The Human Integrin \(\alpha 8\beta 1\) Function as a Receptor for Tenascin, Fibronectin, and Vitronectin*

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Integrins are a class of cell adhesion glycoproteins composed of two noncovalently associated subunits, \(\alpha\) and \(\beta\). Each subunit contains a large extracellular domain, a transmembrane domain, and a short cytoplasmic domain. Integrins are known to bind to a wide variety of extracellular matrix proteins, including fibronectin, vitronectin, collagens, and laminins. The specificity of protein binding is determined by particular combinations of \(\alpha\) and \(\beta\) subunit pairing. The ligand binding site is formed by the extracellular domain of both subunits and requires the presence of divalent cations. Many integrins interact

with ligands through the tripeptide arginine-glycine-aspartic acid (RGD).

The \(\alpha 8\beta 1\) integrin subunit was originally identified by Bossy et al. (1) in the chick embryo nervous system and was shown to be a partner for \(\beta 1\). We have identified human \(\alpha 8\), donated and sequenced the cDNA, raised antibodies to the predicted cytoplasmic domain sequence, and determined its distribution in adult mammalian tissues (2). We found that \(\alpha 8\) is predominantly expressed in a variety of visceral and vascular smooth muscle cells, kidney mesangial cells, and lung myofibroblasts (2).

To gain insight into potential functions of \(\alpha 8\beta 1\) in vivo, we sought to determine potential ligands. We tested various ligands for their ability to direct \(\alpha 8\beta 1\) to focal contacts, to bind to \(\alpha 8\beta 1\) by affinity chromatography, and to support adhesion of \(\alpha 8\)-transfected cells. All of the results provide strong evidence that \(\alpha 8\beta 1\) can function as an RGD-dependent receptor for tenascin, fibronectin, and vitronectin.

**EXPERIMENTAL PROCEDURES**

Materials—Fibronectin was prepared from human plasma as described by Engvall and Ruoslahti (3), and vitronectin was prepared from human plasma according to Yatohgo and co-workers (4). Type I collagen from rat tail, type IV collagen from human placenta, and fibrinogen were purchased from Sigma. Type I collagen was heat-denatured by incubating at 100°C for 15 min (5). Tenascin was purchased from Life Technologies, Inc. Recombinant tenasin fragments were a gift from Dr. Kathryn Crossin, Scripps Research Institute, La Jolla, CA (6). Osteopontin was a gift from Dr. Cecilia Giachelli, University of Washington, Seattle, WA. Laminin was a gift from Dr. Randy Kramer, University of California, San Francisco.

Antibodies and Reagents—Rabbit polyclonal antibody to the human \(\alpha 8\) cytoplasmic domain was previously characterized (2) and shown to cross-react with rat \(\alpha 8\) (see “Results”). Anti-vinculin antibody was purchased from Sigma. Monoclonal antibody L230 (anti-\(\alpha v\)) (7) was prepared in our laboratory from hybridoma cells obtained from American Type Culture Collection (ATCC); P1B5 (anti-\(\alpha 3\)) (8, 9) was obtained from Telios Pharmaceuticals (San Diego, CA); P1H5 (anti-\(\alpha 2\)) (10) and P3D10 (anti-\(\alpha 5\)) were a gift from Dr. William Carter (Fred Hutchison Cancer Center, Seattle, WA); P1F6 (anti-\(\alpha v\)) (11) was a gift from Dr. Elizabeth Wayner (University of Minnesota, Minneapolis, MN). The peptide GRGDSP was obtained from Telios Pharmaceuticals. The peptides ACRGDDWMCG (RGDGW), DSCRR- TAWACRL (CRRETAWAC), and ACDCRGDCCFG (GC) were gifts from Drs. Erikki Koivunen and Erikki Ruoslahti, La Jolla Cancer Research Foundation, La Jolla, CA (12, 13).

