protein is presumably the intact α_4 subunit. The nature of the 130 kDa protein is unknown. It could represent a precursor or a protease cleavage product of the intact α_4 subunit.

To determine whether the α_4 integrin is expressed on the cell surface, cell surface proteins of CHO B2, AA2, AD5 and BA4 cells were biotinylated, and lysates immunoprecipitated with the monoclonal anti-α_4 antibody P4C2. Four biotinylated bands (apparent molecular mass of 150, 130, 80 and 70 kDa) were detected in the precipitates from all three α_4 expressing cell lines but not the CHO B2 precipitate (Fig. 2). The 150 kDa, 80 kDa and 70 kDa bands most likely represent the intact and the proteolytic fragments of the α_4 subunit that have been reported previously (Hemler et al., 1990). The 130 kDa band is the β_1 subunit associated with the α_4 as determined by immunoblot with an anti-β_1 antibody (not shown). The association of the α_4 with the β_1 subunit was confirmed by experiments in which lysates immunoprecipitated by an anti-β_1 cytoplasmic domain antibody were immunoblotted with the anti-α_4 monoclonal P4C2 (not shown). We conclude from these results



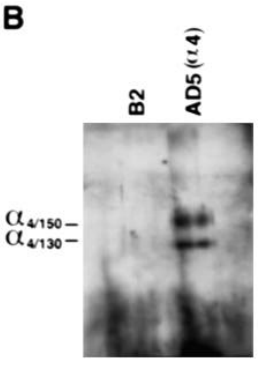


Fig. 1. Immunoblot detection of α_4 integrins produced by α_4 transfectants AA2, AD5 and BA4. Cells from one confluent 100 mm tissue culture dish were lysed with 0.2 ml of lysis buffer. (A) The soluble cellular proteins from CHO B2, BA4, AD5, AA2 or human rhabdomyosarcoma cells were separated on reducing and nonreducing SDS-PAGE gels. (B) The $\alpha_4\beta_1$ integrins from CHO B2 and AD5 lysates were immunoprecipitated with monoclonal anti-α_4 antibody P4C2 and the precipitated proteins were separated on reducing SDS-PAGE. The α_4 subunit was detected by immunoblotting with a rabbit polyclonal anti-α_4 cytoplasmic domain antiserum N11 (1:400 dilution) and the ECL^R detection system.

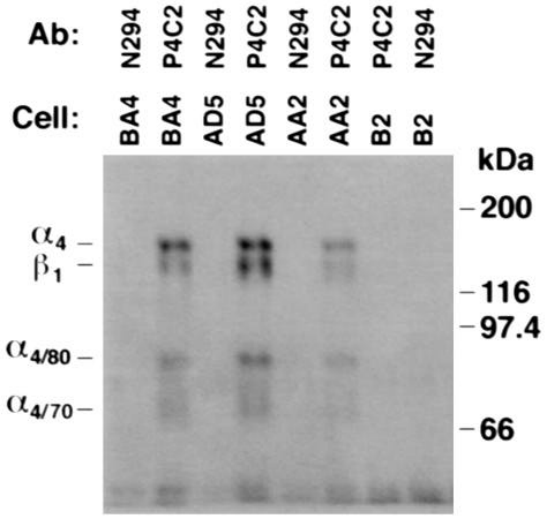


Fig. 2. Detection of cell surface α_4 integrins produced by α_4 transfectants AA2, AD5 and BA4. Cells (the α_4-negative parental CHO B2 cells and the α_4-positive CHO AA2, AD5 and BA4 cells) from one confluent 100 mm tissue culture dish were surface biotinylated, lysed and immunoprecipitated with monoclonal anti-α_4 antibody P4C2 or as a control, anti-Fn monoclonal antibody N294. The precipitated proteins were separated on reducing SDS-PAGE and the biotinylated proteins were detected by avidin-biotinylated horseradish peroxidase complex and the ECL^R detection system.

that the human α_4 is associated with hamster β_1 and expressed on the cell surface of the CHO AA2, AD5 and BA4 cells.

