Integrin Receptors on Aortic Smooth Muscle Cells Mediate Adhesion to Fibronectin, Laminin, and Collagen

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Extracellular matrix receptors on vascular smooth muscle cells help in anchoring the cells during contraction and in promoting cellular migration after vessel injury. We found that rat aortic smooth muscle cells attach to surfaces coated with fibronectin, laminin, and collagen types I and IV. Cell attachment to these substrates appears to be mediated by members of the \( \beta_4 \) integrin family of extracellular matrix receptors. Antibodies to the \( \beta_4 \) subunit not only demonstrated the presence of integrin complexes in focal adhesion plaques but also blocked cell adhesion to the different substrates. Ligand-affinity chromatography and sodium dodecyl sulfate–polyacrylamide gel electrophoresis isolated a series of receptor complexes that were recognized by antisera to \( \beta_4 \) integrin receptors. Each of the receptors appeared to be a heterodimer in which one of several \( \alpha \) subunits shared a common 120-kDa (nonreduced) \( \beta_4 \) subunit protein. The rat aortic smooth muscle cells had one \( \alpha \) subunit (150 kDa nonreduced, 140 kDa reduced) that bound exclusively to fibronectin. There was a second \( \alpha \) subunit (150 kDa nonreduced, 160 kDa reduced) that bound exclusively to collagen type I. In addition, there was a third \( \alpha \) subunit (185 kDa nonreduced, 200 kDa reduced) that was promiscuous and bound to collagen types I and IV as well as to laminin; the 185-kDa \( \alpha \) subunit appeared to bind to collagen more efficiently than it did to laminin. Thus, smooth muscle cells express multiple integrin receptors with different ligand specificities that appear to mediate cell interactions with the extracellular matrix. (Circulation Research 1990;67:175–186)

The mechanisms that regulate smooth muscle cell migration into the arterial intima play an important role in arterial wound repair and atherosclerosis. During arterial remodeling, both nondividing and proliferating smooth muscle cells migrate out of the muscle media and through the internal elastic lamina to increase the intimal smooth muscle cell number. Factors that affect the cell's ability to adhere to the extracellular matrix (ECM) alter the balance between cell stability and cell mobility. Components of the ECM that surround medial smooth muscle cells include fibronectin, laminin, and collagen types I and IV. These ECM proteins not only promote cell adhesion and migration but also influence the phenotypic appearance of the smooth muscle cell.

The response of the smooth muscle cell to the ECM probably depends on specific cell surface receptors. In vivo, aortic smooth muscle cells form dense adhesion plaques at the junction between the ECM and the intracellular contractile microfilament system. These dense plaques include proteins such as vinculin, talin, and \( \alpha \)-actinin. In other cell types, a group of related cell surface glycoproteins, called integrins, have been implicated as surface receptors for mediating cell adhesion to the ECM and to other cells. Each receptor is a heterodimer in which one of several homologous \( \alpha \) subunits associates noncovalently with a \( \beta \) subunit. The integrins require the presence of divalent cations to function; the \( \alpha \) subunits of the receptors contain several repetitive amino acid sequences that are similar to the Ca\(^{2+}\) binding sites of other divalent cation binding proteins. Several of the integrins can interact with a specific amino acid sequence, Arg-Gly-Asp (RGD), which is located in the cell binding region of fibronectin and vitronectin and is also present in laminin, collagen types I and IV, and other adhesive proteins. On the other hand, some integrin receptors appear not to use the RGD...
sequence, although sequences related to RGD may be important in these interactions. In human cells there are at least three major subfamilies of integrin receptors, which are defined by their 6 chains. (There is recent evidence for a fourth[17] and a fifth[18] class of 6 subunits.) The first subfamily comprises at least six related complexes, each consisting of a 6 chain with a distinct companion 6 chain. Members of the 6 subfamily include receptors for fibronectin (6666, 6665), laminin (6665, 6665, and 6665), and collagen types I and IV (6665, 6665, 6665, and 6665). On human cells, these receptors correspond to the very late activation (VLA) heterodimers first described on T-cells.[25,26]

The purpose of the present study was to identify rat aortic smooth muscle (RASM) cell proteins that might act as cell surface receptors for ECM molecules. We used affinity chromatography with immobilized ECM ligands to purify surface receptors followed by immunochemistry to determine their relatedness to the 6 integrins. We compared the structural and functional characteristics of the RASM cell surface glycoproteins with other putative ECM receptors described for cells of different tissues and species.

Materials and Methods

Cell Culture

Smooth muscle cells were isolated as previously described[27] from rat aortas and characterized by their morphology and ability to be recognized by monoclonal antibody against 6 smooth muscle actin (Sigma Chemical Co., St. Louis). The cells were grown in monolayer culture in Dulbecco's modified Eagle's medium (DME) H-16 (Gibco Laboratories, Grand Island, N.Y.) supplemented with 1 g/l glucose, 5% fetal calf serum, 100 units/ml penicillin, and 100 6/ml streptomycin. Cells were passaged at preconfluence after a 2-minute treatment with trypsin-EDTA (0.25 g/100 ml trypsin and 2 mM EDTA in Ca2+/Mg2+-free phosphate buffered saline [PBS] [mM: Na2PO4, 15, KH2PO4, 1.5, NaCl 140, KCl 2]) at room temperature. Cells were used between passages six and 12.

Antibodies

Anti-hamster 6, (variously called anti-GP140 and anti-ECMR), a polyclonal antiserum raised in goats to the 120–160-kDa 6 integrin glycoprotein complexes purified from BHK cells, was the generous gift of Drs. K. Knudsen and C. Damsky.[28,29] This antiserum inhibits adhesion of several mouse and rat cell lines to laminin, fibronectin, and type IV collagen[30,31] but not to thrombospondin. It recognizes the 120-kDa 6 integrin heterodimers.[30,31,33] Anti-hamster 6, serum also has been shown to recognize the 6 subunit of the vitronectin receptor[34] in some cell lines; however, the RASM cells used in our experiments do not express 6 integrins (R. I. Cyman, unpublished observations, 1989).