Cell Lines—Human intestinal smooth muscle cells (HISM), rat embryonic fibroblasts (REF 52), human embryonic kidney cell line 293, and human colon carcinoma cell line SW 480 were obtained from ATCC and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and penicillin/streptomycin. The \(\alpha 9\)-transfected 293 (14) and \(\beta 3\)-transfected 293 cells were previously characterized (15).

Immunofluorescence—Coverslips were coated overnight at 4°C with 10–20 \(\mu\)g/ml of the following extracellular matrix proteins: fibronectin, vitronectin, collagen types I and IV, denatured collagen type I, laminin, and fibrinogen. Coverslips were blocked with 3% bovine serum albumin.
(BSA), phosphate-buffered saline (PBS) at 37°C for 30 min. Cells
grown to 80% confluence were removed from tissue culture plates with
2 mM EDTA, PBS, washed with PBS, resuspended in Serum-free Dul- 
becco’s modified Eagle’s medium, and plated onto the coverslips. Three 
hours after plating, cells were fixed and permeabilized with 2% 
paraformaldehyde, 0.1% Triton X-100 for 10 min. Coverslips were 
blocked with 3% BSA and then incubated with anti-Tenascin C 
(RGDKWSL) and found to be free of mutations that would alter the encoded 
peptide sequence. Coverslips were then incubated with fluorescein 
conjugated streptavidin (1:100) for 15 min at room temperature, washed with PBS, 
and mounted with Vectashield (Vector Laboratories, Burlingame, 
CA).

To block endogenous production of extracellular matrix proteins, 
cells were incubated with the protein synthesis inhibitor cycloheximide 
(30 μg/ml) for 3 h in serum-free medium. Cells were then 
detached with 2 mM EDTA and seeded on fibronectin- and vitronectin-covered 
coverslips in the presence of cycloheximide.

Preparation of cDNA Expression Constructs—A cDNA containing 
the entire coding region of human α8 was initially constructed in 
the pBluescript (Stratagene, La Jolla, CA). The full-length α8 cDNA was 
amplified by PCR using the previously reported cDNA clone HA13A (2) as 
a template. This cDNA contains the signal peptide as well as 321 nucleotides 
in the 5′ untranslated region followed by the ATG initiation codon (double 
underlined) and the sequence encoding the first nine amino acids of 
the α8 signal peptide (ALRVLLEL). The MAC 1F primer has 
20 nucleotide overlap (underlined) with the 3′ end of the Mac 2F 
primer and encodes the remainder of the human αM integrin subunit (16). 
The MAC 2F primer contains a HindIII recognition sequence (underlined), 12 nucleotides of 
the 5′ untranslated region, followed by the ATG initiation codon 
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the αM signal peptide (ALRVLLEL). The Mac 1F primer has 
a 20 nucleotide overlap (underlined) with the 3′ end of the Mac 2F 
primer and encodes the remainder of the αM signal peptide (ALCHG) 
followed by the N-terminal 9 amino acids of the α8 signal peptide (FNLDV). 
The first PCR reaction used forward primer Mac 1F and a α8-specific 3′ reverse 
primer and the first PCR reaction cDNA as a template. In order to 
correct the deletion, we amplified the region from clone HA15-A that 
was previously amplified (Fig. 2A). The amplification product 
was then transferred to protein coated wells (3 g/ml) and anti-vinculin antibody (2 
μg/ml) for 3 h in serum-free medium. Cells were then centrifuged 
at 14,000 rpm for 20 min.

Affinity chromatography—Cells grown to 80% confluence were 
detached with 2 mM EDTA in PBS. Cells were centrifuged and washed 
three times in labeling buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM 
MnCl2, 20 mM glucose). Cells were surface labeled with 125I-sodium 
iodide using the lactoperoxidase method (17).