The $\alpha_4\beta_1$ integrin mediates adhesion and spreading of CHO cells on fibronectin and VCAM-1

We next determined whether the hybrid human α_4-hamster β_1 integrin can mediate cell adhesion and spreading on Fn and VCAM-1. We found that the α_4-expressing CHO AA2, AD5 and BA4 cells adhered to Fn, VCAM-1 and CS1-BSA, while the α_5-positive, α_4-negative CHO B2B4 cells adhered only to Fn. The α_4 and α_5 double negative CHO B2 or Xma cells, which were obtained by transfecting the CHO B2 cells with a CDM8/α_4 vector cleaved in the α_4 coding region, adhered to neither Fn nor VCAM-1. Fig. 3A shows the results with CHO Xma, AD5 and B2B4 cells. The adhesion of the α_4-positive CHO AD5 cells to Fn and VCAM-1 was inhibited by the CS1 peptide. More than 70% of the adhesion to Fn and 55% of that to VCAM-1 was inhibited with 88 μM of the peptide. Increasing CS-1 peptide concentration did not significantly increase the inhibition (Fig. 3B). This inhibitory effect is specific for the CS1 sequence, since no inhibition was seen when the cells were incubated with same concentrations of a scrambled CS1 peptide (CS1S) (not shown). These results suggest that the human α_4/hamster β_1 integrin functions as a receptor supporting cell adhesion on both Fn/CS1 and VCAM-1. In support of this, the adhesion of the AD5 cells to CS1 and VCAM-1 was inhibited by monoclonal anti-α_4 antibody P4C2 by 96% and 84%, respectively (not shown).

We next determined the effect of Mn^{2+} on AD5 adhesion to CS1 and VCAM-1. To do this, we decreased the coating concentration of VCAM-1 to 2.5 μg/ml because the AD5 adhesion on wells coated with 10 μg/ml VCAM-1 was close to saturation. The results demonstrated that the adhesion of the AD5

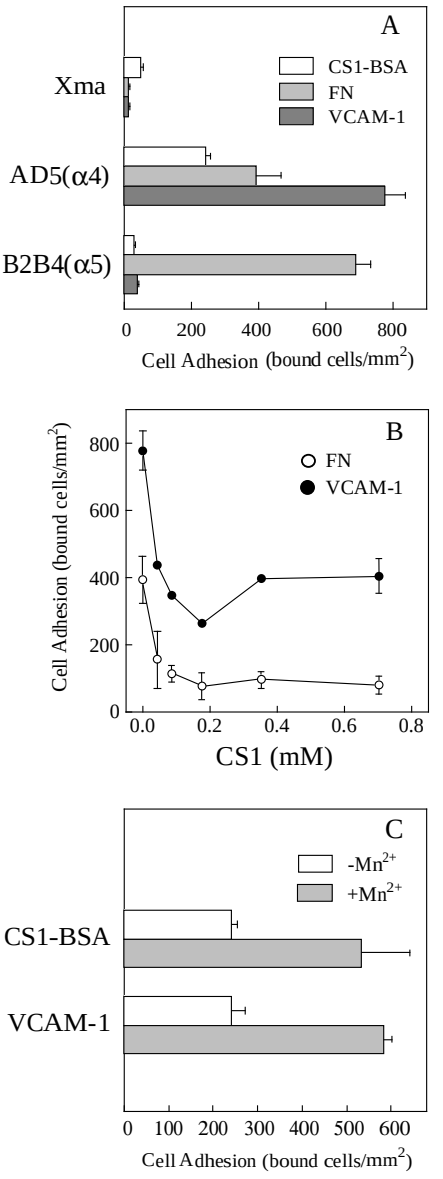


Fig. 3. CHO cells expressing $\alpha_4\beta_1$ integrins adhere to CS1-BSA, Fn and VCAM-1. (A) The Xma cells (α_4, α_5-negative), the AD5 cells (α_4-positive, α_5-negative) and the B2B4 cells (α_4-negative, α_5-positive) were suspended in α-MEM and seeded into wells coated with 100 μg/ml CS1-BSA, 10 μg/ml mouse cellular Fn or 10 μg/ml VCAM-1. (B) The AD5 cells were suspended in α-MEM containing various concentrations of CS1 peptide and seeded into wells coated with 10 μg/ml mouse cellular Fn or 10 μg/ml VCAM-1. (C) The AD5 cells were suspended in α-MEM ($-Mn^{2+}$) or α-MEM supplemented with 0.5 mM Mn^{2+} ($+Mn^{2+}$) and seeded into wells coated with 100 μg/ml CS1-BSA or 2.5 μg/ml VCAM-1. Cell adhesion was quantified as described in Materials and Methods. The data represent means ± S.D. from duplicate wells.

cells to both CS1 and VCAM-1 was greatly enhanced by Mn^{2+} (Fig. 3C). None of the cells adhered to surfaces coated with BSA, either in the presence or absence of Mn^{2+} (not shown).