A rabbit antiserum to a peptide derived from the cytoplasmic domain of the 6 integrin subunit, anti-6, cyt,[35] and a rabbit antiserum to a peptide derived from the cytoplasmic domain of the integrin 66 subunit, anti-6, were the generous gift of Dr. K. Tomasselli and Dr. L. Reichardt (University of California, San Francisco). Anti-human 6, a rabbit antiserum that recognizes the 6 and the 6 subunits of the fibronectin receptor,[35] was the generous gift of Dr. E. Ruoslahti (La Jolla Cancer Foundation, La Jolla, Calif.). Heat-inactivated normal rabbit and normal goat sera were used as controls.

Cell Adhesion Assay

Cell adhesion was measured using a previously described assay.[30] Cells were labeled for 18–24 hours with 2 Ci/ml 5-125Iiodo-2'-deoxyuridine ([125I] IUDR, ICN Biomedicals, Irvine, Calif.). In some experiments, protein synthesis was inhibited by preincubating the cultures with cycloheximide (25 mg/ml) for 3 hours before cell harvest. Cycloheximide was included in the culture media used in the subsequent adhesion assay. The cycloheximide experiments were performed to show that cell adhesion was not due to newly synthesized ECM proteins. The cells were removed from the culture plates by brief incubation (2 minutes) at room temperature with trypsin-EDTA; this method has been used previously in studying integrins.[36,37] The trypsin was inactivated by washing the cells with DME plus 5% fetal bovine serum (FBS) at 4°C. The cell pellet was resuspended in DME plus 1 mg/ml bovine serum albumin (BSA) for the assay. In some experiments, the cells were suspended in a divalent cation–free medium (mM: NaCl 140, KCl 5.4, d-glucose 5.56, HEPES 10; pH 7.4; 1 mg/ml BSA) to study the effects of divalent cations on cell adhesion.

The wells of uncharged polystyrene 96 well microtiter plates (Serocluster, Costar Corp., Cambridge, Mass.) were precoated with poly-L-lysine (M, 500,000, Sigma), wheat germ agglutinin (Calbiochem Corp., La Jolla, Calif.), fibronectin,[38] laminin,[39] type IV collagen,[39] type I collagen (Collagen Corp., Palo Alto, Calif.), gelatin (Sigma), or with BSA in sterile Ca2+/Mg2+-free PBS for 1 hour at 37°C. The wells were then washed with PBS, and nonspecific adherence to the coated wells was blocked with 1 mg/ml BSA in DME (or divalent cation–free medium) for 1 hour at 37°C. Appropriate dilutions of antibodies and peptides (Gly-Arg-Gly-Asp-Ser-Pro [GRGESP] and Gly-Arg-Gly-Glu-Ser-Pro [GRGESP], Peninsula Laboratories, Belmont, Calif.) were added to the wells. The 125I-labeled cells (2 106 cells per well) were allowed to attach to the wells for 15 minutes at 37°C.

Cell Surface Radiiodination and Metabolic Labeling With [3H]Glucosamine

To metabolically label glycoproteins, cells were incubated for 48 hours in DME plus 5% FBS and 50 Ci/ml [3H]glucosamine (New England Nuclear, Boston). After the culture plates were washed four times...
times with PBS, the cells were solubilized in cold lysis buffer (0.5% NP40 [Calbiochem], 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin) at 4°C for 1 hour. The lysates were then centrifuged at 700g for 10 minutes, transferred to new tubes, and centrifuged at 10,000g for 10 minutes.

For surface labeling, the cells were removed from the tissue culture plates with trypsin-EDTA solution and inoculated into 10-cm bacteriologic culture dishes. Collagen-coated microcarrier beads (Cytodex-3, Pharmacia LKB Biotechnology, Piscataway, N.J.) that had previously been swollen and washed in culture medium were added to the plates so that approximately four to eight cells bound to each microcarrier bead. We used 3 ml of swollen beads (approximately 10⁹ carriers) in 30 ml of culture medium. The cells attached to the beads rapidly, and the plates were gently rocked 15 times per minute for 24 hours at 37°C. The microcarriers with cells attached were then washed three times with cold PBS and resuspended in an equal volume of iodination buffer (mM: Tris-HCl 50, pH 7.4, NaCl 150, MnSO₄ 0.1, glucose 20). Iodination was initiated by adding glucose oxidase (Sigma), lactoperoxidase (Calbiochem), and carrier-free Na¹²⁵I (Amersham Corp., Arlington Heights, Ill.) at final concentrations of 0.1 unit/ml, 100 µg/ml, and 500 µCi/ml, respectively. The suspension was mixed by rotation on ice for 25 minutes; the reaction was terminated with excess glucose-free iodination buffer. The microcarriers were recovered by centrifugation and extracted with cold solubilization buffer for 1 hour: (mM) octyl β-D-glucopyranoside 200 (Boehringer Mannheim, Indianapolis), Tris-HCl 50, pH 7.4, MnSO₄ 1. Protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 2 µg/ml aprotinin [Sigma]) were added during solubilization and throughout the subsequent procedures. The suspension was centrifuged for 10 minutes at 2,000g, and the pellet was extracted a second time.

The supernatants of the solubilized whole-cell extracts were combined and used for immunoprecipitation or affinity chromatography. In some experiments, the collagen-coated microcarrier beads, without any attached cells, were iodinated and extracted with solubilization buffer; the solubilized extract of the microcarrier beads was used as a control for the whole-cell extracts during the affinity chromatography experiments.