Cell lysates were prepared for precipitation by incubating with 200 
μg acetylglucoside, 100 μg Tris-HCl, 1 mM dithiothreitol (DTT) 
and 5 (P3D10), or - (P5D2) for 5 min. The 
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correct the deletion, we amplified the region from clone HA15-A that 
was not contain the deletion (2) and used unique restriction sites (BstI 
and HpaI) in the α8 clone in order to ligate the fragment. All polym- 

ase chain reactions were performed with Vent DNA polymerase (New England Biolabs) 
which has been reported to provide significantly 
higher fidelity than Taq polymerase. The plasmid was then completely 
sequenced using Sequenase 2.0 (U.S. Biochemical Corp., Cleveland, 
OH) and found to be free of mutations that would alter the encoded 
amino acid sequence. The plasmid was cut at the unique HindIII and 
XbaI sites in the pBluescript polylinker, and ligated into the mamma- 
lium expression vector pCDNAIneo between the unique HindIII and 
XbaI sites to generate pCDNAα8I.

Transfection of Mammalian Cells—The human embryonic kidney 
cell line 293 and human colon carcinoma cell line SW 480 were trans- 
fected using the Lipofectin reagent (Life Technologies, Inc.) according to 
the manufacturer’s instructions. Stably transfected cell lines were 
selected in medium containing the neomycin analog G418 (0.4 mMg/ml). 
Cells were transfected with either pCDNAα8I (α8-transfected cells) 
or pCDNAIneo alone (mock-transfected cells).

Flow Cytometry—Cells were detached with 2 mM EDTA, 
Washed with PBS, resuspended in normal goat serum and incubated at 4°C 
for 10 min. After centrifugation, cells were re suspended in PBS containing 
the appropriate antibody: P1H5 (anti-α2), P1B5 (anti-α3), P3D10 (anti- 
α5), L230 (anti-αv), P5D2 (anti-β1) and incubated for 20 min on ice. 
After subsequent washes with PBS, cells were incubated with phyco- 
yrithrin-conjugated goat anti-mouse antibody (Boehringer Mannheim)
cells plated on various extracellular matrix proteins as a first step towards identifying ligands for α8β1. In our experiments we used a human smooth muscle cell line (HISM) and a rat embryo fibroblast (REF) cell line, which we found to express α8β1 by immunoprecipitation (Fig. 1, lanes 1 and 2).

HISM cells plated on extracellular matrix proteins were analyzed by double-labeling immunofluorescence microscopy using antibodies to vinculin (to detect focal contacts) and α8 (Fig. 2). Three hours after plating on fibronectin, vitronectin, or collagen, HISM cells were well spread and formed vinculin-containing focal contacts. In cells plated on fibronectin or vitronectin, α8 co-localized to vinculin-containing focal contacts (Fig. 2, A–D). In contrast, in cells plated on collagen type I or denatured collagen type I, α8 did not localize to focal contacts, despite the abundance of focal contacts identified by vinculin staining (Fig. 2, E and F). Cells plated on tenascin adhered, but did not form vinculin-containing focal contacts (data not shown). Cells plated on fibrinogen or laminin attached poorly and did not spread or form focal contacts. No localization of α8 staining was detected in these cells (data not shown). Identical results were obtained using rat embryo fibroblast cells (data not shown). It is unlikely that α8 localization was due to matrix protein secretion by the cells, because control experiments using cells pretreated with the protein synthesis inhibitor, cycloheximide, yielded similar results (Fig. 3). We were not able to demonstrate focal contact formation by α8-transfected or un-transfected 293 cells. However, α8-transfected SW 480 colon carcinoma cells showed new localization of α8 to focal contacts when plated on fibronectin and vitronectin (data not shown). Thus, vitronectin and fibronectin, but not collagen, specifically promote localization of α8 to focal contacts. These data suggest vitronectin and fibronectin are ligands for α8β1.

Heterologous Expression of α8 cDNA—The human embryonic kidney cell line 293 is highly permissive for transfection of heterologous cDNAs and does not express α8 as determined by immunoprecipitation (Fig. 1, lane 3). After transfection of 293 cells with α8 cDNA we obtained four independent clones that expressed α8 as detected by Western blot analysis (data not shown). Immunoprecipitation of surface-labeled cells demonstrated that α8β1 was expressed on the cell surface (Fig. 1, lane 4).