The $\alpha_4\beta_1$ expressing CHO cell lines spread well on Fn, VCAM (Fig. 4A) and on invasin (not shown), an integrin binding protein from *Yersinia pseudotuberculosis* (Isberg and

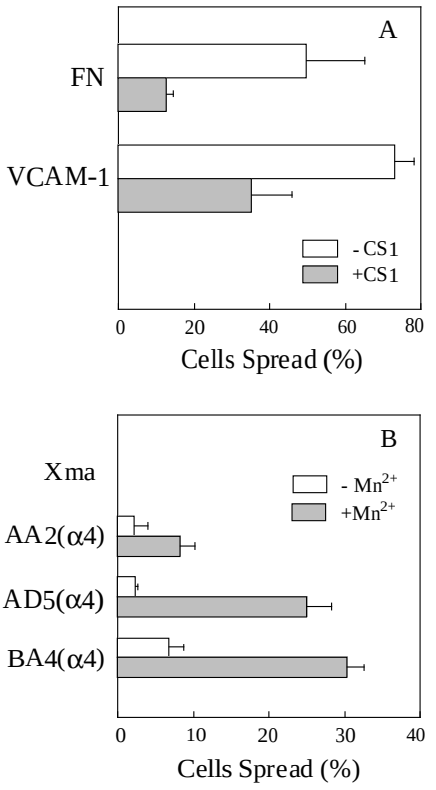


Fig. 4. CHO cells expressing $\alpha_4\beta_1$ integrin spread on Fn, VCAM-1 and CS1-BSA. (A) The α_4-positive AD5 cells were suspended in α-MEM (–CS1) or α-MEM supplemented with 88 μM CS1 peptide (+CS1) and seeded into plates coated with 10 μg/ml mouse cellular Fn or 10 μg/ml VCAM-1. (B) The α_4-negative Xma cells and the α_4-positive AA2, AD5 and BA4 cells were suspended in α-MEM ($-Mn^{2+}$) or α-MEM supplemented with 0.5 mM Mn^{2+} ($+Mn^{2+}$) and seeded into wells coated with 100 μg/ml CS1-BSA. The percentage of cells adopting a well-spread morphology was determined as described in Materials and Methods. The data represent means ± S.D. from duplicate wells.

Leong, 1990). The AD5 spreading on Fn and that on VCAM-1 was decreased by 75% and 52%, respectively, in the presence of soluble CS1 peptide (Fig. 4A). Although the CS1 peptide is an effective inhibitor of $\alpha_4\beta_1$ mediated cell spreading, it has much lower activity in supporting spreading of the CHO cells (Fig. 4B). The cell spreading on CS1-BSA is greatly increased in the presence of 0.5 mM Mn^{2+} (Fig. 4B). Taken together, these results demonstrate that the human α_4/hamster β_1 integrin functions as a receptor supporting cell adhesion and spreading on Fn/CS1 and VCAM-1, and the receptor activities are modulated by Mn^{2+}.

$\alpha_4\beta_1$ Integrins support cell motility on fibronectin and VCAM-1

To determine whether $\alpha_4\beta_1$ integrins support cell motility on Fn or VCAM-1, we compared the ability of the α_4 expressing

