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Integrin $\alpha 8\beta 1$ Promotes Attachment, Cell Spreading, and Neurite Outgrowth on Fibronectin

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The integrin $\alpha 8$ subunit, isolated by low stringency hybridization, is a novel integrin subunit that associates with $\beta 1$. To identify ligands, we have prepared a function-blocking antiserum to the extracellular domain of $\alpha 8$, and we have established by transfection K562 cell lines that stably express $\alpha 8\beta 1$ heterodimers on the cell surface. We demonstrate here by cell adhesion and neurite outgrowth assays that $\alpha 8\beta 1$ is a fibronectin receptor. Studies on fibronectin fragments using RGD peptides as inhibitors show that $\alpha 8\beta 1$ binds to the RGD site of fibronectin. In contrast to the endogenous $\alpha 5\beta 1$ fibronectin receptor in K562 cells, $\alpha 8\beta 1$ not only promotes cell attachment but also extensive cell spreading, suggesting functional differences between the two receptors. In chick embryo fibroblasts, $\alpha 8\beta 1$ is localized to focal adhesions. We conclude that $\alpha 8\beta 1$ is a receptor for fibronectin and can promote attachment, cell spreading, and neurite outgrowth on fibronectin.

INTRODUCTION

The integrins are a large family of heterodimeric cell surface molecules that serve as receptors for extracellular matrix molecules (ECM)[1], for members of the Ig family, and for complement and blood clotting proteins (reviewed in Hynes, 1992). Integrins play an important role in development, wound healing, and immune responses (reviewed in Adams and Watt, 1993). Certain bacteria and viruses use integrins as receptors or coreceptors for entry into the host cell (reviewed in Isberg and Van Nhieu, 1994). Each member of the integrin family is a heterodimer composed of one α and one β subunit. To date, 15 different α subunits and eight different β subunits have been characterized in vertebrate species. Most α subunits appear to associate with only one β subunit but a few form heterodimers with more then one β subunit. Some heterodimers have multiple identified ligands; some have only one; for others, including $\alpha 8\beta 1$, ligands are not well characterized (reviewed in Sonnenberg, 1993). In this paper, we demonstrate that $\alpha 8\beta 1$ is a functionally important receptor for fibronectin (FN).

FN is a major component of the ECM and different alternatively spliced forms are expressed in developmentally regulated patterns (ffrench-Constant and Hynes, 1989). Several different integrins serve as receptors for FN and interact with two principal domains within FN. One of these, the central cell-binding domain, contains the RGD sequence within the 10th FNIII repeat. The second domain is located close to the carboxy terminus of FN and contains the alternatively spliced IIICS domain and an adjacent heparin binding sequence. The integrins $\alpha 5\beta 1$, $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 6$, and perhaps $\alpha 3\beta 1$ and $\alpha 7\beta 1$ bind to the RGD site (Pytela *et al.*, 1985a,b; Cheresh and Spiro, 1987; Charo *et al.*, 1990; Dedhar and Gray, 1990; Vogel *et al.*, 1990; Elices *et al.*, 1991; Busk *et al.*, 1992; Gu *et al.*, 1994; Weinacker *et al.*, 1994). The integrin $\alpha IIb\beta 3$ interacts with both the RGD site and a second site within the 9th FNIII repeat (Pytela *et al.*, 1986; Bowditch *et al.*, 1991, 1994). Sequences located within the 8th and 9th FNIII repeats synergize with the RGD site in promoting $\alpha 5\beta 1$ inte-

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[1] Abbreviations used: BSA, bovine serum albumin; ECM, extracellular matrix; FN, fibronectin; FN40, 40 kDa C-terminal fragment of FN; FN45, 45 kDa N-terminal fragment of FN; FN120, 120 kDa fragment of FN; MAb, monoclonal antibody; PBS, phosphate buffered saline; RGD, arginine-glycine-aspartic acid; RGE, arginine-glycine-glutamic acid; VN, vitronectin.

grin-dependent functions such as cell adhesion and cell spreading (Obara *et al.*, 1988; Aota *et al.*, 1991; Kimizuka *et al.*, 1991; Nagai *et al.*, 1991). The integrin $\alpha4\beta1$ binds to IIICS and heparin binding domains of FN whereas the integrin $\alpha4\beta7$ binds to the IIICS region (Wayner *et al.*, 1989; Elices *et al.*, 1990; Garcia *et al.*, 1990; Guan and Hynes, 1990; Mould *et al.*, 1990; Ruegg *et al.*, 1992). Within IIICS, two sequences named CS-1 and CS-5 have been identified, each promoting $\alpha4\beta1$-dependent adhesion. CS-1 appears to be at least two orders of magnitude more active then CS-5 and is therefore the dominant cell binding site (Humphries *et al.*, 1986, 1987; Mould *et al.*, 1990, 1991). The minimal active sequences within CS-1 and CS-5 has been mapped to the tripeptide LDV and tetrapeptide REDV, respectively (Humphries *et al.*, 1986). Interaction of $\alpha4\beta1$ has also been observed with an IDAPS pentapeptide sequence located within the heparin binding domain of FN (Mould and Humphries, 1991).

Recently it has been shown that the ligand specificity of integrin receptors may be regulated. For example, $\alpha4\beta1$ interacts under certain conditions with the RGD site within FN (Sanchez-Aparicio *et al.*, 1994). Interaction of $\alpha4\beta1$ with RGD was only observed after activation of $\alpha4\beta1$ with an antibody directed against the $\beta1$ subunit (Sanchez-Aparicio *et al.*, 1994). In addition, divalent cations are known to influence ligand specificity of numerous integrins including $\alpha4\beta1$ (Masumoto and Hemler, 1993a,b; Shimizu and Mobley, 1993). Ligand specificity may also be influenced by the lipid environment (Conforti *et al.*, 1990; Smyth *et al.*, 1992). Finally, it may be regulated in a cell type–specific manner. For example, integrin $\alpha2\beta1$ serves as a collagen receptor on some cells and as a collagen and laminin receptor on others (Elices and Hemler, 1989; Kirchhofer *et al.*, 1990; Chan and Hemler, 1993).

The integrin $\alpha8$ is expressed as a heterodimer with $\beta1$ in both primary chicken embryo fibroblasts and primary sensory neurons (Bossy *et al.*, 1991; Varnum-Finney *et al.*, 1995). Immunohistochemical localization of $\alpha8$ in the developing chicken embryo revealed a striking expression pattern. During embryonic development $\alpha8$ is abundantly expressed on several classes of projection axons in the central and peripheral nervous systems, suggesting a role in establishment of axonal projections in the developing embryo. In addition $\alpha8$ is expressed at moderate levels in several epithelia adjacent to basal laminae (Bossy *et al.*, 1991).

To investigate the function of $\alpha8\beta1$ heterodimers, it is essential to identify its ligand(s). For this purpose, we established K562 cell lines expressing $\alpha8\beta1$ heterodimers and generated antibodies to $\alpha8$'s extracellular domain. We used these cells and antibodies to examine possible interactions of $\alpha8\beta1$ with purified ECM and Ig-superfamily members known to be expressed on fasciculating axons in projection tracts. We present evidence here that $\alpha8\beta1$ is a FN receptor that mediates attachment of K562 cells and neurite outgrowth by embryonic sensory neurons. Within FN, $\alpha8\beta1$ recognizes the RGD site. Interestingly, even though both $\alpha5\beta1$ and $\alpha8\beta1$ receptors expressed in K562 cells bind to FN, only the latter promotes extensive cell spreading of these cells, suggesting functional differences between the two receptors.

MATERIALS AND METHODS

Materials

Different preparations of human FN and FN fragments were purchased from Calbiochem (San Diego, Ca) and Life Technologies (Gaithersburg, MD). Human vitronectin was purchased from Calbiochem and human thrombospondin-1 from Life Technologies. Rat collagen I and IV were purchased from Collaborative Research Inc (Bedford, MA). EHS laminin was purified as described (Timpl *et al.*, 1979). Ig molecules and restrictin were a kind gift from Dr. T. Brümmendorf, MPI Tübingen, Tübingen, Germany. They were immunoaffinity purified from embryonic chicken brain as described (Brümmendorf *et al.*, 1993). Axonin-1–expressing cell lines were kindly provided by Dr. P. Sonderegger, University of Zürich, Zürich, Switzerland (Rader *et al.*, 1993). The RGD and RGE peptides were purchased from Life Technologies. Monoclonal antibodies (Mabs) to $\alpha5$ (B1E5 and BIIG2) and $\beta1$ (AIIB2) have been described elsewhere (Werb *et al.*, 1989; Hall *et al.*, 1990) and were a kind gift from Dr. Carolyn Damsky, University of California, San Francisco, CA. The CSAT antibody (Neff *et al.*, 1982) was a kind gift from Dr. A.F. Horwitz, University of Pennsylvania, Philadelphia, PA. The monoclonal antibody Mab16 to $\alpha5$ (Akiyama *et al.*, 1989) was a kind gift from Dr. Kenneth Yamada, National Cancer Institute, Bethesda, MD. The $\beta1$-specific–activating Mab TS2/16 was a kind gift from Dr. M. Hemler (Dana-Farber Cancer Institute, Boston, MA). Antibodies to $\alpha v\beta5$ (P1F6) and vinculin were purchased from Life Technologies and Sigma Chemical (St. Louis, MO), respectively. The $\alpha5$cyto and $\alpha8$cyto antisera raised to the cytoplasmic domain of $\alpha5$ and $\alpha8$, respectively, and CHAV-1, the MAb to chicken αv, have been described earlier (Tomaselli *et al.*, 1988; Bossy *et al.*, 1991; Neugebauer and Reichardt, 1991). Secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). NHS sulfo-biotin was purchased from Pierce Chemical (Rockford, IL). Other reagents for immunological techniques were purchased from Pharmacia (Uppsala, Sweden) or Pierce Chemical. BSA was purchased from Serva (Heidelberg, Germany).

Generation of Polyclonal Antisera against the Extracellular Domain of $\alpha8$

The $\alpha8$ex antibody was raised by immunizing rabbits with a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)–purified glutathione-*S*-transferase $\alpha8$ fusion protein of 93 kDa. This fusion protein was obtained by expressing the plasmid αMFpGEX2T in *Escherichia coli*. The αMFpGEX2T plasmid contains the *Nae*I-*Eco*RI fragment (nucleotide 379-2329) of the chick $\alpha8$ cDNA G4 (Bossy *et al.*, 1991) cloned into the *Sma*I and *Eco*RI sites of the pGEX2T vector (Pharmacia). The nucleotide sequence of the G4 cDNA indicates that this clone has in fact a deletion from nucleotide 654 to 752 of the ckick $\alpha8$ sequence (Bossy *et al.*, 1991). Therefore, the fusion protein corresponds to 26 kDa of glutathione-*S*-transferase fused with 67 kDa of the chick $\alpha8$ subunit. The chick $\alpha8$ part encodes amino acids 62 to 710 of the $\alpha8$ subunit except for amino acids 150 to 185. Immunizations were performed at Caltag Laboratories (South San Francisco, CA).