**Affinity Chromatography**

Affinity columns were prepared by coupling the ligand (BSA, type I collagen, type IV collagen, laminin, or gelatin) to CNBr-activated Sepharose (Pharmacia LKB). The fibronectin affinity column was made from the 120-kDa fragment of fibronectin.⁴⁰ The solubilized whole-cell extract from (7×10⁶)±(4×10⁵) cells (mean±SD) was applied to a BSA-Sepharose precolumn (1x1 cm) previously equilibrated with 50 mM octylglucoside, 50 mM Tris-HCl, pH 7.4, 1 mM MnSO₄, 1 µg/ml BSA, and 0.005% NaN₃ (wash buffer A). The BSA-Sepharose precolumn was eluted with four column volumes of wash buffer A, and the eluate was passed repeatedly through a substrate-Sepharose column (1x5 cm) over 1.5 hours. The substrate-Sepharose column was then washed with five column volumes of wash buffer A (25 ml). Bound receptors were eluted first with one column volume of GRGDS (1 mg/ml) followed by three column volumes of wash buffer A; second, with four column volumes of 0.2 M NaCl in wash buffer A; third, with four column volumes of 10 mM EDTA in wash buffer A without divalent cations; and finally, with three column volumes of 1 M NaCl in wash buffer A. The substrate-Sepharose columns (fibronectin, laminin, and type I and type IV collagen) were reused up to five times. Twenty-two microliters of 1 M MgCl₂ was added immediately to each 1-ml fraction of the EDTA eluate. In some of the affinity studies, 1 mM MnSO₄ was replaced with 1 mM CaCl₂ in both the solubilization solution and the wash buffer A.

**Immunoprecipitation**

Whole-cell extracts in either lysis buffer or solubilization buffer and column eluates were first pre-cleared by incubating 1 ml of the whole-cell extract or column eluate with 50 µl of packed protein A-Sepharose beads (Sigma) for 30 minutes at 4°C, then centrifuging the mixture. Next, 10 µl of antiserum was added to the 1-ml supernatants and incubated for 2 hours at 4°C. Immune complexes were recovered by incubating the supernatants for 2 hours with 50 µl of packed protein A-Sepharose. The protein A beads with bound immune complexes were washed as described.³⁰ Control immunoprecipitation performed with preimmune normal goat or normal rabbit serum produced negligible radioactivity.

**Electrophoresis**

Eluates from the affinity columns and samples from the immunoprecipitation experiments were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli.⁴¹ Samples were solubilized in sample buffer, reduced with 5% 2-mercaptoethanol, and heated at 100°C for 4 minutes. Nonreduced samples were treated as above except that 10 mM N-ethylmaleimide was substituted for 2-mercaptoethanol. The samples were separated on polyacrylamide gels and detected by autoradiography.³ H-labeled proteins were visualized by equilibrating the gel with 2.5-diphenyloxazole, scintillation grade (National Diagnostics, Manville, N.J.). Prestained protein standards (Sigma) were used to calculate the apparent molecular mass and consisted of α₂-macroglobulin (180 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactate dehydrogenase (36.5 kDa), and triosephosphate isomerase (26.6 kDa).
Immunofluorescent Staining

Glass coverslips were coated with 100 μg/ml of fibronectin, laminin, and collagen types I and IV in PBS for 1 hour at 37°C. Cells were suspended in serum-free culture medium containing 0.1% BSA and overlaid (2×10⁴ cells) on coverslips. After 3 hours incubation at 37°C, the cells were fixed and permeabilized as previously described. After incubation with 1% normal goat serum, the samples were incubated for 1 hour with anti-human β₁ (1:2000) and mouse antivinculin (1:100) (ICN). The samples were then incubated for 1 hour with secondary antisera coupled to fluorescein isothiocyanate or rhodamine. The coverslips were mounted in Fluoromount (Fisher Scientific Co., Pittsburgh) and viewed on a Nikon microscope equipped with epiluminescence optics.

Results

Cell Adhesion Studies

RASM cells adhered to wells coated with laminin, fibronectin, and types I and IV collagen in both a dose-dependent and a time-dependent manner (Figure 1). RASM cells also readily attached to poly-L-lysine and wheat germ agglutinin (Figure 1). In contrast, the cells attached poorly to wells coated with gelatin and failed to attach appreciably to wells coated with BSA.

Figure 2 examines the requirements for divalent cations in the adhesion of RASM cells to different substrates. Without any divalent cations in the incubation medium, there was negligible cell binding to laminin or collagen type I or IV; adhesion to fibronectin was also markedly reduced. The requirement for divalent cations in the medium was specific for ECM-derived substrates; adhesion of RASM cells to poly-L-lysine or to wheat germ agglutinin was not affected by their absence. Both Mn²⁺ and Mg²⁺ were equally effective in promoting adhesion to fibronectin, laminin, and collagen types I and IV. The presence of Ca²⁺ alone was 70–90% as effective as Mn²⁺ in supporting adhesion to fibronectin and laminin; however, Ca²⁺ alone could not meet the divalent cation needs of RASM cells adhering to collagen I and IV.

RASM cell attachment to laminin, fibronectin, and collagen I and IV was studied in the presence of a hexapeptide, GRGDSP, derived from the cell binding domain of fibronectin, and a control hexapeptide, GRGESP, with glutamic acid substituted for arginine. When RASM cells were exposed to 1 mg/ml soluble GRGDSP peptide, cell adhesion to fibronectin-coated wells was decreased by 27±7% (mean±SD; p<0.05, paired t test, n=6). The GRGESP peptide had no detectable effect on cell binding to fibronectin. Neither peptide had a noticeable effect on RASM cell attachment to laminin or collagen type I or IV.

A 1% concentration of the anti-hamster β₁ serum inhibited cell adhesion to fibronectin and to collagen I and IV by greater than 90% and inhibited adhesion to laminin by 71% (Figure 3). The same concentration of normal goat serum did not inhibit adhesion. The effects of anti-hamster β₁ were concentration dependent. The inhibition produced by anti-hamster β₁ was specific for ECM-derived substrates because attachment to poly-L-lysine was not affected by similar concentrations of the antiserum.

Immunofluorescent Staining with Anti-Human β₁ Serum

Using anti-human β₁ antibody, we performed immunofluorescence staining of cells adhering to fibronectin, laminin, and collagens I and IV (Figure 4). Intense staining was localized in linear deposits at the marginal edge of the cells on all four protein
substrates. This pattern of staining was most distinct on the cells adhering to fibronectin. These condensations of β1 receptor complexes colocalized with sites of vinculin positive adhesion plaques.