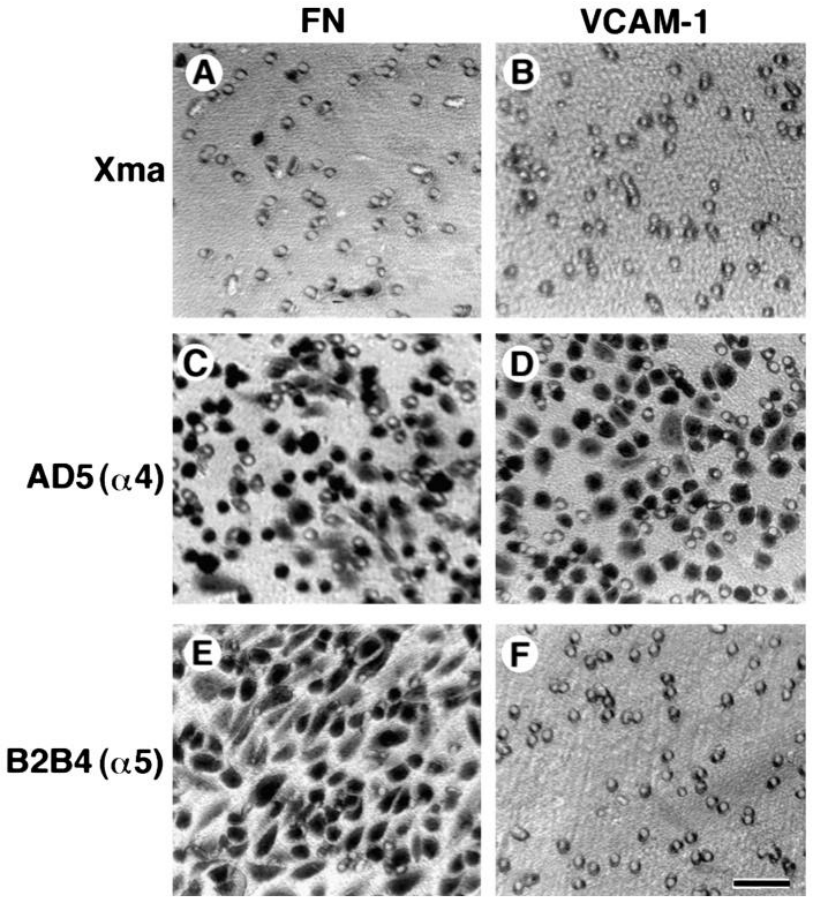


Fig. 5. CHO cells expressing $\alpha_4\beta_1$ integrin migrate in response to Fn and VCAM. The undersurfaces of Transwell motility chamber inserts were coated with 10 μg/ml mouse cellular Fn (A,C and E) or 5 μg/ml VCAM-1 (B, D and F). The α_4-, α_5-negative Xma cells (A and B), the α_4-positive AD5 cells (C and D) and the α_4-negative, α_5-positive B2B4 cells (E and F) were added to the upper chambers of Transwell and incubated as described in Materials and Methods. At the end of the incubation, the cells on the upper surface of the membrane were removed and the cells on the undersurface were stained. Bar in F, 50 μm.

CHO cells to migrate on Fn and VCAM-1 with the α_4-negative CHO cells. The α_4-, α_5-negative CHO B2 or Xma cells did not migrate on Fn (Fig. 5A) or on VCAM-1 (Fig. 5B), whereas the α_4-positive, α_5-negative CHO AD5 cells were able to migrate on either Fn (Fig. 5C) or VCAM-1 (Fig. 5D). The α_4-negative, α_5-positive CHO B2B4 cells migrate on Fn (Fig. 5E) but not VCAM-1 (Fig. 5F). These results demonstrate that the $\alpha_4\beta_1$ integrins support cell motility in response to Fn or VCAM-1 in the absence of the $\alpha_5\beta_1$ integrin, whereas $\alpha_5\beta_1$ integrins mediate motility only on Fn, not VCAM-1.

The $\alpha_4\beta_1$ integrin mediated cell motility on Fn was reduced by approximately 60% in the presence of 0.7 mM CS1 peptide (Fig. 6). This inhibitory effect was specific for the CS1 sequence, since no inhibition was seen when the cells were incubated with the same concentration of the scrambled CS1 peptide (CS1S) (Fig. 6). Thus, the CS1 sequence is involved in the motility of the $\alpha_4\beta_1$ expressing CHO cells on Fn. On the other hand, the CS1 peptide only inhibited cell motility on VCAM-1 by approximately 25% (Fig. 6).