Transfections and Selection of α8-Expressing Cell Lines

K562 cells were maintained as suspension cultures in RPMI 1061 supplemented with 10% fetal bovine serum. To generate α8-expressing sublines, the chicken α8 cDNA (Bossy *et al.*, 1991) was cloned into pAWneo3′, a eukaryotic expression vector containing the Friend spleen focus–forming virus LTR as a promoter and a neomycin resistance gene (kindly provided by Dr. Arthur Weiss, University of California, San Francisco, CA). A full length chicken α8 cDNA was first constructed by ligating the *Eco*RI-*Ava*II fragment (nucleotide 1 to 569) of the G4 cDNA, the *Ava*II-*Bgl*II fragment (nucleotide 570-1256) of the C4 cDNA, and the *Bgl*II-*Bal*I fragment (nucleotide 1257–3420) of the #1.6 cDNA (Bossy *et al.*, 1991) into the *Eco*RI site and a blunted *Hin*dIII site of the bluescript KS$^-$ vector (Stratagene, La Jolla, CA). A 3.4-kb *Sma*I-*Sal*I subfragment of this subclone containing the full length α8 cDNA was recloned into pAWneo3′. K562 cells were transfected with the Lipofectin reagent (Life Technologies) and neomycin-resistant pools were expanded and screened by RNA blot. Positive pools were single cell cloned by limited dilution. Individual single cell-derived subclones were expanded and further screened by Western blot analysis with the α8cyto antibody. Some of the positive clones were analyzed for α8β1 cell surface expression by immunprecipitation of cell surface-labeled cells.

Cell Surface Labeling

Cell surface iodination of K562 and KA8 cells was carried out as described previously (Tomaselli *et al.*, 1988). For cell surface biotinylation, 10^7 cells were harvested, washed twice with PBS, and resuspended in 1 ml PBS supplemented with 20 mM Na-*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5. NHS sulfobiotin was added to a final concentration of 200 μg/ml and cells were incubated for 30 min at room temperature. The cells were washed twice with PBS and lysed in 2 ml lysis-buffer (50 mM Tris-HCl, pH 7.5, 50 mM *N*-octyl-β-D-glucopyranoside, 15 mM NaCl, 1 mM phenylmethylsulfonyl fluoride). Cell debris was removed by centrifugation and extracts were stored at −80°C.

Affinity Purification of α8ex Antiserum

Inclusion bodies from bacteria expressing a GST-α8 fusion protein containing almost the complete extracellular domain of α8 (amino acid 62 to 710 without an internal deletion) were purified according to Harlow and Lane (1988). The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and the area containing α8-GST (as identified by Western blot analysis with GST antibodies and by staining with Ponceau S) was cut out from the filters. The filters were incubated for 1 h in PBS supplemented with 3% BSA to block nonspecific binding sites. Filters were pre-eluted for 15 min at room temperature with 100 mM sodium-glycine, pH 2.5, followed by multiple washes in PBS. The protein A–purified IgG fraction from α8ex sera was incubated for 2 h at room temperature with the filters containing the α8-GST fusion protein. The filters were washed multiple times with PBS and bound IgG molecules were eluted by incubation for 15 min at room temperature in 100 mM Na-glycine, pH 2.5. The eluate was immediately adjusted to neutral pH with 1 M Tris-HCl, pH 8.3. The eluate was dialyzed against Tris-buffered saline (TBS; 24 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl) and stored in aliquots at −20°C. Typically <1% of the input IgG fraction was recovered as affinity-purified α8-specific antiserum.

Affinity Purification of Integrin α8β1

Integrin α8β1 was purified from KA8 cells by affinity chromatography over α8cyto antiserum coupled to protein A beads. Before coupling of the antiserum to protein A beads, the antiserum itself was first affinity purified against the peptide that was used to generate the antiserum. All procedures were carried out as described by Harlow and Lane (1988), with minor modifications. Briefly, 10 mg of peptide containing most of the α8 cytoplasmic domain (Bossy *et al.*, 1991) was coupled to 1.2 ml CNBr-activated Sepharose beads. Between 3 and 5 mg of protein A–purified IgG preparations of α8cyto were applied to the peptide column, eluted, dialyzed, and coupled with dimethylpimelimidate (Pierce Chemical) to protein A beads. KA8 cells (4×10^8 cells/purification) were cell surface biotinylated and extracts were prepared in lysis buffer as described above. Antibody affinity columns were equilibrated in lysis buffer; the extracts were passed three times through the column by gravity flow, the column was washed extensively with column buffer, and bound proteins were eluted with lysis buffer supplemented with 2 mg/ml α8 cyto-peptide. Eluate fractions were stored for 3 to 4 days at 4°C or for extended periods at −70°C.

Immunoprecipitations, Gel Electrophoresis, and Western Blots

Protein gel electrophoresis, Western blot analysis, and immunoprecipitation experiments were carried out as described (Müller *et al.*, 1992) with the following modifications for cell surface biotinylated molecules. Antibodies for immunoprecipitation of cell surface biotinylated molecules were covalently coupled to protein A or G beads with dimethylpimelimidate (Harlow and Lane, 1988). After immunoprecipitation, gel electrophoresis, and transfer of the proteins to nitrocellulose membranes, the membranes were blocked for 1 h with solution I (3% BSA, 5% nonfat dry milk, 0.1% Tween 20 in PBS). Filters were incubated for 1 h with streptavidin-horse radish peroxidase (Zymed, South San Francisco, CA) in solution I, followed by three 10-min washes with solution II (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20). Incubation with enhanced chemiluminescence reagents and autoradiography followed the instructions of the manufacturer (Amersham, Arlington Heights, IL).

Immunofluorescence

Breast muscle chicken embryo fibroblasts were prepared as described (Rein and Rubin, 1968) and cultured in DME-H21 supplemented with 10% fetal bovine serum. Multiwell glass slides (Nunc, Naperville, IL) were precoated for at least 1 h at room temperature with 1 mg/ml poly-D-lysine and washed multiple times with PBS before use. Where indicated, the poly-D-lysine precoated slides were further coated for 3 h at 37°C with 5 μg/ml FN, followed by a 1-h incubation at 37°C with 10 mg/ml BSA. Chicken embryo fibroblasts were trypsinized, washed once in growth medium, and plated either overnight in growth medium on poly-D-lysine–coated slides or plated for 2–4 h in the absence of serum onto FN-coated slides. Cells were incubated at 37°C in a 10% $C0_2$ incubator. Cells were fixed for 10 min at 0°C with 4% paraformaldehyde, blocked for 1 h in solution A (10% normal goat serum, 0.1% Tween 20, 1% glycine, 2% BSA in PBS), incubated for 1 h at room temperature with primary antibody in solution A, washed five times with solution A, incubated for 1 h at room temperature with secondary antibody, washed five times with solution A, embedded in FITC-Guard (TESTOG, Chicago, IL), and analyzed by immunofluorescence microscopy.

Cell Adhesion and Cell Spreading Assays

Cell adhesion assays were essentially carried out as described elsewhere (Hall *et al.*, 1987; Neugebauer and Reichardt, 1991). Linbro titertek 96-well plastic dishes (Flow Laboratories, McLean, VA) were coated for 3 h at 37°C or O/N at 4°C with the substrate molecules and diluted with PBS to the indicated concentration. A total vol of 100 μl substrate solution was applied to each well. Nonspecific binding sites were blocked by incubating coated wells for 1 h at 37°C with 10 mg/ml BSA in PBS. Cells were counted, harvested, washed once with PBS supplemented with 1 mM EDTA, washed once with TBS (24 mM Tris, pH7.4, 137 mM NaCl, 2.7 mM

KCl), and resuspended in TBS containing 0.1% BSA, 2 mM glucose, and divalent cations at varying concentrations. Cells (10^5) were plated out per well. Cells were incubated for 1.5 h at 37°C, washed three to five times with TBS containing the appropriate divalent cation, and fixed for 15 min with 2% paraformaldehyde. Cells were stained for 5 min with 2.5% crystal violet in 20% ethanol, washed three times with water, and retained dye was solubilized in 1% SDS and quantitated by A_{540} in a microtiter plate reader (Flow Laboratories). Values were normalized against BSA-coated wells. Usually, determinations were carried out in duplicates, triplicates, or quadruplicates and most experiments were carried out more than three times. Error bars indicate the standard error of the mean.

For antibody inhibition experiments, cells were preincubated for 15 min on ice with the antibody. Antibody was present throughout the duration of the adhesion assay. All antibodies with the exception of the commercial anti-αv MAb P1F6 were purified over protein A or G columns before use in adhesion assays. The β1-specific AIIB2 antibody was either used as an IgG preparation or as ascites supernatant with identical results. The α8ex antibody was affinity purified against the α8 fusion protein before use (see above). Antibody concentrations for inhibitions were as follows: AIIB2 (anti-β1) 0.1–1.0 μg/ml, B1E5 (anti-α5) 1–2.5 μg/ml, α8ex (anti-α8) 130 μg/ml, and unrelated polyclonal IgG 150 μg/ml. Antibody P1F6 (anti-αvβ5) was supplied as ascites. It effectively blocked αvβ5-mediated vitronectin adhesion of K562 cells at a 1:200 dilution and was used at 1:100 or 1:200 dilution for function inhibition studies on FN.

Spreading assays were carried out under the same conditions as attachment assays except that cells were plated at lower density for 30 to 45 min onto 24-well plates.

Neurite Outgrowth Assays

For neurite outgrowth assays, embryonic day 7–8 chick dorsal root ganglia were dissected and dissociated by incubation for 10 min at 37°C in 0.05% trypsin/PBS followed by trituration. The cells were washed in F12 medium, resuspended in F12 medium supplemented with 10% fetal bovine serum, and plated for 1–3 h onto tissue culture dishes. Nonneuronal cells tend to attach to these dishes whereas neuronal cells could be removed from the dishes with the medium. Neuronal cells were harvested by centrifugation and resuspended in F12 medium supplemented with 1% BSA and 100 ng/ml nerve growth factor. The cells were plated onto 96-well plates (10^3 cells/well) that had been precoated with nitrocellulose (Lagenaur and Lemmon, 1987) followed by coating with FN or a fragment of FN. The cells were gently centrifuged onto the dish and incubated for 4–7 h at 37°C in a 5% CO_2 atmosphere. Where indicated, antibodies were added to the wells simultaneously with the cells. To assay effects of various antibodies, the fraction of cells with neurites extending at least two cell diameters was quantified.

RESULTS

Characterization of α8β1-Expressing K562 Cell Lines

K562 cells are suitable for the identification of novel integrin ligands because they appear to express only two endogenous integrin receptors: moderate levels of α5β1, which binds FN (Arroyo *et al.*, 1992; Chan and Hemler, 1993; Delwel *et al.*, 1993), and low levels of αvβ5, which binds vitronectin (VN) and possibly FN (Pasqualini *et al.*, 1993). Function-inhibiting antibodies to each of these receptors exist, making it possible to express additional integrins and examine binding to any ECM adhesive glycoprotein, including FN or VN. The K562 cell lines employed here express very low levels of αvβ5 and exhibit αvβ5-dependent adhesion to VN, but not to FN (see below). To identify ligands for α8β1, we transfected K562 cells with an α8 cDNA in an expression vector and established clones derived from single cells. Multiple α8-expressing K562 cell lines were identified by antigen blot analysis employing a polyclonal antiserum raised against the cytoplasmic domain of α8. In each cell line, the antiserum recognized a 160-k protein, the reported M_r for α8 (Bossy *et al.*, 1991), which was not present in parental K562 cells (our unpublished observations).