**Immunoprecipitation of Proteins Recognized by Anti-Hamster β1 Serum**

Analysis of the whole-cell detergent extracts of RASM cells by SDS-PAGE under nonreducing conditions identified numerous radiiodinated membrane proteins (Figure 5, lane 1). When the whole-cell extracts were immunoprecipitated with anti-hamster β1, we found a limited series of major, closely migrating proteins with apparent molecular masses centered at 120, 140, 165, and 185 kDa (nonreduced) (Figure 5, lane 2). Two minor bands were seen at 83 and 97 kDa. When the immunoprecipitated whole-cell extracts were examined under reducing conditions (Figure 5, lane 4), the major 120-kDa band exhibited decreased mobility, and the 120- and 140-kDa bands appeared to merge into a diffuse band centered at 140 kDa. There was also decreased mobility under reducing condition of the major bands at 165 kDa (to 175 kDa) and 185 kDa (to 200 kDa). In immunodepletion experiments, anti-serum to the cytoplasmic domain of the integrin β1

**Figure 2.** Rat aortic smooth muscle cell adhesion requirements for divalent cations. Cells labeled with 5-[125I]iodo-2'-deoxyuridine were suspended in divalent cation-free medium and plated into wells precoated with bovine serum albumin (BSA) (1 mg/ml), fibronectin (FN) (8.8 μg/ml), laminin (LN) (20 μg/ml), type I collagen (I) (5 μg/ml), type IV collagen (IV) (0.5 μg/ml), poly-L-lysine (PLL) (10 μg/ml), or wheat germ agglutinin (WGA) (10 μg/ml). CaCl2, MgCl2, and MnSO4 or MnCl2 were added to the wells to make the final concentrations shown in the figure. Cells were allowed to attach to the wells for 15 minutes at 37°C. Values represent the mean percentage (±SD) of the radioactivity that adhered to the well in triplicate wells. The percentage of cells adhering to BSA-coated wells (in 1 mM CaCl2 and 1 mM MgCl2 solutions) was 0±0%. This experiment was repeated twice and produced the same findings.

**Figure 3.** Effect of anti-hamster β1 serum on rat aortic smooth muscle cell attachment to extracellular matrix proteins. Cells were labeled with 5-[125I]iodo-2'-deoxyuridine and plated into wells precoated with bovine serum albumin (BSA) (1 mg/ml), fibronectin (FN) (8.8 μg/ml), laminin (LN) (20 μg/ml), type I collagen (I) (0.8 μg/ml), type IV collagen (IV) (0.5 μg/ml), or poly-L-lysine (PLL) (10 μg/ml). The assay was performed in the presence of control medium, goat anti-hamster β1 serum (diluted 1:100, 1:200, 1:400, 1:800), and normal goat serum (diluted 1:100). Cells were allowed to attach to the wells for 15 minutes at 37°C. The percentage of cells adhering to BSA-coated wells was 1±0%. Values represent the mean percentage (±SD) of the radioactivity that adhered to the well in triplicate wells. This experiment was repeated three times and produced the same findings.
subunit recognized the same protein bands as the anti-hamster β1 serum (data not shown).

When RASM cells were labeled with [3H]glucosamine, the anti-hamster β1 serum specifically recognized a major complex of polypeptides with molecular masses of 120, 140–160, and 185 kDa under nonreducing conditions (data not shown). In addition, there were minor bands at 97, 200, and 220 kDa. The 165-kDa band observed on the surface-iodinated cells was not detected in the metabolically labeled cells. Under reducing conditions, the 120- and 140–160-kDa complex appeared to migrate as a single diffuse band of 140 kDa; the 185-kDa band migrated at 195 kDa, and the 200–220-kDa band migrated at 240 kDa. The [3H]glucosamine-labeled cell extracts were also subjected to immunoprecipitation with antibodies to the human fibronectin receptor, anti-human β1 antiserum. The recovered radiolabeled proteins comigrated with those immunoprecipitated with the anti-hamster β1 antiserum under both nonreducing and reducing conditions; however, the minor bands at 200 and 220 kDa (nonreduced conditions) were not observed with the anti-human β1 antiserum (not shown).

**Figure 4.** Localization of β1 integrin receptors in focal adhesion plaques. Rat aortic smooth muscle cells were permitted to adhere to coverslips coated with fibronectin (panels A and B), laminin (panels C and D), collagen IV (panels E and F), or collagen I (panels G and H) as described in "Materials and Methods." At the time of fixation, the cells were in various stages of spreading and migration. The cell shapes were not unique to any of the coating substrates. After the samples were fixed and permeabilized, they were stained with antibodies to vinculin (panels A, C, E, and G) and human β1 (panels B, D, F, and H). Arrows point to representative adhesion plaques identified by antibodies to human β1.

**Figure 5.** Immunoprecipitation of solubilized whole-cell extracts with anti-hamster β1 serum and affinity chromatography on fibronectin-Sepharose columns. Cells were surface labeled, solubilized in octyl β-D-glucopyranoside, and immunoprecipitated with anti-hamster β1 serum. Samples were analyzed under nonreducing (lanes 1 and 2) or reducing conditions (lanes 3 and 4) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7%). Immunoprecipitates from the whole-cell extracts are shown in lanes 2 and 4. The cell extracts also were applied to the fibronectin-Sepharose column and successively eluted with wash buffer A (see "Materials and Methods"), 0.2 M NaCl buffer, GRGDSP (1 mg/ml), and 10 mM EDTA buffer. Column fractions (2 ml) were examined under nonreducing (lanes 5 and 6) conditions. Material eluted with GRGDSP: lane 5. Material eluted with EDTA (lane 6) was subjected to immunoprecipitation with antiserum to the α5 subunit (lanes 7 and 8) and with anti-hamster β1 serum (lanes 9 and 10). The immune complexes were examined under nonreducing (lanes 7 and 9) and reducing conditions (lanes 8 and 10). After treating a sample of the EDTA eluate at 100°C in the presence of 1 M/100 ml SDS, the sample was subjected to immunoprecipitation with anti-hamster β1 serum (lane 11), antiserum to the β1 cytoplasmic subunit (lane 12), and antiserum to the α5 cytoplasmic subunit (lane 13) and examined under nonreducing conditions (lanes 11–13). Molecular mass markers in kilodaltons are shown on the left of each gel.
Identification of Fibronectin-Binding Proteins