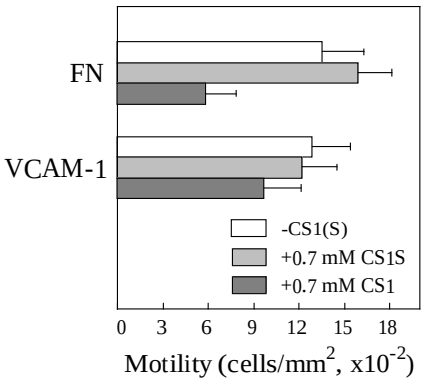


Fig. 6. Inhibition of the $\alpha_4\beta_1$ integrin mediated cell motility on Fn and VCAM-1 by CS1 peptide. The AD5 cells were suspended in 1% BSA-αMEM (–CS1(S)), 1% BSA-αMEM containing 0.7 mM CS1S (+0.7 mM CS1S) or CS1 peptide (+0.7 mM CS1). The cell suspensions were then added to the upper chamber of the Transwell inserts in which the undersurface of membranes was coated with 10 μg/ml mouse cellular Fn or 5 μg/ml VCAM-1. 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 the cells/mm^2 of the microscopic field (error bars =S.D.).

The CS1 sequence is not only involved in $\alpha_4\beta_1$ mediated cell motility on Fn, it is also sufficient to support $\alpha_4\beta_1$ mediated cell motility. Fig. 7 shows that the $\alpha_4\beta_1$-positive CHO BA4 cells migrate on CS1 peptide conjugated to rabbit IgG (CS1-IgG) whereas no cells migrate on CS1S-IgG (Fig. 7A,B). As we expected, the $\alpha_4\beta_1$-negative Xma cells migrated neither on CS1-IgG nor on CS1S-IgG (Fig. 7C,D). The $\alpha_4\beta_1$-mediated cell motility on immobilized CS1-IgG was almost completely inhibited by soluble CS1 peptide (Fig. 8).

$\alpha_4\beta_1$ Integrins cannot functionally substitute for $\alpha_5\beta_1$ integrins in fibronectin matrix assembly

We showed previously that α_5 deficient CHO B2 cells were unable to incorporate exogenously supplied Fn into the extracellular matrix, whereas expression of the α_5 integrin in the CHO B2 cells restored Fn matrix assembly activity. To test whether $\alpha_4\beta_1$ integrins can substitute for $\alpha_5\beta_1$ integrins in Fn matrix assembly, we determined the abilities of the Xma cells (α_4-, α_5-negative), the AD5 cells (α_4-positive, α_5-negative) and the B2B4 cells (α_4-negative, α_5-positive) to assemble a Fn-containing extracellular matrix. Fig. 9 shows that neither the Xma (Fig. 9A) nor the AD5 (Fig. 9B) cells are capable of assembling a Fn matrix, while the α_5-positive CHO B2B4 cells readily assembled a fibrillar Fn matrix under the same culture conditions (Fig. 9C). Increasing the Fn concentration in the culture medium to 300 μg/ml did not result in assembly of a fibrillar Fn matrix by the AD5 cells (not shown). The failure of the α_4-positive, α_5-negative AD5 cells to assemble a Fn matrix was confirmed by analyzing 2% deoxycholate insoluble fractions (Wu et al., 1993) prepared from the CHO cells. Like diploid fibroblasts (Barry and Mosher, 1988; McDonald, 1988; Quade and McDonald, 1988), CHO B2B4 cells deposit Fn into a matrix form that resists solubilization in deoxycholate containing solutions (Fig. 10). No Fn was detected in the 2% deoxycholate insoluble fractions prepared from the Xma (α_4-, α_5-negative) or AD5 cells (α_4-positive, α_5-negative) cultured