To analyze cell surface expression of α8 and α8β1 heterodimers, integrins were immunoprecipitated from membrane extracts of cell surface biotinylated K562 cells and α8 transfectants. Figure 1 shows the data for one representative clone, KA8, but similar results were obtained with additional clones. Either β1-specific or an α5-specific Mab precipitated α5β1 heterodimers from parental K562 cells and KA8 cells. The α5 subunit was resolved into three bands presumably representing differentially glycosylated forms of α5 (Figure 1A, lane 1). Anti-β1, but not anti-α5 precipitated an additional protein of M_r 160 k from KA8, but not K562 membrane extracts (Figure 1A, lane 4). This protein must be the α8 subunit for several reasons:

1) The M_r 160-k protein was only observed in transfectants, but not in parental cell lines. Its size is consistent with the reported size for α8 (Bossy *et al.*, 1991). 2) A protein of the same M_r was specifically immunoprecipitated from KA8 but not K562 cells by two antisera raised against separate domains of α8 (Figure 1B). An antibody to the cytoplasmic domain (α8cyto) coprecipitated bands of M_r 160 k and 115 k, the expected sizes of α8 and β1. An antibody to the extracellular domain of α8 (α8ex) precipitated the M_r 160-k, but not the M_r 115-k band, suggesting that it disrupted the association between α8 and β1. Other anti-integrin antibodies have previously been shown to dissociate integrin heterodimers (Horwitz *et al.*, 1985). 3) The β1-associated M_r 160-k protein was completely removed by an α8cyto antibody column (Figure 1C, lane 6). Before coupling, the α8cyto antibody was affinity purified against the peptide used to generate the antibody to ensure absolute specificity. Although all of the α8β1 heterodimer was retained on the column, all the α5β1 heterodimer was in the flow-through (Figure 1C, lanes 5 and 6). 4) The electrophoretic mobility of the M_r 160-k protein decreased in the presence of the reducing agent dithiothreitol, consistent with the previously reported behavior of chick α8, which suggests that the mature α8 subunit is cleaved into two fragments linked by disulfide bonds (Bossy *et al.*, 1991; our unpublished observations).

We conclude that KA8 cells express both α5β1 and α8β1 integrin heterodimers on the cell surface. Both heterodimers appear to be expressed at similar levels

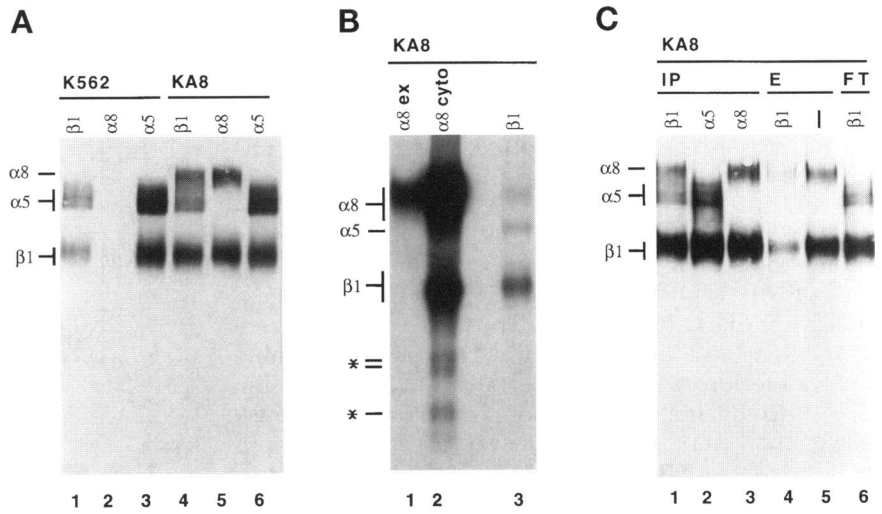

Figure 1. Integrin expression in K562 cells and α8 transfectants. (A) Extracts from cell surface biotinylated K562 and KA8 cells were immunoprecipitated with the indicated antibodies (anti-β1 [AIIB2] and anti-α8 [α8cyto]; anti-α5 [B1E5]) separated on 6% polyacrylamide gels, transferred by blotting to nitrocellulose, reacted with streptavidin-horseradish peroxidase conjugates, and visualized by chemiluminescence. (B) KA8 cells were cell surface iodinated and integrins were precipitated with the indicated antibodies. After electrophoretic separation, proteins were visualized by autoradiography. The * marks potential breakdown products. (C) Extracts from cell surface biotinylated KA8 cells were passed over an α8cyto antibody column. The flow through fraction (FT) and the eluate (E) were analyzed by immunoprecipitation with β1 antibody (lanes 4 and 6) or the eluate was loaded directly onto the gel without immunoprecipitation (lane 5). As a control, input material (IP) was also analyzed by immunoprecipitation with the indicated antibodies (lanes 1–3). After electrophoresis and transfer to nitrocellulose, filters were reacted with streptavidine-horseradish peroxidase conjugates and proteins were visualized by chemiluminescence.

(Figure 1A). No other β1 class integrins were detected on the cell surface. Antigen blot analysis with antibodies specific for β1, α5, αv, and β5 indicated that each subunit is expressed at similar levels in K562 and KA8 cells (our unpublished observations). In agreement with earlier findings (Arroyo *et al.*, 1992; Chan and Hemler, 1993; Delwel *et al.*, 1993) we did not detect any expression of α3 or α4 in K562 cells.

Screen for α8β1 Ligands

The expression pattern of α8 within epithelia suggested that it may interact with components of basement membranes. In addition, its expression within major axonal tracts on fasciculating axons raises the possibility that α8 may interact with counter receptors on axonal surfaces. To identify possible α8β1 ligands, several purified ECM molecules and immuno-affinity–purified Ig-superfamily members known to be expressed on axons were assayed in cell adhesion assays (Table 1). As previously reported, endogenous K562 integrins show low ligand binding efficacy in Mg^{2+} but ligand binding can be activated by Mn^{2+} or by the β1-specific–activating Mab TS2/16 (Arroyo *et al.*, 1992; Chan and Hemler, 1993). Therefore, cell adhesion assays were carried out in the presence of different concentrations (1 to 10 mM) of either Mg^{2+}, Ca^{2+}, Mn^{2+}, or a combination of these ions. Similar experiments were performed in the presence of Mg^{2+} or Ca^{2+} and the β1-activating antibody TS2/16.

Parental K562 cells and α8 transfectants adhered to several preparations of FN, VN, F11, DM-GRASP, and restrictin. Adhesion to F11, DM-GRASP, and restrictin appeared to be mediated by receptors other than α8β1 because it was not inhibited by antibodies to either the α8 or β1 subunit (our unpublished observations). These interactions were therefore not further analyzed. α8β1-mediated interactions were observed with substrates coated with FN (see below). In some assays interactions were also observed with VN; however, these interactions showed great variability depending on the VN preparation. Clearly, the VN preparations were not contaminated with FN as determined by Western blot analysis with FN-specific antibodies (our unpublished observations). The reason for this incon-

Table 1. Ig-superfamily molecules and ECM components analyzed for interactions with α8β1

Extracellular matrix molecules		
Fibronectin	human	+
Vitronectin	human	?
Laminin (EHS-sarcoma-derived)	mouse	−
Collagen I	rat	−
Collagen IV	rat	−
Restrictin	chicken	−
Thrombospondin-1	human	−
Ig-superfamily		
N-CAM	chicken	−
Ng-CAM	chicken	−
Nr-CAM	chicken	−
F11	chicken	−
DM-GRASP	chicken	−
Axonin-1	chicken	−
Neurofascin	chicken	−

Adhesion assays were carried out with parental K562 cells and α8 transfectants. +, α8β1 dependent adhesion; −, no detectable α8β1 dependent adhesion; ?, α8β1 dependent adhesion was observed only in some experiments (see text). Interactions with axonin-1 were analyzed by cell aggregation assays employing an axonin-1 expressing transfectant (Rader *et al.*, 1993).

sistency is at present unclear, but raises the possibility that $\alpha 8\beta 1$ is a receptor for VN in addition to FN.

Adhesion to FN

In the presence of Mg^{2+}, both K562 and KA8 cells adhered weakly but significantly to FN. In the presence of Ca^{2+}, adhesion was close to background levels (Figure 2A). In the presence of either cation, KA8 cells often showed slightly stronger adhesion. In the presence of Mn^{2+}, both cell lines adhered strongly to FN. Levels of adhesion to FN in the presence of Mn^{2+} were defined as 100% because they were indistinguishable from adhesion to the control substrate poly-D-lysine (our unpublished observations). In dose response curves, half maximal binding was observed for both cell lines at 5 μg/ml or <1 μg/ml coating concentration in the presence of Mg^{2+} or Mn^{2+}, respectively (Figure 2B). The curves did not differ significantly between the two cell lines.

To identify which integrins mediate interactions of these cells with FN, effects of subunit-specific antibodies were examined. Results in Figure 3 show that MAbs directed either against $\beta 1$ (MAb AIIB2) or $\alpha 5$ (MAb B1E5) almost completely inhibit binding of K562 cells to FN. Anti-$\alpha v\beta 5$ (MAb P1F6) and several other unrelated polyclonal antisera (our unpublished observations) did not interfere with adhesion. Adhesion of KA8 cells to FN was also eliminated by the anti-$\beta 1$ MAb. In contrast, however, anti-$\alpha 5$ MAbs were not effective in blocking KA8 cell adhesion. Three different anti-$\alpha 5$ MAbs (B1E5, BIIG2, and Mab16) failed to block adhesion of KA8 and two additional $\alpha 8$-expressing cell lines (our unpublished observations).

To analyze interactions mediated by $\alpha 8\beta 1$, the effect of anti-$\alpha 8$ex antibody was tested in the same assay (Figure 3). The antibody was affinity purified using the $\alpha 8$ fusion protein to ensure specificity. Incubation with affinity-purified $\alpha 8$ex alone did not inhibit binding of K562 or KA8 cells to FN; however, the antibodies did inhibit attachment of KA8 cells to FN significantly when used in combination with an $\alpha 5$ MAb (B1E5). As controls, other antibody combinations were also

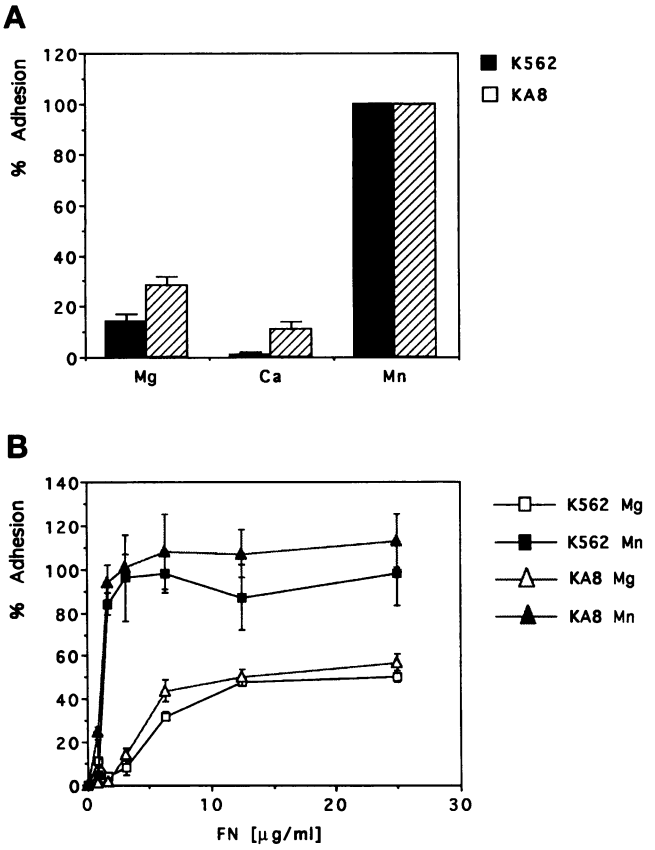

Figure 2. Adhesion to FN of K562 and KA8 cells. Adhesion assays were carried out with K562 and KA8 cells in the presence of either 1 mM Mg^{2+}, Ca^{2+}, or Mn^{2+} after coating of tissue culture plastic with (A) 5 μg/ml FN or (B) different coating concentrations between 0.8 and 25 μg/ml FN.