125I-labeled proteins eluted from a fibronectin affinity column were identified by SDS-PAGE and autoradiography (Figure 5). No labeled proteins were eluted from the fibronectin column by the 0.2 M NaCl buffer wash (data not shown). Bound material was eluted with the RGD-containing hexapeptide (Figure 5, lane 5). After separation under nonreducing conditions, two broad polypeptide bands of 120 and 150 kDa were detected. These bands were immunoprecipitated by the anti-hamster β1 serum (data not shown) and corresponded to the 120- and 140–160-kDa bands in the anti-hamster β1 and anti-human β1 immunoprecipitates from the whole-cell extracts (compare with Figure 5, lane 2). Subsequent washing with EDTA buffer eluted a similar dimeric protein complex (Figure 5, lane 6) that had the same apparent molecular mass as the complex eluted with GRGDSP and was immunoprecipitated with antisemur to the cytoplasmic domain of the α5 subunit (Figure 5, lanes 7 and 8) and with anti-hamster β1 serum (Figure 5, lanes 9 and 10). After reduction with 2-mercaptoethanol, the apparent molecular mass of the two subunits shifted so that they comigrated at 140 kDa (Figure 5, lanes 8 and 10), as did the corresponding bands on immunoprecipitation of the whole-cell extract (see Figure 5, lane 4). If the dimeric protein complex, eluted with EDTA, was treated at 100°C in the presence of 1 g/100 ml SDS to disrupt noncovalent associations, then anti-hamster β1 serum (Figure 5, lane 11) and antiserum to the cytoplasmic domain of the β subunit (Figure 5, lane 12) precipitated only the 120-kDa band (nonreduced); antiserum to the cytoplasmic domain of α5 precipitated only the 150-kDa band (nonreduced) (Figure 5, lane 13).

No additional labeled proteins could be eluted with 1 M NaCl buffer.

Identification of Type IV Collagen-Binding Proteins

GRGDSP eluted a polypeptide from the type IV collagen-Sepharose column that had an apparent molecular mass of 58 kDa (nonreduced) and 84 kDa (reduced). Similarly, 0.2 M NaCl buffer eluted a broad band with apparent molecular mass of 58 kDa (nonreduced). Neither the 58-kDa polypeptide released by GRGDSP nor the material eluted with 0.2 M NaCl buffer was immunoprecipitated by anti-hamster β1 serum. In addition, when 125I-labeled proteins from microcarrier beads alone (without any cells) were loaded onto type IV collagen-Sepharose columns and eluted with 0.2 M NaCl buffer, the same bands were observed (58 kDa, nonreduced, and 84 kDa, reduced; data not shown).

When the type IV collagen-Sepharose column was eluted with EDTA, two polypeptides were recovered with apparent molecular mass (nonreduced) of 120 and 185 kDa (Figure 6, lane 1). Upon reduction, these peptides migrated at 135 and 200 kDa (Figure 6, lane 3). The set of proteins eluted with EDTA was recognized by the anti-hamster β1 serum (Figure 6, lane 2) and appeared to correspond to the 120- and 185-kDa bands seen on the immunoprecipitation of the whole-cell extract (Figure 5, lane 2). The high affinity binding of the 120/185-kDa proteins to the type IV collagen-Sepharose column required the presence of Mn2+ and was not detected in buffers.

Figure 6. Affinity chromatography of detergent-solubilized rat aortic smooth muscle cells on type IV (lanes 1–3) and type I (lanes 4–6) collagen-Sepharose columns and laminin-Sepharose columns (lanes 7–16). Proteins eluted from the type IV collagen-Sepharose column with 10 mM EDTA buffer were immunoprecipitated with anti-hamster β1 serum (lane 2, nonreduced). The original sample was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7%) under nonreducing (lane 1) and reducing (lane 3) conditions. Proteins eluted from the type I collagen-Sepharose column with 10 mM EDTA buffer were immunoprecipitated with anti-hamster β1 serum. The original sample was examined under nonreducing conditions (lane 4), and the immune complex (lanes 5 and 6) was examined under nonreducing (lane 5) and reducing (lane 6) conditions. Lanes 7–16: Proteins eluted from the laminin-Sepharose columns with 10 mM EDTA buffer. Aliquots from the first four 2-ml fractions from the EDTA buffer elution were analyzed under nonreducing (lanes 7–10) and reducing (lanes 11–14) conditions. EDTA eluates in lanes 11 and 12 were pooled (lane 15, reduced) and immunoprecipitated with anti-hamster β1 serum (lane 16, reduced). Molecular mass markers (in kilodaltons) are shown on the left of each gel.
containing Ca\(^{2+}\) without the presence of Mg\(^{2+}\) or Mn\(^{2+}\) (data not shown).

**Identification of Type I Collagen-Binding Proteins**

When cell extracts were loaded onto a type I collagen-Sepharose affinity column, a different elution pattern was obtained. Both GRGDSP and the 0.2 M NaCl buffer failed to elute any radiolabeled material (data not shown). Three bands were eluted from the type I collagen-Sepharose column with EDTA (Figure 6, lanes 4 and 5): two major bands at 185 and 120 kDa and a minor band at 150 kDa. On reduction, the 185- and 120-kDa bands shifted to 200 and 135 kDa, respectively. The band at 150 kDa shifted to 160 kDa on reduction (Figure 6, lane 6). All three bands eluted with EDTA were recognized by the anti-hamster \(\beta_1\) serum (Figure 6, lane 5). EDTA effectively removed all of the polypeptides that specifically bound to the collagen type I–Sepharose column because no further material in this region could be eluted with buffer containing 1 M NaCl (not shown). The polypeptides that bound to collagen type I–Sepharose were specific for native collagen because they bound only minimally (if at all) to gelatin-Sepharose columns.