To determine whether α8 transfectants have altered levels of other β1-associated subunits, we performed fluorescein-activated cell sorting analysis using a panel of anti-integrin antibodies (Fig. 4). Mock-transfected and α8-transfected 293 cells contained similar amounts of cell surface α2, α3, α5, and αv-containing integrins. Thus, changes in adhesive properties of α8-transfected cells are not due to changes in the surface expression of other integrins.

Adhesion of α8-transfected 293 Cells—To determine whether α8β1 can mediate cell adhesion to fibronectin and vitronectin, we compared the adhesive properties of mock-transfected and α8-transfected 293 cells. Fibronectin and vitronectin adhesion were not significantly affected by α8 expression (Fig. 5), although a slight increase in adhesion to 3 μg/ml vitronectin was noted. When endogenous receptors for fibronectin and vitronectin were blocked with monoclonal antibodies, the contribution of α8β1 was more apparent (Figs. 6 and 7). The adhesion of wild type or mock-transfected cells to fibronectin was almost completely inhibited (>90%) by either anti-β1 antibody (P5D2) or anti-α5 antibody (P5D10) (Fig. 6A). In contrast, α8-transfected cell adhesion was only partially blocked by anti-α5 antibody (33%), but was completely blocked by anti-β1 antibody. These data suggest that the α8-transfected cells are using α8β1 in addition to α5β1 to adhere to fibronectin.

Vitronectin adhesion of mock-transfected and wild type 293 cells was almost completely inhibited by the blocking anti-αv antibody, L230 (Fig. 7A), consistent with previous reports (15, 20). In contrast, vitronectin adhesion in α8-transfected cells was only partially inhibited (19%) by the anti-αv antibody, L230, and inhibited by 41% using the anti-β1 antibody, P5D2. Vitronectin adhesion was completely abolished using both antibodies in combination. These data suggest that α8-transfected cells are using α8β1 in addition to αv-containing integrins, to adhere to vitronectin.

To further elucidate the binding characteristics of α8β1 to fibronectin and vitronectin, adhesion assays were performed in the presence of three different synthetic peptides (Figs. 6B and 7B). Although integrins can interact through a common RGD site in the ligand, conformationally constrained peptides can discriminate between various RGD binding integrins. The cyclic peptide, CRRETAWAC, has recently shown to be highly selective for α5β1 (13). At concentrations sufficient to block adhesion of α5β1 to fibronectin, CRRETAWAC does not block αvβ1 fibronectin adhesion or αv-mediated vitronectin adhesion (13). In an analogous fashion, the cyclic peptide 4C selectively inhibits αv-mediated adhesion (21). In contrast, the peptides GRGDSP and RGDGW are able to block both α5- and αv-
mediated adhesion (12). We took advantage of these selective peptides to further define the binding characteristics of α8β1. We found that adhesion of α8-transfected cells to fibronectin was inhibited by the peptide RGDGW, but not by the CRRETAWAC peptide (Fig. 6B). The addition of the α5 blocking antibody, P3D10, to CRRETAWAC, did not significantly decrease fibronectin adhesion (data not shown). In contrast, adhesion of mock-transfected 293 cells to fibronectin was inhibited by either RGDGW or CRRETAWAC (Fig. 6B). Adhesion of mock-transfected and α8-transfected 293 cells to fibronectin was not affected by the αv-selective 4C peptide (data not shown). These results suggest that α8β1 interacts with fibronectin by mechanisms that are similar to, but distinguishable from, those used by α5β1.

Adhesion of α8-transfected cells to vitronectin was inhibited by the peptide RGDGW, but not by the 4C peptide (Fig. 7B). The addition of the αv blocking antibody, L230, to 4C did not further inhibit vitronectin adhesion (data not shown). In contrast, adhesion of mock-transfected 293 cells to vitronectin was inhibited by either RGDGW or 4C (Fig. 7B). Adhesion of mock-transfected and α8-transfected 293 cells to vitronectin were not

**FIG. 3.** Focal contact formation by HISM cells in the presence of cycloheximide. Cells were preincubated with cycloheximide (30 μg/ml) for 3 h and then allowed to spread on 10 μg/ml fibronectin (A and B) and vitronectin (C and D) for 3 h in the presence of cycloheximide. Cells were fixed, permeabilized, and double-labeled with anti-α8 antibody (A and C) and anti-vinculin antibody (B and D).

