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Sodium Dodecyl Sulfate-Stable Complexes of Echistatin and RGD-Dependent Integrins: A Novel Approach to Study Integrins

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ABSTRACT

This study shows that disintegrins, echistatin as a model, can be used as a radiolabeled probe to simultaneously detect the presence of individual RGD-dependent integrins on cardiac fibroblasts. Binding of ^{125}I-echistatin to fibroblasts was proportional to cell number, time dependent, reversible, saturable, specific, and membrane bound. SDS-polyacrylamide gel electrophoresis and autoradiograms revealed that ^{125}I-echistatin was associated with three radioactive protein bands of 180, 210, and 220 kDa that were identified by RGD affinity chromatography, immunoblotting, and immunoneutralization as $\alpha_v\beta_3$, $\alpha_3\beta_1/\alpha_5\beta_1/\alpha_v\beta_1$, and $\alpha_8\beta_1$ heterodimeric integrins, respectively. These results suggest that echistatin binds to RGD-dependent integrins, forming SDS-stable complexes in the absence of chemical cross-linkers, reducing conditions and heating. As assessed by radioligand-binding filtration, disintegrins displayed binding characteristics with an IC_{50} ranging from 0.044 to 1.1 nM, but with slope factors lower than 1, indicating the presence of several binding sites. Resolved by SDS-polyacrylamide gel electrophoresis to reveal echistatin-integrin complexes, disintegrins and RGD peptides displayed different binding affinities to individual RGD-dependent integrins present on cardiac fibroblasts. Elegantin and flavostatin demonstrated the highest affinity toward integrins, whereas flavoridin and acPen-RGDC had a greater specificity toward $\alpha_v\beta_3$-integrin. In summary, echistatin forms SDS-stable complexes with RGD-dependent integrins. This model offers a novel way to visualize RGD-dependent integrins, to investigate their activation state, and to determine the integrin specificity of RGD peptides.

Integrins are heterodimeric proteins consisting of 1 α- and 1 β-subunit. More than 16 α- and 8 β-subunits have been identified that can form more than 20 functional combinations. Integrins are cell surface receptors permitting cell-cell and cell-extracellular matrix (ECM) interactions. These receptors thus contribute to the state of cells through their adhesion to ECM and to cellular dynamic changes such as mobility, growth, and proliferation (for reviews see Meredith et al., 1996; Giancotti, 1997). Integrins are subdivided into different families based on their structural composition, their expression in specific cell types, or their affinity toward certain groups of ECM proteins. Among them, several integrins, namely, $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, and, under special conditions, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_2\beta_1$, and $\alpha_1\beta_1$, have been documented to bind through the RGD motif present in proteins such as fibronectin, fibrinogen, von Willbrand factor, vitronectin, osteopontin, and others (Ruoslahti, 1997).

Whereas the functional importance of integrins in cellular growth has been well established, there are few means of investigating their presence and functionality on the cell surface. Snake venom contains toxins among which are peptides with the potential to inhibit platelet aggregation by interacting and inhibiting $\alpha_{IIb}\beta_3$-integrin. Therefore, they have been termed disintegrins (Gould et al., 1990). All disintegrins possess in their central core an RGD motif framed by disulfide bridges and conserved sequences surrounding this motif. Disintegrins have been used in radioligand-binding assays to evaluate the density of $\alpha_{IIb}\beta_3$-integrin on platelets or $\alpha_v\beta_3$ on vascular smooth muscle cells (McLane et al., 1994; Marcinkiewicz et al., 1996; Kumar et al., 1997). However, this type of assay cannot discriminate between different RGD-dependent integrins if several of them are present on the cell surface. Although disintegrins have the potential to interact with all RGD-dependent integrins, their use as probes to study cell surface integrins has not been extensively exploited. The present study demonstrates that ^{125}I-echistatin, through the formation of SDS-stable disintegrin-

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ABBREVIATIONS: ECM, extracellular matrix; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis.

integrin complexes, can be an effective pharmacological tool to evaluate the presence of individual RGD-dependent integrins on the surface of cardiac fibroblasts.