To demonstrate that the 185/120-kDa material in the EDTA eluate from the collagen type I–Sepharose was the same complex as found in the EDTA eluate from the type IV collagen–Sepharose column, we first passed the whole-cell extract over a type I collagen–Sepharose column and then passed the void and wash volume from the type I–Sepharose column over a type IV collagen–Sepharose column. After the whole-cell extract was passed over the collagen type I column, there was no 185/120-kDa (EDTA-eluted) set of proteins binding to the type IV collagen–Sepharose column; this was consistent with the removal of the 185/120-kDa material by the type I collagen column (not shown).

**Identification of Laminin-Binding Proteins**

Application of 0.2 M NaCl buffer to a laminin-Sepharose affinity column released a diverse group of polypeptides with apparent molecular masses less than 90 kDa. The major band observed had a nonreduced mobility of 58 kDa, which shifted to 84 kDa on reduction (data not shown). These proteins were also observed when the extracts of microcarrier beads, without cells, were passed over laminin-Sepharose columns and eluted with 0.2 M NaCl buffer. GRGDSP released a negligible amount of labeled polypeptides (data not shown), which had the same electrophoretic mobility as the material released by 0.2 M NaCl buffer. None of the low molecular mass peptides released by GRGDSP or 0.2 M NaCl buffer was immunoprecipitated by anti-hamster \(\beta_1\) serum.

When the column was eluted with EDTA, at least five diffuse polypeptide bands were eluted: (nonreduced conditions) 49, 56, a broad band of 120, 185, and 210 kDa (Figure 6, lane 7). On reduction, the electrophoretic mobilities shifted to 57, 60, 100, 110, a major band at 135, 200, and 240 kDa (Figure 6, lane 11). When the EDTA eluate from the laminin column was immunoprecipitated with either anti-hamster \(\beta_1\), anti-human \(\beta_1\), or anti-\(\beta_1\) cyt sera and run on SDS-PAGE under reducing conditions, the sharp band at 240 kDa and the minor bands at 57 and 100 kDa (Figure 6, lane 16) were not precipitated. The major band at 135 kDa (reduced) as well as the bands at 60, 110, and 200 kDa (reduced) were precipitated by the antisera (Figure 6, lane 16, data not shown for anti-human \(\beta_1\) or anti-\(\beta_1\) cyt sera). When the EDTA eluate from the laminin column was run on a two-dimensional PAGE gel (first dimension nonreduced; second dimension reduced), we found that a sharp 125-kDa band (nonreduced), which comigrated with the broad 120-kDa band, corresponded to the 110-kDa band reduced (not shown); similarly, the 50–60-kDa bands (nonreduced) corresponded to the bands around 60 kDa (reduced). The bands at 120 and 185 kDa (nonreduced) migrated at 135 and 200 kDa (reduced), respectively.

EDTA removed the polypeptides from the laminin-Sepharose column with different efficiencies. The 185-kDa band (nonreduced) was eluted in the earlier EDTA fractions (Figure 6, lanes 7 and 11), whereas the minor 125/110-kDa (nonreduced/reduced) band was eluted in subsequent EDTA fractions (Figure 6, lanes 12–14).

**Discussion**

The RASM cells attached avidly to the ECM proteins laminin, fibronectin, and collagen types I and IV (but not denatured collagen), as well as to non-ECM substrates (poly-l-lysine and wheat germ agglutinin) (Figure 1). Differences in adherence between the substrates may represent differences in the affinities of the cells for the substrates or differences in the binding of the substrates to the assay wells.43 Several features of RASM cell attachment to ECM proteins were compatible with its mediation by integrinlike receptors of the \(\beta_1\) family: their requirement for divalent cations and their inhibition by the anti-hamster \(\beta_1\) serum. The presence of divalent cations was necessary for RASM cell attachment to the ECM proteins, but not to the non-ECM substrates (Figure 2). Although Ca\(^{2+}\) alone would permit cell attachment to laminin and fibronectin, it would not support attachment to collagen types I and IV. As was observed in other cell lines,11,44,45 there was greater cell attachment in the presence of Mn\(^{2+}\) and Mg\(^{2+}\) than Ca\(^{2+}\) (Figure 3). These results are compatible with the observation that the known integrin receptors require divalent cations for binding to their ligands.11,12,46

The specific peptide sequence RGD found in many ECM proteins has been implicated as a recognition sequence for fibronectin,16,42 laminin,47,48 and collagen.49 Although mammalian-RGD–directed receptors are typically among the integrin superfamily,10 non-integrinlike binding proteins that appear to bind to the RGD sequence also have been found.49 The soluble RGD hexapeptide GRGDSP did inhibit
RASM cell attachment to fibronectin; however, RASM cell adhesion to fibronectin was inhibited by only 27%. In addition, high concentrations of the hexapeptide GRGDSP were required to inhibit cell attachment. This has been observed by others. In general, cell surface receptors have only a moderate affinity for fibronectin and other adhesive proteins (approximately $10^{-6}$ M).\textsuperscript{50} When the size of the cell-binding fragment has been reduced to the size of the GRGDSP region, its affinity for the cell surface is decreased significantly.\textsuperscript{50} The synthetic RGD peptide did not have a noticeable effect on adhesion to laminin or collagen types I and IV. Our data are consistent with the hypothesis that other receptors, not dependent on RGD-like sequences, may play a role in cell anchorage\textsuperscript{12,51,52} or that sequences outside of the minimum RGD recognition site may play important roles in the efficient binding of RASM cells to ECM proteins.\textsuperscript{50,53} Before any conclusion is drawn about the lack of specificity of the RGD sequence in mediating cell attachment, however, a more extensive panel of peptides should be tried.\textsuperscript{16,49}

Anti-hamster $\beta_1$ serum interfered with RASM cell adhesion to fibronectin, laminin, and collagen types I and IV. The anti-hamster $\beta_1$ serum is a polyclonal antibody that probably binds to several sites on the $\beta_1$ subunit. Although the exact antigenic determinants are not known, presumably the receptor site (or sites) is included in the antigenic site(s), or its conformation is changed when the antibody binds to the $\beta_1$ subunit. It is unlikely that antibody-induced cytotoxicity was involved because prior studies with several different rodent cell lines have shown that the inhibitory effects of anti-hamster $\beta_1$ serum are completely reversible.\textsuperscript{28,29,30,33} In addition, in the presence of antiserum, the RASM cells were able to attach to the non-ECM substrate poly-L-lysine (Figure 3).