**FIG. 4.** Flow cytometry of α8-transfected 293 cells (black bars) and mock-transfected 293 cells (white bars). The results represent an average of four different clones of α8-transfected 293 cells and mock-transfected 293 cells. Cells were incubated with the following antibodies: P1H5 (anti-α2), P1B5 (anti-α3), P3D10 (anti-α5), L230 (anti-αv), and P5D2 (anti-β1) and were analyzed using a FACScan. The y axis represents fluorescence intensity (in arbitrary units).

**FIG. 5.** Adhesion of mock-transfected (black squares) and α8-transfected (open squares) 293 cells to increasing concentrations (0.3, 1, 3, 10, and 20 μg/ml) of (A) fibronectin and (B) vitronectin. The y axis represents the absorbance at 595 nm after staining attached cells with crystal violet.
affected by the α5-selective peptide, CRRETAWAC (data not shown). Thus, the 4C peptide, at the concentration used, blocks αv but not αβ1-mediated adhesion to vitronectin, suggesting that αβ1 interacts with vitronectin through mechanisms distinct from those used by αv integrins.

Affinity Chromatography—To determine whether αβ1 binds directly to fibronectin and vitronectin, we performed affinity chromatography using octylglucoside lysates of 125I-labeled, α8-transfected 293 cells. We passed cell lysates over fibronectin-Sepharose (Fig. 8A) or vitronectin-Sepharose (Fig. 8B) columns, washed with loading buffer (lanes 2–8), and eluted with GRGDSP (lanes 9–12) and EDTA (lanes 13–16). The eluate of the fibronectin-Sepharose column contained three labeled proteins with sizes corresponding to putative fibronectin receptor subunits (Fig. 8A, arrows): 120 kDa (β1), 150 kDa (α5), and 170 kDa (α8). To determine whether the 170-kDa/120-kDa protein is indeed identical with αβ1, we performed immunoprecipitations with anti-α5 antibody (Fig. 9A, lanes 1–3). The putative αβ1 heterodimer (170 kDa/120 kDa) was detected in the cell lysate (lane 1), was not detectable in wash fractions (lane 2), and was again present in the GRGDSP eluate (lane 3). To confirm the identity of the 150-kDa/120-kDa protein as α5β1, we performed an immunoprecipitation of the GRGDSP eluate with P3D10 (anti-α5) antibody (lane 4).

The GRGDSP eluate of the vitronectin-Sepharose column contained major bands of 150 kDa and 95 kDa (Fig. 8B, arrows). However, immunoprecipitation of the eluted fractions with anti-α8 antibody demonstrates the 170-kDa/120-kDa complex corresponding to αβ1 was specifically eluted from the vitronectin-Sepharose column by GRGDSP (Fig. 9B, lanes 1–3).

Adhesion of αβ1 to Additional RGD-containing Proteins—Because the above experiments suggested that αβ1 was binding to the RGD sites in fibronectin and vitronectin, we examined additional RGD-containing proteins for the ability to bind to αβ1. We tested the ability of mock- and α8-transfected 293 cells to adhere to tenasin, fibrinogen, thrombospondin, osteopontin, and denatured collagen type I (Fig. 10). We found that α8-transfected 293 cells adhered and spread well on tenasin, whereas mock-transfected 293 cells did not adhere (Fig. 10). Neither mock-transfected nor α8-transfected 293 cells adhered to fibrinogen, thrombospondin, osteopontin, and denatured collagen type I (Fig. 10).