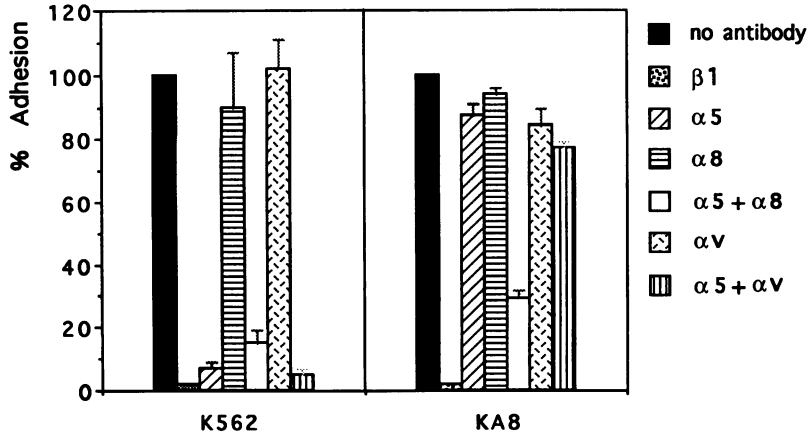

Figure 3. Effect of integrin subunit specific antibodies on Mn^{2+} stimulated adhesion. Adhesion to FN (5 μg/ml coating concentration) was measured in the presence of 1 mM Mn^{2+}. The antibodies were as follows: anti-$\beta 1$ (AIIB2), anti-$\alpha 5$ (B1E5), anti-$\alpha 8$ ($\alpha 8$ex), and anti-$\alpha v\beta 5$ (P1F6). For antibody concentrations see MATERIALS AND METHODS. Adhesion in the absence of antibody was set at 100% and adhesion to BSA (usually less then 1%) was subtracted.

tested. Simultaneous addition of anti-α5 (MAb B1E5) and anti-αvβ5 (MAb P1F6) did not significantly reduce adhesion of KA8 cells to FN (Figure 3). Adhesion was also not inhibited by a combination of anti-α5 (MAB B1E5) and IgG preparations of several unrelated polyclonal antisera (our unpublished observations). Thus, these data provide strong evidence that two different integrin heterodimers, α5β1 and α8β1, mediate KA8 cell adhesion to FN. The integrin αvβ5 does not appear to have contributed to FN adhesion in these assays. The αvβ5 MAb employed here was clearly active, because in parallel assays it efficiently inhibited adhesion of K562 cells to VN (our unpublished observations).

Anti-β1 MAb TS2/16 Activated Adhesion to FN

β1-integrin–dependent adhesion of K562 cells to ECM molecules in the presence of Mg^{2+} can be enhanced by TS2/16, an anti-β1 MAb (Arroyo *et al.*, 1992; Chan and Hemler, 1993). Adhesion of either K562 or KA8 cells to FN was strongly up-regulated by TS2/16 (Figure 4). TS2/16-promoted adhesion of K562 cells to FN was completely inhibited by a MAb against the α5 subunit (B1E5). In contrast, KA8 adhesion to FN was partially, but not completely inhibited by the α5 MAb. Although anti-α8ex alone had only weak inhibitory affects, in combination with anti-α5 it virtually eliminated adhesion of KA8 cells to FN. A MAb to αvβ5 (P1F6) did not reduce adhesion significantly. These data also demonstrate that KA8 cells use both α5β1 and α8β1 heterodimers as receptors for FN.

In the presence of Mg^{2+} and TS2/16, anti-α5 was partially effective alone in blocking FN adhesion of KA8 cells; however, in the presence of Mn^{2+}, anti-α5 alone was ineffective (compare Figures 3 and 4). This suggests that adhesion of KA8 cells to FN in the presence of TS2/16 is more strongly dependent on α5β1 than adhesion in the presence of Mn^{2+}. As one possible interpretation, Mn^{2+} may activate α5β1 and α8β1 heterodimers equally well, whereas MAb TS2/16 may be much more effective at activating the α5β1 heterodimer.

Cell Spreading on FN

To analyze cell spreading and other possible morphological responses, the parental K562 cells and three different α8 transfectants were plated at low density on FN; representative results are shown for subclone KA8 (Figure 5). In the presence of Mg^{2+}, both K562 and KA8 cells adhered weakly to FN and the cells maintained a rounded morphology (Figure 5, panels a and b). In the presence of Mn^{2+}, K562 cells spread slightly on FN (Figure 5, panel c). In contrast, KA8 cells spread strongly on FN and elaborated numerous lammelopodia-like protrusions (Figure 5, panel d). Spreading was evident within 30 min after plating. Spreading of KA8 cells was inhibited by anti-β1 (Figure 5, panel e) or by affinity-purified α8ex antibody (Figure 5, panel f), but not by anti-α5 (Figure 5, panel g) or an unrelated polyclonal antibody (Figure 5, panel h). Extensive cell spreading of KA8 cells but not K562 cells was also induced in the presence of Mg^{2+} and the β1-activating antibody TS2/16 (Figure 6, a and b). Again, spreading was inhibited by anti-α8 (Figure 6, panel d) but not anti-α5 antibody (Figure 6, panel c).

We conclude that integrin α8β1 but not α5β1 can promote extensive cell spreading of K562 cells on FN. Importantly, both heterodimers were expressed at similar levels on the cell surface of KA8 cells (Figure 1). Thus, the results suggest that the two integrins α5β1 and α8β1 are functionally different.

Focal Adhesion Localization

We were unable to detect α8β1 or α5β1 in focal adhesions in K562 cells because very few, if any such structures are present, as assessed by staining with

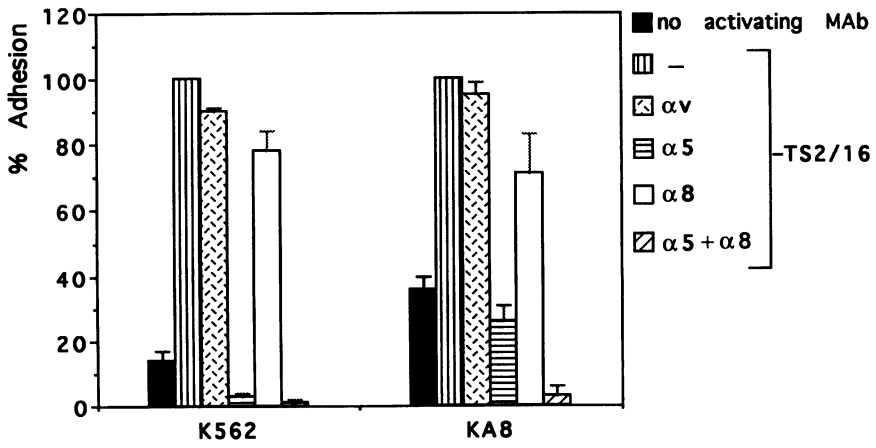

Figure 4. Inhibition of TS2/16-stimulated adhesion to FN. Adhesion to FN (5 μg/ml coating concentration) was measured in the presence of 1 mM Mg^{2+} and the integrin-activating antibody TS2/16. The blocking antibodies were as follows: anti-β1 (AIIB2), anti-α5 (B1E5), anti-α8 (α8ex), and anti-αvβ5 (P1F6). The values were normalized as indicated in Figure 3. For antibody concentrations see MATERIALS AND METHODS.

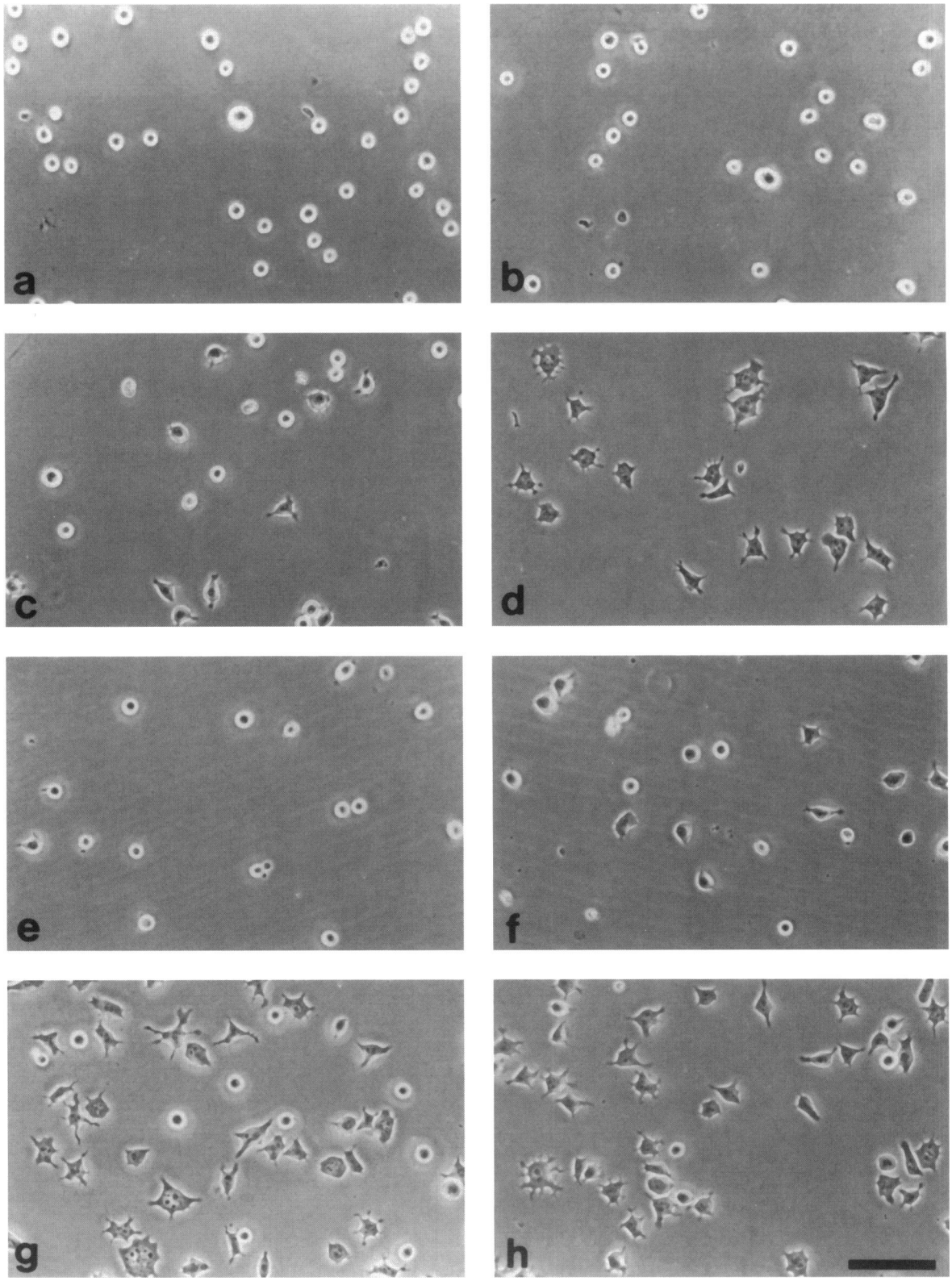

Figure 5. Spreading assay. K562 cells (a and b) or KA8 cells (c-h) were plated onto FN (5 μg/ml coating concentration) in the presence of either 1 mM Mg^{2+} (a and c) or 1 mM Mn^{2+} (b and d-h); in the absence of antibody (a-d); or in the presence of an anti-β1 antibody AIIB2 (e), anti-α8 antibody α8ex (f), anti-α5 antibody B1E5 (g), unrelated polyclonal IgG (h). Cells were photographed 30–45 min after plating. For antibody concentrations see MATERIALS AND METHODS. Scale bar, 100 μm.