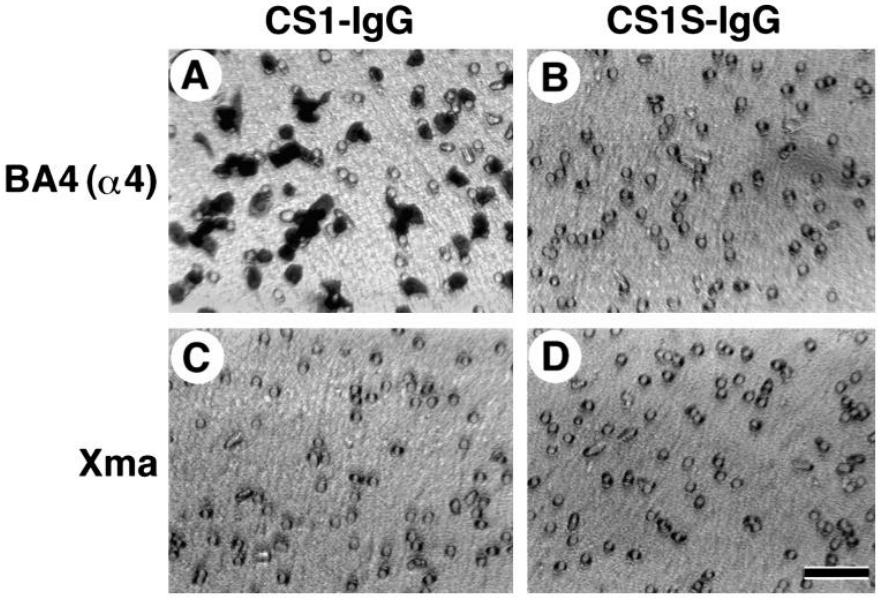


Fig. 7. The CS1 sequence supports α_4 integrin mediated cell motility. The undersurfaces of Transwell motility chamber inserts were coated with 100 μg/ml CS1-IgG (A and C) or 100 μg/ml CS1S-IgG (B and D). The α_4-positive BA4 cells (A and B) and the α_4-negative Xma cells (C and D) were added to the upper chambers of the Transwell and the motility assay was performed as described in Materials and Methods. Bar in D, 50 μm.

with exogenous plasma Fn (Fig. 10). Therefore, although the $\alpha_4\beta_1$ integrins mediate CHO cell attachment, spreading and motility on Fn, they cannot substitute for $\alpha_5\beta_1$ integrins in Fn matrix assembly.

DISCUSSION

The ability of cells to attach and to migrate in response to extracellular stimuli is critical during normal embryological development and many pathological processes (Humphries et al., 1991). Cell migration along solid substrata requires cell surface receptors interacting with both substratum and cytoskeleton. Previous studies have shown that the $\alpha_5\beta_1$ integrin is involved in motility of many types of cells on Fn (Akiyama et al., 1989; Schreiner et al., 1989; Giancotti and Ruoslahti, 1990; Bauer et al., 1992). A major finding of this study is that $\alpha_4\beta_1$ integrins mediate cell motility on Fn independent of $\alpha_5\beta_1$. The $\alpha_4\beta_1$ integrin recognizes the alternatively spliced CS1 site on Fn, and we have shown that this site alone is sufficient to promote $\alpha_4\beta_1$ integrin-mediated cell motility. Therefore, like cell adhesion, cell motility on Fn may be modulated by both the types of Fn receptors ($\alpha_4\beta_1$ or $\alpha_5\beta_1$ or both) and by isoforms of Fn containing or lacking the CS1 sequence.

Cells derived from α_5-null mutant embryos were able to migrate in response to Fn (Yang et al., 1993). Our results provide a possible explanation for that observation, namely that $\alpha_4\beta_1$ is sufficient to mediate cell motility on Fn. Of course, we cannot rule out the possibility that other Fn receptors also support cell motility. Not all Fn binding receptors are capable of mediating cell motility on Fn. For example, the $\alpha_v\beta_1$ integrin expressed in the α_5 deficient CHO B2 cells binds Fn and mediates cell adhesion and spreading on Fn but does not support cell motility (Zhang et al., 1993).