To identify adhesion receptors for fibronectin, collagen types I and IV, and laminin, we looked at the $^3$H-labeled cell surface proteins that both bound to the respective affinity columns (with dependence on divalent cations) and were recognized by the anti-hamster $\beta_1$ serum. Several groups have examined the effectiveness of moderate salt concentrations in eluting the low-affinity receptors\textsuperscript{50} that bind to the ECM proteins.\textsuperscript{20,24,49,54,56} We found that when integrinlike cell surface proteins were bound to substrate affinity columns in the presence of $\text{Mn}^{2+}$, they could not be eluted with 0.2 M $\text{NaCl}$ alone. Other, low molecular weight proteins that were not recognized by anti-hamster $\beta_1$ serum were frequently eluted from the columns with 0.2 M $\text{NaCl}$. However, these low molecular weight proteins were probably derived from proteins on the microcarrier beads or serum proteins trapped within the beads during the iodination. To test whether additional surface proteins on RASM cells might bind to the specific ECM-affinity column in a divalent cation-independent manner, we washed the affinity columns with EDTA buffer and followed this with 1 M $\text{NaCl}$. No new proteins were detected in the 1 M $\text{NaCl}$ eluates.

Specificity of binding to the ECM ligand affinity columns was tested using resin coupled with BSA. None of the polypeptides recognized by the anti-hamster $\beta_1$ serum bound to the BSA column; in contrast, they could be selectively and completely removed from the solubilized cell extracts by passing them over a series of laminin, fibronectin, and collagen type I and IV affinity columns.

Both the GRGDSP peptide and EDTA eluted a broad protein dimer complex from the fibronectin affinity column that was recognized by anti-hamster $\beta_1$ serum and had a molecular mass of 120 and 150 kDa (nonreducing conditions). In our hands, EDTA was much more effective than GRGDSP (1 mg/ml) in eluting the protein from the column (not shown). This may have been due to our use of $\text{Mn}^{2+}$, rather than $\text{Ca}^{2+}$ or $\text{Mg}^{2+}$, as the divalent cation in our solutions.\textsuperscript{31} This protein complex had strong similarities to the classical fibronectin receptor ($\alpha_\text{V}\beta_3$).\textsuperscript{19,35,57} When the complex was dissociated by SDS, antiserum to the cytoplasmic domains of the $\beta_1$ and $\alpha_\text{V}$ subunits precipitated the 120- and 150-kDa bands, respectively. On reduction, the 120-kDa band of the fibronectin binding complex demonstrated a disulfide-dependent upward shift in molecular mass; this is typical of the $\beta$ subunit of the integrin class of cell surface receptors\textsuperscript{28} and suggests the presence of multiple intramolecular disulfide-rich domains in this protein band. The fibronectin receptor complex isolated from RASM cells bound only to fibronectin. This behavior is similar to that of the $\alpha_\text{V}\beta_3$ receptor and differentiates the RASM cell fibronectin receptor from promiscuous receptors like the $\alpha_\text{V}\beta_1$ receptor, which also binds to fibronectin but binds to laminin and collagen types I and IV as well.\textsuperscript{20,21}

A heterodimer complex (185/120 kDa), recognized by anti-hamster $\beta_1$ serum, was eluted from the type IV collagen affinity column with EDTA. This integrinlike receptor bound to collagen in the presence of $\text{Mn}^{2+}$ but not in the presence of $\text{Ca}^{2+}$. The effect of $\text{Ca}^{2+}$ and $\text{Mn}^{2+}$ on the protein binding to the affinity column parallels the ability of these divalent cations to alter RASM cell adhesion to immobilized collagen type IV (Figure 2). This 185/120-kDa complex appears to be a promiscuous receptor because it could also bind to collagen type I as well as laminin (Figure 6). On reduction, the 120-kDa $\beta_1$ chain increased its apparent molecular mass to 135 kDa (as it did in the fibronectin receptor); the 185-kDa $\alpha$ band increased to 200 kDa. By analogy, this 185-kDa protein has approximately the same SDS-PAGE mobilities in both reducing and nonreducing conditions as the $\alpha$ subunit of the VLA-1 ($\alpha_\text{V}\beta_3$) heterodimer that has been characterized in human cell lines.\textsuperscript{25} The VLA-1 integrin receptor recently has been found to bind to both type IV and type I collagen in human melanoma cells.\textsuperscript{23}

Neither the VLA-1 collagen receptor in melanoma cells nor the 185/120-kDa collagen/laminin receptor complex from rat cells could be eluted from the collagen/laminin affinity columns with the amino acid
sequence RGD (see “Results” and Figure 6).23,24 This indicates that the 185/120-kDa VLA-1-like integrin interacts specifically with collagen and laminin and that a unique recognition signal other than RGD may be involved.

A unique set of radiolabeled proteins was observed to bind to collagen type I affinity columns. In addition to the 185/120-kDa integrin complex that bound to collagen IV and to laminin, there was also a second integrin heterodimer complex (150/120 kDa) that only bound to type I collagen and could be eluted with EDTA. This 185/150/120-kDa complex of receptors that bind to collagen type I was observed to bind efficiently to native collagen but poorly to gelatin. This correlates with the poor adhesion of the cells to gelatin substrates. The RASM cell 150-kDa protein has approximately the same SDS-PAGE mobility pattern under both reducing and nonreducing conditions as the α subunit of the αβ integrin heterodimer that has been characterized in human cell lines.25 In the past, the αβ integrin receptor has been found to bind to both collagen type I and type IV.20,21,23,58,59 We found that this αβ integrin preferentially bound to collagen type I.