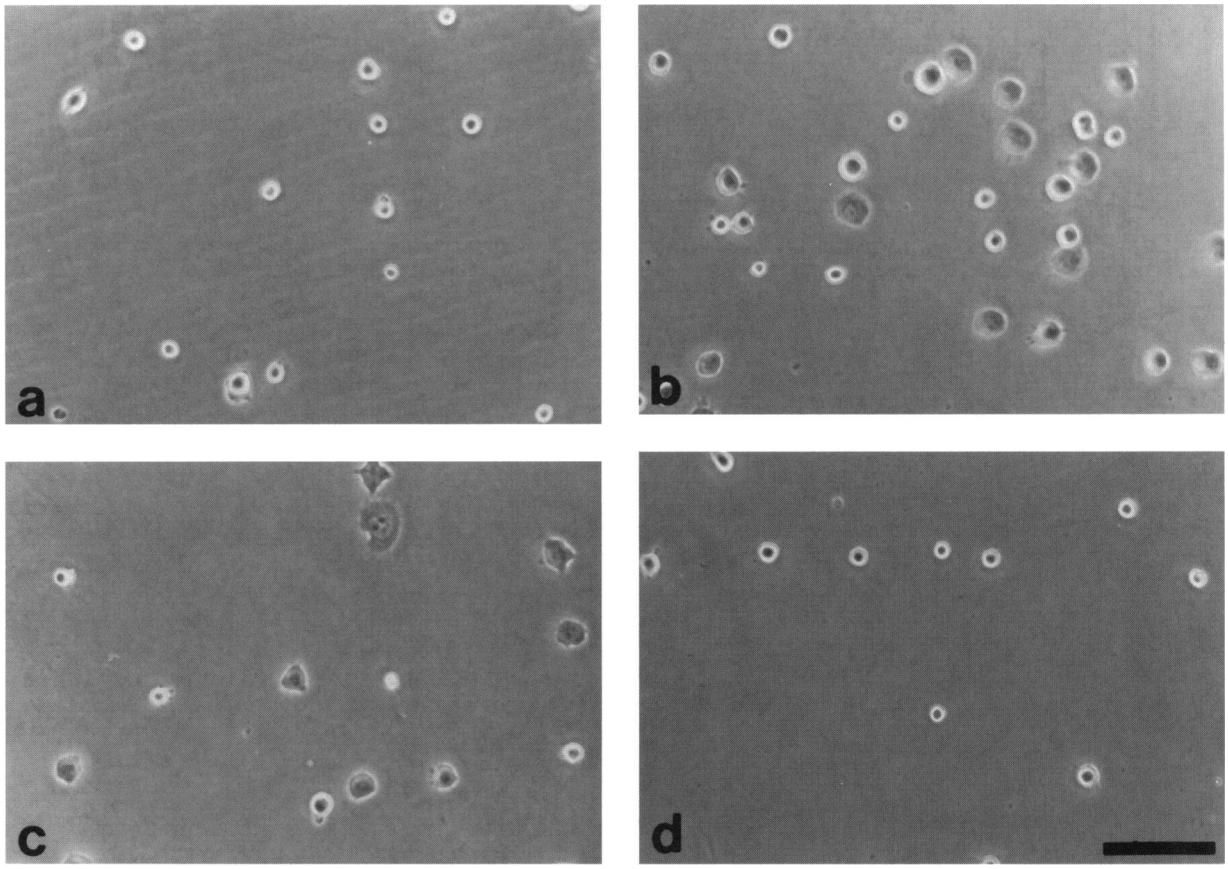

Figure 6. Spreading assay. K562 cells (a) or KA8 cells (b-d) were plated onto FN (5 μg/ml coating concentration) in the presence of 1 mM Mg^{2+} and the β1-activating antibody TS2/16; in the absence of function-blocking antibodies (a and b); in the presence of anti-α5 antibody B1E5 (c); in the presence of anti-α8 antibody α8ex (d). Cells were photographed 30–45 min after plating. For antibody concentrations see MATERIALS AND METHODS. Scale bar, 100 μm.

vinculin antibodies (our unpublished observations). We therefore analyzed the localization of α8 in primary chick embryo fibroblasts, which have previously been shown to express α8β1 heterodimers (Bossy *et al.*, 1991) and assemble numerous focal adhesions (reviewed in Burridge *et al.* 1990). Accordingly, chick embryo fibroblasts were allowed to attach and spread on FN for 2–4 h. By immunofluorescence α8 and vinculin were colocalized in focal adhesions (Figure 7, a and b). Interestingly, α8 was predominantly observed within focal adhesions at the edges of cells but rarely in more central regions of the cells. Localization of α8 in focal adhesions was also observed when the fibroblasts were plated overnight on poly-D-lysine–coated cover slips (Figure 7, c and d). Under these conditions, these fibroblasts synthesized high quantities of FN and assembled it into an extensive matrix (our unpublished observations). Thus it seems likely that endogenously synthesized FN accounts for the formation of focal adhesions containing α8 in this experimental paradigm.

Integrin α8β1 Recognizes the RGD Site within FN

To identify the binding site(s) for α8β1 within FN, adhesion assays were carried out with various chymotryptic FN fragments (40 kDa, 45 kDa, 120 kDa) in the presence of Mn^{2+}. The FN120 fragment contains the RGD sequence recognized by many different integrins including α5β1. The C-terminal FN40 fragment contains binding sites for integrin α4β1 and α4β7. The N-terminal FN45 fragment contains sites that mediate FN matrix assembly and collagen binding. No integrin has been shown to bind within this region of FN (for a review see Sonnenberg, 1993).

In the presence of Mn^{2+} (Figure 8) or the β1-activating antibody TS2/16 (our unpublished observations) both K562 and KA8 cells adhered strongly to FN120 but not significantly to FN40 or FN45 (our unpublished observations). Antibody inhibition experiments confirmed that the adhesion to FN120 was dependent on both α5β1 and α8β1 (Figure 8).

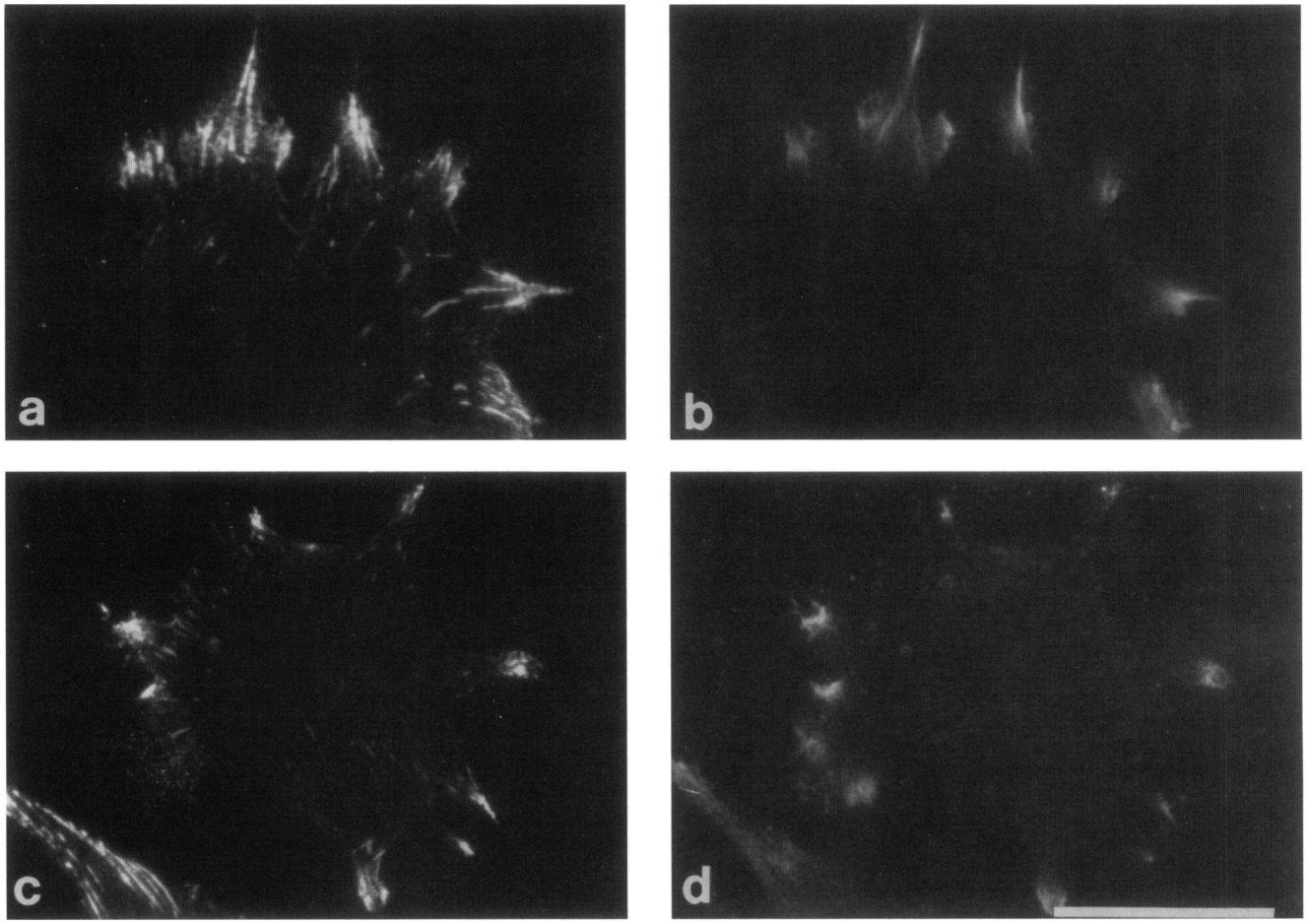

Figure 7. Focal adhesion localization. Chicken embryo fibroblasts were plated for 4 h on FN-coated surfaces (a and b) or for 16 h on poly-D-lysine–coated surfaces (c and d). Cells were fixed and stained for either vinculin (a and c) or integrin α8 (b and d) and analyzed by immunofluorescence microscopy. Vinculin was visualized with a fluorescein-coupled secondary antibody and α8 with a texas-red coupled secondary antibody. Scale bar, 100 μm.