Previous studies have shown that the α_5 deficient CHO B2 cells do not adhere, spread or migrate (Schreiner et al., 1989; Bauer et al., 1992) in response to Fn nor can they assemble a fibrillar Fn matrix (Wu et al., 1993). Expression of $\alpha_5\beta_1$ integrins in the B2 cells restored all these cellular activities (Bauer et al., 1992; Wu et al., 1993) whereas expression of $\alpha_v\beta_1$ integrins restored only cell adhesion and spreading on Fn but not cell motility or Fn matrix assembly (Zhang et al., 1993). The data presented herein demonstrate that expression of $\alpha_4\beta_1$ integrins restored cell adhesion, spreading and motility in response to Fn but not assembly of a fibrillar Fn matrix. The level of $\alpha_4\beta_1$ expressed on CHO AD5 cell surface is less than that of $\alpha_5\beta_1$ on CHO B2B4 surface but more than that of $\alpha_5\beta_1$ on CHO 1-23 surface (Wu and McDonald, unpublished observation). Our previous studies have shown that the CHO 1-23 cells are capable of assembling a Fn matrix (Wu et al., 1993). Thus, the impaired ability of the CHO AD5 cells to assemble a Fn matrix cannot be simply explained by lack of enough Fn-binding integrins on the cell surface. Instead, these results strongly suggest that there are true functional differences between $\alpha_4\beta_1$ and $\alpha_5\beta_1$ Fn receptors in Fn matrix assembly.

How these two Fn binding integrins function differently in Fn matrix assembly is currently unknown, but several reasonable hypotheses, which are not mutually exclusive, can be delineated. One possibility is that $\alpha_4\beta_1$ has an unfavorable binding affinity and/or kinetics compared to $\alpha_5\beta_1$ to retain sufficient Fn on the cell surface to assemble a Fn matrix. This is the case for $\alpha_v\beta_1$, which has a four-fold lower affinity for the 110 kDa cell binding fragment of Fn than $\alpha_5\beta_1$ (Zhang et al., 1993). The second possibility is that binding of $\alpha_5\beta_1$ to the RGD containing cell adhesive domain of Fn induces a conformational change in Fn, facilitating Fn-Fn interaction during Fn matrix assembly, whereas binding of $\alpha_4\beta_1$ to the CS1 site of Fn does not. The third possibility is that $\alpha_5\beta_1$, but not $\alpha_4\beta_1$, functions in Fn matrix assembly not only by binding to the cell adhesive domain of Fn, but also by modulating other cell surface activity, such as binding of the 29 kDa amino-terminal domain of Fn (Wu et al., 1993), that is critical for Fn matrix assembly (McDonald, 1988). It is possible that $\alpha_4\beta_1$ and $\alpha_5\beta_1$ transduce different signals into the cells and up- or down-regulate expression of certain genes coding for molecules that

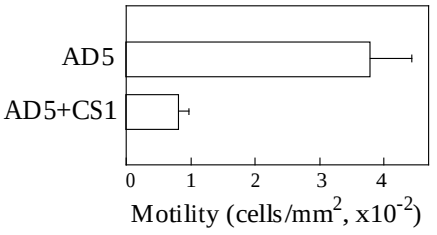


Fig. 8. Inhibition of the $\alpha_4\beta_1$ integrin mediated cell motility on CS1-IgG by soluble CS1 peptide. The AD5 cells were suspended in 1% BSA-αMEM (AD5) or 1% BSA-αMEM containing 0.7 mM CS1 (AD5+CS1). The cell suspensions were then added to the upper chambers of the Transwell inserts in which the undersurfaces of membranes were coated with 100 μg/ml CS1-IgG. 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 the cells/mm^2 of the microscopic field (error bars =S.D.).