The 185/120- and 150/120-kDa integrin αβ heterodimers described in the present work differ from other collagen binding proteins: Wayner and Carter20 and, more recently, Gehlsen et al60,61 have shown that another β integrin, αβ, can mediate human cell adhesion to collagen and laminin as well as to fibronectin. Although the RASM cell 185/120-kDa complex may mimic the αβ integrin receptor in its ability to bind to collagen and laminin, neither its pattern of electrophoretic migration on reduction nor its inability to bind to fibronectin are consistent with the αβ integrin receptor. Dedhar et al69 identified three surface proteins (M, 250, 70, and 30 kDa) on human osteosarcoma cells that might mediate cell adhesion to type I collagen; these collagen-binding proteins could be inhibited by a specific Gly-Arg-Gly-Asp-Thr-Pro (GRGDTP) peptide, but were not related to the integrins. Gullberg et al62 found a glycoprotein in rat hepatocytes that mediated adhesion to collagen type I but not to type IV; however, the protein complex they identified had an M of 115/105 kDa under nonreducing conditions and 130/115 kDa under reducing conditions, which differed from those reported here.

RASM cells appear to have several proteins that bind to laminin. The anti-hamster β1 serum inhibited most of the cell binding to laminin, which suggests that the major cell receptors for laminin are integrinlike proteins or that β1 integrins are essential for mediating cell adhesion. Several integrinlike proteins of the β1 subfamily have been shown to act as potential laminin receptors in other cell lines. Cell substrate attachment antigen, located on avian cells, is reported to bind to laminin as well as to fibronectin and collagen.63 In mammalian cells, the αβ integrin binds to laminin as well as to fibronectin and collagen types I and IV.20,21,25,60,61 Sonnenberg et al22 have found that αβ, which is present in a variety of human and rodent cell types, appears to mediate adhesion to laminin. Rat B50 cells use an αβ integrin family that binds to laminin.

The major protein band that elutes with EDTA from the laminin-Sepharose column is the 120-kDa β1 subunit. The 185/120-kDa αβ integrin complex that binds to collagen I and IV also binds to laminin. Recently, Ignatius and Reichardt24 have identified a 200/120-kDa laminin receptor in rat B50 cells that also appears to behave like an αβ dimer. This “laminin receptor” from rat B50 cells also binds to collagen type I and therefore may be the same receptor as the 185/120-kDa complex reported here (M.J. Ignatius, L.M. Goetzl, L.F. Reichardt, personal communication, 1988). We have found that in the RASM cells, the αβ integrin receptor binds much more efficiently to collagen than it does to laminin (unpublished observations). In human cells, αβ binds preferentially to collagens23 but does exhibit some affinity for laminin (R.I. Clyman, unpublished results, 1989). A sharp protein band of 210 kDa (nonreduced) was also consistently eluted from the laminin column. This band did not immunoprecipitate with either the anti-hamster β1, the anti-human β1, or the anti-β cyt sera. The failure to precipitate this band may mean that this putative α subunit is only loosely associated with the β1 subunit so that the complex dissociated during immunoprecipitation24,65,66 or that it is another, non-β integrin protein similar to those described by Kleinman et al55 or Smalheiser and Schwartz.56

Two other bands were weakly recognized by the anti-hamster β1 and anti-human β1 sera (56 and 125 kDa, nonreduced conditions, or 60 and 110 kDa, reduced conditions). These proteins appear to be similar to those seen on human melanoma cells,64 but additional studies will be needed to characterize these bands. Although the 60-kDa (reduced) protein has a similar electrophoretic pattern when run under nonreducing conditions as the nonintegrin, 68-kDa monomer,54,67 its ability to be immunoprecipitated by the anti-hamster β1 serum makes it unlikely that they are the same protein.30 Although we do not believe that there is a 68-kDa laminin binding protein on RASM cells, we have not tried to elute the columns or block cell adhesion on laminin with the pentapeptide Tyr-Ile-Gly-Ser-Arg.67

ECM receptors on smooth muscle cells play an important role in anchoring the cells during vasoconstriction. The expression of several integrin-related receptor complexes specific for different ECM components would also provide a means for regulating the smooth muscle cell migration that occurs after injury to the arterial wall. We have identified a series of β1 integrinlike complexes on RASM cells that may be responsible, in part, for the cellular interactions with fibronectin, laminin, and collagen types I and IV. These receptors appear to be similar to members...
of the β1-integrin family of ECM receptors. Our conclusions are based on the recognition of these glycoproteins by several antibodies to the β1 subunit, by their substrate affinities, and by their electrophoretic mobilities on SDS-PAGE. This series of receptor complexes shares a common 120-kDa (nonreduced) β1-subunit protein. There is a 185-kDa (nonreduced) α subunit that is promiscuous and involved in binding to collagen types I and IV as well as to laminin. Interestingly, this has strong parallels to the α5 subunit in its mobility and relative affinity for laminin and collagen types I and IV. In these smooth muscle cells, the 185-kDa (nonreduced) α subunit appears to bind to collagen much more efficiently than it does to laminin. In addition, there is a 150-kDa (nonreduced) α subunit that binds exclusively to collagen type I and a 150-kDa (nonreduced) α subunit that binds exclusively to fibronectin. We have also identified two proteins that are associated with the β1 subunit and bind only to laminin. By immunofluorescent staining, the β1 integrins were localized in linear deposits at the marginal edge of the cells on all four of the substrates tested. The clustering of these receptors in focal adhesion plaques (demonstrated by the costaining of these sites with antibodies to vinculin) suggests that these receptors are important in cell adhesion to the different substrates. Further studies will reveal what role these different glycoprotein receptor complexes have in the adhesion process.

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Integrin receptors on aortic smooth muscle cells mediate adhesion to fibronectin, laminin, and collagen.

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