These results were virtually identical to those obtained with full length FN (Figure 3). RGD peptide competition experiments revealed that adhesion of both cell lines to FN120 was completely inhibited by an RGD peptide, but not by a control RGE peptide (Figure 9). This data suggests that α8β1 recognizes the same RGD sequence within FN as is recognized by α5β1.

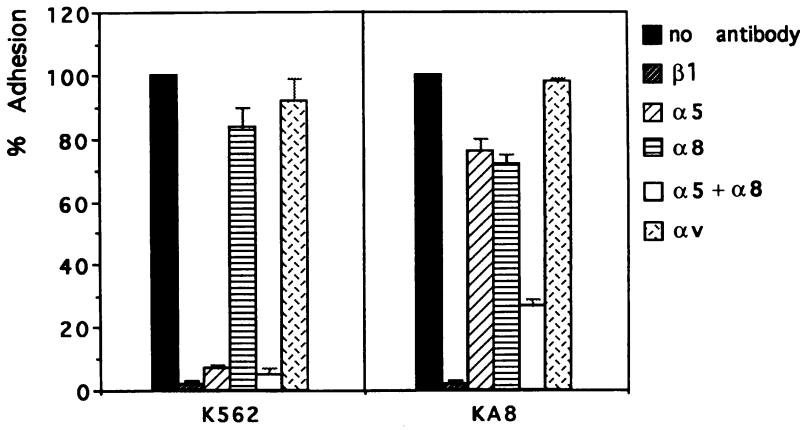

Figure 8. Antibody inhibition of adhesion to FN120. Adhesion of K562 and KA8 cells to FN120 (5 μg/ml coating concentration) was measured in the presence of 1 mM Mn^{2+} and the indicated antibodies: anti-β1 (AIIB2), anti-α5 (B1E5), anti-α8 (α8ex), and anti-αv (P1F6). The values were standardized as indicated in Figure 3. For antibody concentrations see MATERIALS AND METHODS.

Integrin α8β1-Dependent Neurite Outgrowth of Sensory Neurons

Integrin α8β1 is highly expressed on axons of embryonic sensory neurons within the developing nervous system (Bossy *et al.*, 1991). To analyze a possible function of α8 in neuronal differentiation, we performed neurite outgrowth assays of embryonic day 7 and 8 dorsal root ganglion neurons in culture on full length FN and FN fragments (Table 2). It has previously been demonstrated that dorsal root ganglion neurons respond with robust neurite outgrowth when plated onto different ECM substrates such as laminin or FN (Rogers *et al.*, 1983; Tomaselli *et al.*, 1986; Humphries *et al.*, 1988). These cells express integrin α8β1 heterodimers (Varnum-Finney *et al.*, 1995). After plating in the presence of nerve growth factor, dorsal root ganglion neurons extended neurites on FN, FN120, or FN40 within 4 h. Neurite outgrowth on FN and FN120 was partially inhibited by affinity-purified α8ex antiserum, suggesting that α8β1 is involved in neurite outgrowth on FN. Neurite outgrowth on FN40 was not inhibited by α8ex consistent with the observation that α8β1 expressing KA8 cells do not adhere to FN40 (our unpublished observations).

Neurite outgrowth assays on FN120 were also carried out in the presence of antibodies to αv and β1. Both antibodies partially inhibited neurite outgrowth. When added together, the αv and β1 antibodies completely inhibited neurite outgrowth. This suggests that β1 and αv integrins account for most and perhaps all dorsal root ganglion neurite outgrowth on FN120. The data also indicate that αv must associate with a β subunit distinct from β1 on these cells. Antibodies specific for α8 and αv together also had an additive but partial inhibitory effect on neurite outgrowth. This suggests that α8β1 and at least one additional β1 integrin is involved in neurite outgrowth on FN120. A likely candidate would be α5β1, but lack of a function blocking anti-chick α5 antibody made it unfeasible to test this possibility. In conclusion, α8β1, together with unidentified β1 and αv integrins, promotes neurite outgrowth by dorsal root ganglion neurons on FN 120.

DISCUSSION

The major objective of this study was to identify ligands for integrin α8β1. We conclude that α8β1 is a functionally important FN receptor that recognizes the RGD site within the central cell binding domain of FN. This conclusion is based on the following observations: 1) FN adhesion of K562 transfectants expressing integrin α5β1 and α8β1 (Figure 1) was completely inhibited by an anti-β1 MAb (Figures 3 and 4). Adhesion was also strongly inhibited by a combination of anti-α5 and anti-α8 antibodies but not by either antibody alone (Figures 3 and 4). 2) Adhesion of α8 transfectants to a FN fragment spanning the central cell binding domain was dependent on integrin α5β1 and α8β1 and completely inhibited by RGD peptide (Figures 8 and 9). 3) α8 transfectants spread substantially better on FN then parental K562 cells. Spreading was inhibited by anti-β1 or anti-α8 but not by anti-α5 antibodies (Figures 5 and 6). This suggests that α8β1 but not α5β1 promotes substantial cell spreading of K562 cells. 4) Neurite outgrowth of primary sensory neurons on FN was partially dependent on integrin α8 (Table 2).

α8β1-dependent adhesion to FN had a similar requirement for specific divalent cations as has been described for other β1 class integrins heterologously expressed in K562 cells (Arroyo *et al.*, 1992; Chan and Hemler, 1993; Delwel *et al.*, 1993). Minimal adhesion was observed in the presence of Mg^{2+} or Ca^{2+}. Adhesion was strongly enhanced by Mn^{2+} or by the β1-specific–activating antibody TS2/16 (Figures 2–4). Interestingly, cell adhesion in the presence of Mn^{2+} was inhibited by a combination of anti-α5 and anti-α8 antibodies but not by either antibody alone (Figure 3). In contrast, adhesion in the presence of TS2/16 was partially sensitive to anti-α5 MAb (Figure 4). This observation is consistent with the interpretation that MAb TS2/16 preferentially activated integrin α5β1 whereas Mn^{2+} activated α5β1 and α8β1 equally well. Previous studies have shown that β1-specific MAbs can affect individual integrin heterodimers differently. For example, a MAb to chicken β1 inhibits cell adhesion to

Table 2. Inhibiton of neurite outgrowth by sensory neurons on FN

	Antibody Added					
	cont	α8	αv	β1	αv+α8	αv+β1
FN	100	60 ± 1	65 ± 15	36 ± 9	n.d.	n.d.
FN120	100	52 ± 4	51 ± 9	25 ± 6	33 ± 9	1 ± 1
FN40	100	99 ± 4	113 ± 7	55 ± 11	103 ± 7	54 ± 8

Sensory dorsal root ganglion neurons were isolated from E8 chicken embryos and cultured on intact FN, FN120, or FN40. To assay effects of various antibodies, the fraction of neurons with neurites extending at least two cell diameters was quantified. Antibodies and antibody concentration were as follows: anti-α8 (α8ex, 20 μg/ml); anti αv (CHAV, 100 μg/ml); and anti-β1 (W1B10, 50 μg/ml).

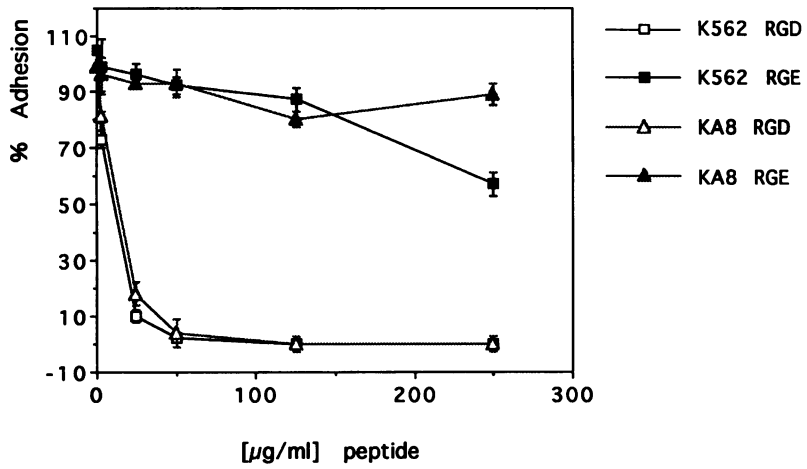

Figure 9. Effects of RGD peptides on adhesion to FN120. Adhesion of K562 or KA8 cells to FN120 was carried out in the presence of the indicated amounts of RGD or RGE peptide. Coating concentrations for FN120 were 5 μg/ml. Adhesion assays were carried out in the presence of 1 mM Mn^{2+}.

VN but promotes adhesion to laminin and collagen types I and IV (Neugebauer and Reichardt, 1991).

Adhesion to FN in the presence of Mn^{2+} was inhibited strongly but not completely by a combination of α5 and α8 antibodies. Several criteria support the fact that the residual adhesion in the presence of these antibodies was not caused by expression of an additional integrin. First, adhesion to FN was completely inhibited by an anti-β1 MAb (Figures 3 and 4); α5β1 and α8β1 were the only β1-class integrins expressed (Figure 1). Expression of other potential FN binding integrins such as α3β1, α4β1, or αvβ1 was not detectable by us or by other groups (Figure 1 and our unpublished observations; Arroyo *et al.*, 1992; Chan and Hemler, 1993; Delwel *et al.*, 1993; Pasqualini *et al.*, 1993). Second, we observed low level expression of integrin αv (our unpublished observations), which presumably forms a heterodimer with β5 as previously described for K562 cells (Pasqualini *et al.*, 1993). Antibodies to integrin αv (our unpublished observations) or αvβ5 (Figures 3 and 4) did not affect adhesion to FN, even in the presence of anti-α5 antibodies (Figure 3), suggesting that αv-containing integrins were not involved. We can currently only speculate on the nature of the residual adhesion. Clearly, the anti-α5 antibody can completely inhibit α5 function (Figures 3 and 4). This suggests that the anti-α8 antibody may only partially block interaction of α8 with the substrate. Integrins are known to exist in multiple different conformational states that are recognized with different affinity by integrin antibodies (reviewed in Loftus *et al.*, 1994). It is possible that in the presence of Mn^{2+}, α8β1 may exist in multiple different conformers, some of which may be recognized with low affinity by the α8-specific antibody. Interestingly, in the presence of the β1-activating antibody TS2/16, adhesion was completely inhibited (Figure 4), suggesting that TS2/16 may induce an α8β1 conformation preferentially recognized by the antibody. Further studies are necessary to resolve these issues.