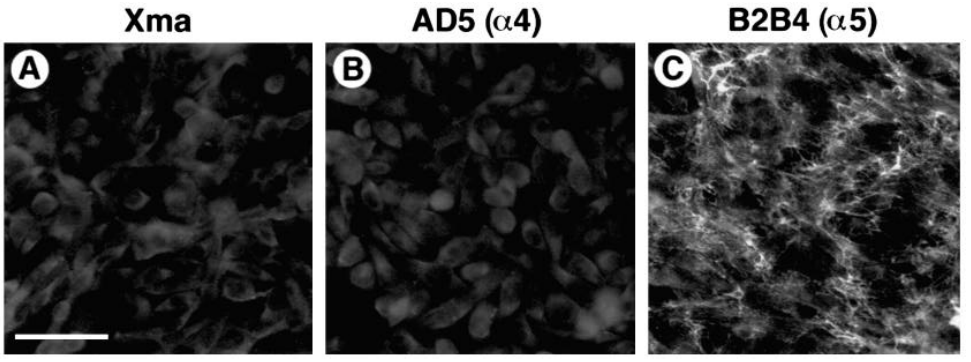


Fig. 9. CHO AD5 cells (α_4-positive, α_5-negative) do not assemble a fibrillar Fn-containing matrix whereas CHO B2B4 cells (α_4-negative, α_5-positive) do. CHO Xma cells (A), AD5 cells (B) and B2B4 cells (C) were stained with a rabbit IgG anti-fibronectin antibody and FITC-conjugated goat anti-rabbit IgG antibodies as described in Materials and Methods. The cells were observed under epifluorescence microscopy. Bar in A, 50 μm, applies to all panels.

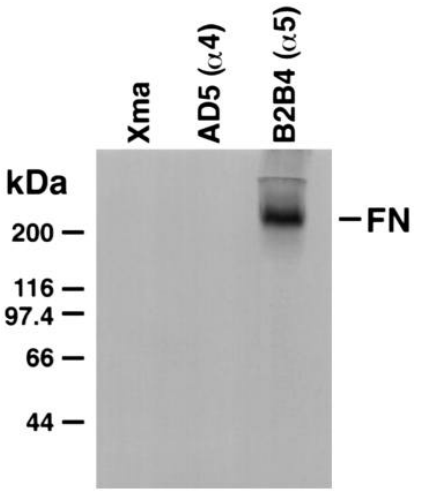


Fig. 10. CHO AD5 cells (α_4-positive, α_5-negative) do not deposit a Fn matrix resisting detergent solubilization whereas CHO B2B4 cells (α_4-negative, α_5-positive) do. CHO Xma cells, AD5 cells and B2B4 cells were grown in media supplemented with 50 μg/ml bovine plasma Fn. Fn in the deoxycholate-insoluble ECM fractions was detected by immunoblot as described in Materials and Methods. Each lane was loaded with the matrix fraction corresponding to 100 μg of the 3% Triton X-100 soluble cellular proteins.

are directly or indirectly involved in organizing the 29 kDa amino-terminal fragment binding sites. Alternatively, $\alpha_5\beta_1$, but not $\alpha_4\beta_1$, is capable of associating with other molecules to form a cell surface complex that binds to the amino-terminal domain of Fn. The latter possibility is supported by recent observations that $\alpha_5\beta_1$ and the amino-terminal fragment of Fn were co-localized in focal adhesions (Peters and Mosher, 1994). The α_4-positive, α_5-negative CHO cells described in this report, together with the previously described α_4-negative, α_5-positive CHO cells, will be excellent model systems for testing these hypotheses.

The data presented herein show that expression of $\alpha_4\beta_1$ integrins in the CHO cells enables the cells to adhere, spread and migrate in response to VCAM-1. The cell adhesion and cell motility mediated by $\alpha_4\beta_1$/VCAM-1 were inhibited by CS1 peptide by 55% (Fig. 3B) and 25% (Fig. 6), respectively, indicating that the binding of $\alpha_4\beta_1$ to VCAM-1 is related to the binding of $\alpha_4\beta_1$ to CS1. The difference in inhibition is probably caused by the difference between the two assays. The cell adhesion assay measures the ability of a cell to resist detachment by shear forces. The CS-1 peptide occupies the ligand binding site of $\alpha_4\beta_1$ integrins and reduces the number of receptors binding to VCAM. This reduction in strength results in cell detachment from the substratum. However, the reduced cell-VCAM interaction may still be sufficient for most cells to generate the adhesion required for cell migration in our cell motility assay. The inhibition of cellular processes mediated by $\alpha_4\beta_1$/VCAM-1 by CS1 peptide is consistent with results from recent studies. For example, Makarem et al. (1994) showed recently using solid-phase competition assays that the CS1 inhibition of the VCAM-1-$\alpha_4\beta_1$ interaction was competitive, suggesting that the VCAM-1 and Fn/CS1 binding sites on the $\alpha_4\beta_1$ integrin are spatially close and possibly identical. The cell lines described herein are useful tools in studying the binding mechanisms of the two ligands as well as in identification of compounds blocking $\alpha_4\beta_1$ integrin mediated cell adhesion and migration.

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