Tenasin is a modular protein that contains several fibronectin type III repeats. An RGD site located in the third fibronectin type III repeat of tenasin (TNfn3) has been shown to mediate adhesion of several integrins, including αvβ3 and probably αvβ6 (22). To determine whether αβ1 was mediating adhesion through this RGD site, we tested the adhesion of α8-
The anti-a mentsoftenasc in (Fig. 11). The aden and mock-transfected 293 cells to various recombinant fragments. The a-transfected cells adhered to the RGD-containing fragment, TNfn3, in a concentration-dependent manner. As expected, mock-transfected 293 cells did not adhere to TNfn3. In contrast, a-transfected 293 cells did not adhere to a fragment containing the fourth through sixth fibronectin-type III repeats, TNfn4–6 (Fig. 11). To confirm that a-transfected cells were binding through the RGD site of TNfn3, we tested adhesion of fibronectin type III repeats, TNfn4–6 (Fig. 11). To confirm that a-transfected cells did not adhere to a fragment containing the fourth through sixth fibronectin-type III repeats, TNfn4–6 (Fig. 11). We found that this abolished adhesion of a-transfected cells to a mutant TNfn3 in which the RGD site had been altered to RAA (TNfn3RAA). We found that this abolished adhesion of a-transfected cells to a mutant TNfn3 in which the RGD site had been altered to RAA (TNfn3RAA).

Adhesion to TNfn3 by a-transfected cells was abolished by the anti-β1 antibody, P5D2 (Fig. 12). We also tested the ability of the selective, synthetic peptides to block adhesion to TNfn3. The peptides, GRGDSP and RGDGW blocked adhesion of a-transfected cells to tenascin. In contrast, the cyclic CRRETAWAC peptide did not inhibit adhesion and the cyclic 4C peptide partially inhibited adhesion to TNfn3 (Fig. 12). Adhesion of β3-transfected cells to tenascin was abolished by the 4C peptide (data not shown). Taken in concert, these results suggest that a8β1 is binding to the RGD site in tenascin. However, this interaction must be somewhat distinct from the αV-tenascin interactions because it is not completely inhibited by the peptide 4C.

In summary, we have demonstrated that a8β1 can bind to tenascin, fibronectin, and vitronectin by interacting with the RGD sites on these ligands. We also show that a8β1 is capable of localizing to focal contacts on fibronectin and vitronectin on fibroblasts and smooth muscle cells. a8β1 is eluted from both fibronectin and vitronectin affinity columns by an RGD-containing peptide. Our fibronectin adhesion data are in agreement with the recently published observations that chicken αβ1 is able to support attachment, spreading, and neurite outgrowth by transfected cells (23). Two other β1-containing integrins are known to interact with RGD sites: α5β1 and αvβ1. These α subunits, along with αIIb, are the most closely

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to cleaved, and do not contain I domains. Despite the similarities to RGD-containing peptides, are post-translationally targeted collagen. Additionally, a subfamily of a5b1 is more promiscuous and can bind to vitronectin and tenascin as well as fibronectin. In addition, binding by a8b1 is not affected by the peptide CRRETAWAC, which efficiently blocks a5b1. When compared to a5b3, the binding repertoire of a8b1 is more limited. Although both a8b1 and a5b3 bind to tenasin, fibronectin, and vitronectin, a8b1 does not bind to several other a5b3 ligands, including fibrinogen, thrombospondin and denatured collagen. Additionally, a8-mediated adhesion is not affected by the peptide 4C. Thus, the binding characteristics of a8b1 are unique and distinguishable from both a5b1 and a5b3.

In adult mammalian tissues, a8b1 is prominently expressed in vascular and visceral smooth muscle cells, kidney mesangial cells, and lung myofibroblasts. Tenascin, fibronectin, and vitronectin are thought to play a role in the response to injury and inflammation. Thus, a8b1 may contribute to the functional changes that occur in smooth muscle cells during tissue repair. Since smooth muscle cells also express other fibronectin, vitronectin, and tenasin receptors, such as a5b1, a5b5, and a5b3, it will be important to determine the specific functional contribution of a8b1 to smooth muscle cell behavior.

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