In some assays we observed α8β1-dependent adhesion of α8 transfectants to VN (Table 1). Adhesion was clearly sensitive to α8 and β1 antibodies and it was inhibited by RGD peptides, suggesting that α8β1 binds to the RGD site within VN (our unpublished observations); however, adhesion to VN was only observed with some but not other preparations of VN. The cause for this variability is at present not clear. The VN preparations were not contaminated with FN as analyzed by Western blotting, and no other major contaminants were detected by staining gels after electrophoretic separation of VN preparations (our unpublished observations). As one possibility, the RGD site within VN may have a very defined conformation that is sensitive to denaturation during purification. α8β1 may not recognize the denatured RGD site. Further experiments are necessary to address this point.

α8β1 is an additional member of the extensive family of integrins that bind to FN. One may ask why there are so many different receptors for a single ECM ligand. Functional redundancy between receptors cannot be excluded, but recent observations suggest that different integrins may have different functions. For example, Zhang *et al.* (1993) showed that the integrin α5β1 can promote attachment, matrix assembly, and migration of CHO cells on FN whereas the integrin αvβ1 functions only as an adhesion receptor. In another study, the integrin α5β1 and an αv-containing heterodimer were shown to contribute to cell adhesion and spreading of endothelial cells on FN, but a rise in intracellular Ca^{2+} was only observed for αv-mediated interactions (Schwartz and Denninghoff, 1994). Our data suggest that binding of α5β1 or α8β1 to FN may lead to receptor-specific cellular responses: integrin

α8β1 but not integrin α5β1 induced substantial spreading of K562 cells (Figures 5 and 6). It seems likely that integrin α5 and α8 cytoplasmic domains may specifically interact with or modulate the activity of different cytoplasmic components. Indeed, different integrin cytoplasmic domains have been shown to execute distinguishable functions. Chan *et al.* (1992) expressed in RD cells integrin chimeras containing the extracellular and transmembrane domain of α2 linked to different α cytoplasmic domains. The cytoplasmic domain of integrin α4 conferred enhanced motility to the cells while cytoplasmic domains of α2 and α5 allowed enhanced collagen gel contraction. Pasqualini and Hemler (1994) showed by a similar approach that the β1 cytoplasmic domain promotes adhesion, proliferation, and focal adhesion localization but not migration of CHO cells on FN; in contrast, the β5 cytoplasmic domain promotes adhesion and migration but not focal adhesion localization or proliferation. It will be interesting to construct chimeras between integin α5 and α8 to analyze possible functional differences caused by the cytoplasmic domains of these receptors.

During embryonic development integrin α8 is highly expressed in the developing nervous system on a number of projection axons. Expression is also observed in several epithelial cells adjacent to basal lamina where FN may be its primary ligand (Bossy *et al.*, 1991). FN is found in the pathway of migrating neural crest cells and supports migration of these cells (Newgreen and Thiery, 1980; Krotoski *et al.*, 1986; Dufour *et al.*, 1988). FN also supports neurite outgrowth of numerous peripheral neurons when analyzed in vitro (Rogers *et al.*, 1983; Tomaselli *et al.*, 1986; Humphries *et al.*, 1988). It is up-regulated at sites of nerve injury, suggesting a possible function in regeneration (Lefcort *et al.*, 1992). Our data suggest that embryonic sensory neurons use multiple integrins, including the integrin α8β1 to extend neurites on FN (Table 2); however, FN is a rather poor substrate for in vitro neurite outgrowth of many neurons derived from the central nervous system (Rogers *et al.*, 1983). The striking axonal expression pattern of α8 in the central nervous system (Bossy *et al.*, 1991) has tempted us to analyze possible interactions of α8β1 with members of the Ig-superfamily known to be expressed on fasciculating axons (reviewed in Sonderegger and Rathjen, 1992). We did not observe an interaction of α8β1 with any of the Ig-superfamily members tested (Table 1). Recent experiments in our laboratory show that α8β1 can promote neurite outgrowth of dorsal root ganglion neurons and motor neurons on tenascin-C (Varnum-Finney *et al.*, 1995). Tenascin is highly expressed in the central nervous system (Grumet *et al.*, 1985; Chuong *et al.*, 1987), suggesting that α8β1 may mediate some of the interactions of central neurons with this ECM glycoprotein. Studies are in progress to identify additional ligands.

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REFERENCES

Adams, J.C., and Watt, F.M. (1993). Regulation of development and differentiation by the extracellular matrix. Development *117*, 1183–1198.

Akiyama, S.K., Yamada, S.S., Chen, W.T., and Yamada, K.M. (1989). Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. J. Cell Biol. *109*, 863–875.

Aota, S., Nagai, T., and Yamada, K.M. (1991). Characterization of regions of fibronectin besides the arginine-glycine-aspartic acid sequence required for adhesive function of the cell-binding domain using site-directed mutagenesis. J. Biol. Chem. *266*, 15938–15943.

Arroyo, A.G., Sanchez, M.P., Campanero, M.R., Martin, P.I., Dejana, E., and Sanchez, M.F. (1992). Regulation of the VLA integrin-ligand interactions through the beta 1 subunit. J. Cell Biol., *117*, 659–670.

Bossy, B., Bossy-Wetzel, E., and Reichardt, L.F. (1991). Characterization of the integrin alpha 8 subunit: a new integrin beta 1-associated subunit, which is prominently expressed on axons and on cells in contact with basal laminae in chick embryos. EMBO J. *10*, 2375–2385.

Bowditch, R.D., Halloran, C.E., Aota, S., Obara, M., Plow, E.G., Yamada, K.M., and Ginsberg, M.H. (1991). Integrin alpha IIb beta 3 (platelet GPIIb-IIIa) recognizes multiple sites in fibronectin. J. Biol. Chem. *266*, 23323–23328.

Bowditch, R.D., Hariharan, M., Tominna, E.G., Smith, J.W., Yamada, K.M., Getzoff, E.D., and Ginsberg, M.H. (1994). Identification of a novel integrin binding site in fibronectin: differential utilization by beta 3 integrins. J. Biol. Chem. *269*, 10856–10863.

Brümmendorf, T., Hubert, M., Treubert, U., Leuschner, R., Tarnok, A., and Rathjen, F.G. (1993). The axonal recognition molecule F11 is a multifunctional protein: specific domains mediate interactions with Ng-CAM and restrictin. Neuron *10*, 711–727.

Burridge, K., Nuckolls, G., Otey, C., Pavalko, F., Simon, K., and Turner, C. (1990). Actin-membrane interaction in focal adhesions. Cell Differ. Dev. *32*, 337–342.

Busk, M., Pytela, R., and Sheppard, D. (1992). Characterization of the integrin alpha v beta 6 as a fibronectin-binding protein. J. Biol. Chem. *267*, 5790–5796.

Chan, B.M., and Hemler, M.E. (1993). Multiple functional forms of the integrin VLA-2 can be derived from a single alpha 2 cDNA clone: interconversion of forms induced by an anti-beta 1 antibody. J. Cell Biol. *120*, 537–543.

Chan, B.M., Kassner, P.D., Schiro, J.A., Byers, H.R., Kupper, T.S., and Hemler, M.E. (1992). Distinct cellular functions mediated by different VLA integrin alpha subunit cytoplasmic domains. Cell *68*, 1051–1060.

Charo, I.F., Nannizzi, L., Smith, J.W., and Cheresh, D.A. (1990). The vitronectin receptor alpha v beta 3 binds fibronectin and acts in concert with alpha 5 beta 1 in promoting cellular attachment and spreading on fibronectin. J. Cell Biol. *111*, 2795–2800.

Cheresh, D.A., and Spiro, R.C. (1987). Biosynthetic and functional properties of an Arg-Gly-Asp–directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen, and von Willebrand factor. J. Biol. Chem. *262*, 17703–17711.

Chuong, C.M., Crossin, K.L., and Edelman, G.M. (1987). Sequential expression and differential function of multiple adhesion molecules during the formation of cerebellar cortical layers. J. Cell Biol. *104*, 331–342.

Conforti, G., Zanetti, A., Pasquali, R.I., Quaglino, D.J., Neyroz, P., and Dejana, E. (1990). Modulation of vitronectin receptor binding by membrane lipid composition. J. Biol. Chem. *265*, 4011–4019.

Dedhar, S., and Gray, V. (1990). Isolation of a novel integrin receptor mediating Arg-Gly-Asp–directed cell adhesion to fibronectin and type I collagen from human neuroblastoma cells: association of a novel beta 1–related subunit with alpha v. J. Cell Biol. *110*, 2185–2193.

Delwel, G.O., Hogervorst, F., Kuikman, I., Paulsson, M., Timpl, R., and Sonnenberg, A. (1993). Expression and function of the cytoplasmic variants of the integrin alpha 6 subunit in transfected K562 cells: activation-dependent adhesion and interaction with isoforms of laminin. J. Biol. Chem. *268*, 25865–25875.

Dufour, S., Duband, J.L., Humphries, M.J., Obara, M., Yamada, K.M., and Thiery, J.P. (1988). Attachment, spreading and locomotion of avian neural crest cells are mediated by multiple adhesion sites on fibronectin molecules. EMBO J. *7*, 2661–2671.

Elices, M.J., and Hemler, M.E. (1989). The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others. Proc. Natl. Acad. Sci. USA *86*, 9906–9910.

Elices, M.J., Osborn, L., Takada, Y., Crouse, C., Luhowskyj, S., Hemler, M.E., and Lobb, R.R. (1990). VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. Cell *60*, 577–584.

Elices, M.J., Urry, L.A., and Hemler, M.E. (1991). Receptor functions for the integrin VLA-3: fibronectin, collagen, and laminin binding are differentially influenced by Arg-Gly-Asp peptide and by divalent cations. J. Cell Biol. *112*, 169–181.

ffrench-Constant, C., and Hynes, R.O. (1989). Alternative splicing of fibronectin is temporally and spatially regulated in the chicken embryo. Development *106*, 375–388.

Garcia, P.A., Wayner, E.A., Carter, W.G., and Ferreira, O.J. (1990). Human B lymphocytes define an alternative mechanism of adhesion to fibronectin: the interaction of the $\alpha 4\beta 1$ integrin with the LHGPEILDVPST sequence of the type III connecting segment is sufficient to promote cell attachment. J. Immunol. *144*, 3361–3366.

Grumet, M., Hoffman, S., Crossin, K.L., and Edelman, G.M. (1985). Cytotactin, an extracellular matrix protein of neural and non-neural tissues that mediates glia-neuron interaction. Proc. Natl. Acad. Sci. USA *82*, 8075–8079.

Gu, M., Wang, W., Song, W.K., Cooper, D.N., and Kaufman, S.J. (1994). Selective modulation of the interaction of alpha 7 beta 1 integrin with fibronectin and laminin by L-14 lectin during skeletal muscle differentiation. J. Cell Sci. *107*, 175–181.

Guan, J.L., and Hynes, R.O. (1990). Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor alpha 4 beta 1. Cell *60*, 53–61.

Hall, D.E., Neugebauer, K.M., and Reichardt, L.F. (1987). Embryonic neural retinal cell response to extracellular matrix proteins: developmental changes and effects of the cell substratum attachment antibody (CSAT). J. Cell Biol. *104*, 623–634.

Hall, D.E., Reichardt, L.F., Crowley, E., Holley, B., Moezzi, H., Sonnenberg, A., and Damsky, C.H. (1990). The alpha 1/beta 1 and alpha 6/beta 1 integrin heterodimers mediate cell attachment to distinct sites on laminin. J. Cell Biol. *110*, 2175–2184.

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Horwitz, A., Duggan, K., Greggs, R., Decker, C., and Buck, C. (1985). The cell substrate attachment (CSAT) antigen has properties of a receptor for laminin and fibronectin. J. Cell Biol. *101*, 2134–2144.

Humphries, M.J., Akiyama, S.K., Komoriya, A., Olden, K., and Yamada, K.M. (1986). Identification of an alternatively spliced site in human plasma fibronectin that mediates cell type-specific adhesion. J. Cell Biol. *103*, 2637–2647.

Humphries, M.J., Akiyama, S.K., Komoriya, A., Olden, K., and Yamada, K.M. (1988). Neurite extension of chicken peripheral nervous system neurons on fibronectin: relative importance of specific adhesion sites in the central cell-binding domain and the alternatively spliced type III connecting segment. J. Cell Biol. *106*, 1289–1297.

Humphries, M.J., Komoriya, A., Akiyama, S.K., Olden, K., and Yamada, K.M. (1987). Identification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type-specific adhesion. J. Biol. Chem. *262*, 6886–6892.

Hynes, R.O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11–25.

Isberg, R.R., and Van Nhieu, D.T. (1994). Binding and internalization of microorganisms by integrin receptors. Trends Microbiol. *2*, 10–14.

Kimizuka, F., Ohdate, Y., Kawase, Y., Shimojo, T., Taguchi, Y., Hashino, K., Goto, S., Hashi, H., Kato, I., Sekiguchi, K., and Titani, K. (1991). Role of type III homology repeats in cell adhesive function within the cell-binding domain of fibronectin. J. Biol. Chem. *266*, 3045–3051.

Kirchhofer, D., Languino, L.R., Ruoslahti, E., and Pierschbacher, M.D. (1990). Alpha 2 beta 1 integrins from different cell types show different binding specificities. J. Biol. Chem. *265*, 615–618.

Krotoski, D.M., Domingo, C., and Bronner-Fraser, M. (1986). Distribution of a putative cell surface receptor for fibronectin and laminin in the avian embryo. J. Cell Biol. *103*, 1061–1071.

Lagenaur, C., and Lemmon, V. (1987). An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. Proc. Natl. Acad. Sci. USA *84*, 7753–7757.

Lefcort, F., Venstrom, K., McDonald, J.A., and Reichardt, L.F. (1992). Regulation of expression of fibronectin and its receptor, alpha 5 beta 1, during development and regeneration of peripheral nerve. Development *116*, 767–782.

Loftus, J.C., Smith, J.W., and Ginsberg, M.H. (1994). Integrin-mediated cell adhesion: the extracellular face. J. Biol. Chem. *269*, 25235–25238.

Masumoto, A., and Hemler, M.E. (1993a). Multiple activation states of VLA-4: mechanistic differences between adhesion to CS1/fibronectin and to vascular cell adhesion molecule-1. J. Biol. Chem. *268*, 228–234.

Masumoto, A., and Hemler, M.E. (1993b). Mutation of putative divalent cation sites in the alpha 4 subunit of the integrin VLA-4: distinct effects on adhesion to CS1/fibronectin, VCAM-1, and invasin. J. Cell Biol. *123*, 245–253.

Mould, A.P., and Humphries, M.J. (1991). Identification of a novel recognition sequence for the integrin alpha 4 beta 1 in the COOH-terminal heparin-binding domain of fibronectin. EMBO J. *10*, 4089–4095.

Mould, A.P., Komoriya, A., Yamada, K.M., and Humphries, M.J. (1991). The CS5 peptide is a second site in the IIICS region of fibronectin recognized by the integrin alpha 4 beta 1: inhibition of alpha 4 beta 1 function by RGD peptide homologues. J. Biol. Chem. *266*, 3579–3585.

Mould, A.P., Wheldon, L.A., Komoriya, A., Wayner, E.A., Yamada, K.M., and Humphries, M.J. (1990). Affinity chromatographic isolation of the melanoma adhesion receptor for the IIICS region of fibronectin and its identification as the integrin alpha 4 beta 1. J. Biol. Chem. *265*, 4020–4024.

Müller, U., Kleinberger, T., and Shenk, T. (1992). Adenovirus E4orf4 protein reduces phosphorylation of c-Fos and E1A proteins while simultaneously reducing the level of AP-1. J. Virol. *66*, 5867–5878.

Nagai, T., Yamakawa, N., Aota, S., Yamada, S.S., Akiyama, S.K., Olden, K., and Yamada, K.M. (1991). Monoclonal antibody characterization of two distant sites required for function of the central cell-binding domain of fibronectin in cell adhesion, cell migration, and matrix assembly. J. Cell Biol. *114*, 1295–1305.

Neff, N.T., Lowrey, C., Decker, C., Tovar, A., Damsky, C., Buck, C., and Horwitz, A.F. (1982). A monoclonal antibody detaches embryonic skeletal muscle from extracellular matrices. J. Cell Biol. *95*, 654–666.

Neugebauer, K.M., and Reichardt, L.F. (1991). Cell-surface regulation of beta 1-integrin activity on developing retinal neurons. Nature *350*, 68–71.

Newgreen, D., and Thiery, J.P. (1980). Fibronectin in early avian embryos: synthesis and distribution along the migration pathways of neural crest cells. Cell Tissue Res. *211*, 269–291.

Obara, M., Kang, M.S., and Yamada, K.M. (1988). Site-directed mutagenesis of the cell-binding domain of human fibronectin: separable, synergistic sites mediate adhesive function. Cell *53*, 649–657.

Pasqualini, R., Bodorova, J., Ye, S., and Hemler, M.E. (1993). A study of the structure, function and distribution of beta 5 integrins using novel anti-beta 5 monoclonal antibodies. J. Cell Sci. *105*, 101–111.

Pasqualini, R., and Hemler, M.E. (1994). Contrasting roles for integrin beta 1 and beta 5 cytoplasmic domains in subcellular localization, cell proliferation, and cell migration. J. Cell Biol. *125*, 447–460.

Pytela, R., Pierschbacher, M.D., Ginsberg, M.H., Plow, E.G., and Ruoslahti, E. (1986). Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors. Science *231*, 1559–1562.

Pytela, R., Pierschbacher, M.D., and Ruoslahti, E. (1985a). A 125/115-kDa cell surface receptor specific for vitronectin interacts with the arginine-glycine-aspartic acid adhesion sequence derived from fibronectin. Proc. Natl. Acad. Sci. USA *82*, 5766–5770.

Pytela, R., Pierschbacher, M.D., and Ruoslahti, E. (1985b). Identification and isolation of a 140 kd cell surface glycoprotein with properties expected of a fibronectin receptor. Cell *40*, 191–198.

Rader, C., Stoeckli, E.T., Ziegler, U., Osterwalder, T., Kunz, B., and Sonderegger, P. (1993). Cell-cell adhesion by homophilic interaction of the neuronal recognition molecule axonin-1. Eur. J. Biochem. *215*, 133–141.

Rein, A., and Rubin, H. (1968). Effects of local cell concentrations upon the growth of chick embryo cells in tissue culture. Exp. Cell Res. *49*, 666–678.

Rogers, S.L., Letourneau, P.C., Palm, S.L., McCarthy, J., and Furcht, L.T. (1983). Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. Dev. Biol. *98*, 212–220.

Ruegg, C., Postigo, A.A., Sikorski, E.E., Butcher, E.C., Pytela, R., and Erle, D.J. (1992). Role of integrin alpha 4 beta 7/alpha 4 beta P in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. J. Cell Biol. *117*, 179–189.

Sanchez-Aparicio, P., Dominguez-Jimenez, C., and Garcia-Pardo, A. (1994). Activation of the alpha 4 beta 1 integrin through the beta 1 subunit induces recognition of the RGDS sequence in fibronectin. J. Cell Biol. *126*, 271–279.

Schwartz, M.A., and Denninghoff, K. (1994). Alpha v integrins mediate the rise in intracellular calcium in endothelial cells on fibronectin even though they play a minor role in adhesion. J. Biol. Chem. *269*, 11133–11137.

Shimizu, Y., and Mobley, J.L. (1993). Distinct divalent cation requirements for integrin-mediated $CD4^+$ T lymphocyte adhesion to ICAM-1, fibronectin, VCAM-1, and invasin. J. Immunol. *151*, 4106–4115.

Smyth, S.S., Hillery, C.A., and Parise, L.V. (1992). Fibrinogen binding to purified platelet glycoprotein IIb-IIIa (integrin alpha IIb beta 3) is modulated by lipids. J. Biol. Chem. *267*, 15668–15677.

Sonderegger, P., and Rathjen, F.G. (1992). Regulation of axonal growth in the vertebrate nervous system by interactions between glycoproteins belonging to two subgroups of the immunoglobulin superfamily. J. Cell Biol. *119*, 1387–1394.

Sonnenberg, A. (1993). Integrins and their ligands. Curr. Top. Microbiol. Immunol. *184*, 7–35.

Timpl, R., Rhode, H., Gehron-Robey, P., Rennard, S., Foidart, J.M., and Martin, G. (1979). Laminin, a glycoprotein from basement membranes. J. Biol. Chem. *254*, 9933–9937.

Tomaselli, K.J., Damsky, C.H., and Reichardt, L.F. (1988). Purification and characterization of mammalian integrins expressed by a rat neuronal cell line (PC12): evidence that they function as alpha/beta heterodimeric receptors for laminin and type IV collagen. J. Cell Biol. *107*, 1241–1252.

Tomaselli, K.J., Reichardt, L.F., and Bixby, J.L. (1986). Distinct molecular interactions mediate neuronal process outgrowth on non-neuronal cell surfaces and extracellular matrices. J. Cell Biol. 2659–2672.

Varnum-Finney, B., Venstrom, K., Müller, U., Kypta, R., Backus, C., Chiquet, M., and Reichardt, L.F. (1995). The integrin receptor $\alpha 8\beta 1$ mediates interations of embryonic chick motor and sensory neurons with tenascin-C. Neuron (*in press*).

Vogel, B.E., Tarone, G., Giancotti, F.G., Gailit, J., and Ruoslahti, E. (1990). A novel fibronectin receptor with an unexpected subunit composition (alpha v beta 1). J. Biol. Chem. *265*, 5934–5937.

Wayner, E.A., Garcia, P.A., Humphries, M.J., McDonald, J.A., and Carter, W.G. (1989). Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. J. Cell Biol. *109*, 1321–1330.

Weinacker, A., Chen, A., Agrez, M., Cone, R.I., Nishimura, S., Wayner, E., Pytela, R., and Sheppard, D. (1994). Role of the integrin alpha v beta 6 in cell attachment to fibronectin: heterologous expression of intact and secreted forms of the receptor. J. Biol. Chem. *269*, 6940–6948.

Werb, Z., Tremble, P.M., Behrendtsen, O., Crowley, E., and Damsky, C.H. (1989). Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J. Cell Biol. *109*, 877–889.

Zhang, Z., Morla, A.O., Vuori, K., Bauer, J.S., Juliano, R.L., and Ruoslahti, E. (1993). The alpha v beta 1 integrin functions as a fibronectin receptor but does not support fibronectin matrix assembly and cell migration on fibronectin. J. Cell Biol. *122*, 235–242.