Experimental Procedures

Materials. Echistatin, GRGDSP, GRGDTP, acPenRGDC, cycloRGDSPA, cycloRGDdFV, GRGDdSP, and GRGESP were purchased from Bachem California, Inc. (Torrance, CA). Elegantin, flavoridin, and flavostatin were purified from *Trimeresurus elegans* and *Trimeresurus flavoviridis* snake venom, bought from Miami Serpentarium (Miami, FL). Purification was performed by Bio-Gel P-30 and by reverse-phase high-performance liquid chromatography (Scarborough et al., 1993; Maruyama et al., 1997) with purity and concentration being assessed by amino acid analysis and sequencing. Antibodies against rat integrins were generously provided by Dr. R.O. Hynes (Howard Hughes Medical Institute, Cambridge, MA; anti-α_3, no. 8-4; anti-β_1, no. 130), by Dr. Lynn M. Schnapp (Department of Medicine, Mount Sinai School of Medicine, NY; anti-α_8), by Dr. Lou Reichardt (Howard Hughes Medical Institute, University of California, San Francisco, CA; anti-α_8, no. 2415), or were bought from Chemicon International, Inc. (Temecula, CA; anti-α_1, AB1934; anti-α_2, AB1936; anti-α_5, AB1928; anti-α_v, AB1930; anti-α_4, MAB1396 and MAB1383; anti-β_5, AB1926) or from Pharmingen Canada (Mississauga, ON, Canada; anti-β_3, F11; anti-β_1, Ha2/5). Horseradish peroxidase-coupled anti-mouse or anti-rabbit Ig antibody was from Bio-Rad Laboratories (Hercules, CA). Streptavidin-horseradish peroxidase and Hybond ECL were from Amersham Canada, Ltd. (Oakville, ON). X-OMAT AR5 films were from Eastman Kodak Co. (Rochester, NY).

Cardiac Fibroblasts. Fibroblasts were obtained by collagenase digestion of cardiac ventricles from 200- to 250-g Sprague-Dawley rats as described previously (Fareh et al., 1997). Digested cells were plated in plastic dishes in Dulbecco's modified Eagle's medium supplemented with 0.1% bovine serum albumin and 10% fetal bovine serum. The cells were grown for 7 to 10 days after seeding and used at confluency. Only primary cultures were used. Characterization of cultured cells with histological markers indicated that more than 95% of them were indeed fibroblasts.

Iodination of Echistatin and Radioligand-Binding Filtration Assay. Echistatin was iodinated by the lactoperoxidase method with 15 μg of echistatin in the presence of 1 mCi of $Na^{125}I$. The monoiodinated product was purified by reverse-phase high-performance liquid chromatography on a C_4 Vydac column (The Separations Group, Hesperia, CA) with a CH_3CN gradient in 0.1% trifluoroacetic acid. Between 8 and 12 $\times$ 10^8 cpm were usually collected with a specific activity of 800 to 1200 cpm/fmol.

Radioligand-binding filtration assay was performed in duplicate. Fibroblasts were prepared by digestion with a 0.05% trypsin-EDTA solution, counted, and diluted in 0.05 M HEPES, pH 7.4. Alternatively, cells were scraped with a plastic policeman and passed 10 times through a 21 gauge needle. Twenty thousand to 40,000 fibroblasts (unless otherwise specified) were incubated for 90 min at room temperature in a total volume of 250 μl in 0.05 M HEPES, pH 7.4, containing 5 mM $MnCl_2$ in the presence of 250,000 cpm of ^{125}I-echistatin and increasing concentrations (10^{-13} to 10^{-5} M) of peptides. Nonspecific binding was determined by the addition of 10 mM EDTA. Incubation was terminated by rapid filtration on no. 34 glass fiber paper (Schleicher & Schuell, Keene, NH) and washing three times with 3 ml of 0.05 M Tris-HCl, pH 7.4, and 0.154 M NaCl on a 30-well cell harvester (Brandel, Gaithersburg, MD). Filters were presoaked for 1 h in washing buffer containing 5% dry skim milk (Carnation, Nestlé, Don Mills, ON, Canada) to reduce nonspecific adsorption. Radioactivity was counted in a gamma counter with an efficiency of 80%.

Solubilized fibroblasts were obtained by the addition of 0.1 ml/cm^2 of 0.05 M HEPES, pH 7.4, 1% Nonidet P-40 (NP-40), 1 mM $CaCl_2$, and 1 mM $MgCl_2$. After the sample stood on ice for 10 to 20 min, material was collected and centrifuged at 15,000 rpm for 3 min. Proteins were measured by the Bradford assay (Bio-Rad Laboratories). Five to 15 μg of proteins were incubated in a final volume of 25 μl in 0.05 M HEPES, pH 7.4, containing 5 mM $MnCl_2$ in the presence of 25,000 cpm of ^{125}I-echistatin, and increasing concentrations (10^{-13} to 10^{-5} M) of peptides. After 90 min of incubation at room temperature, SDS sample buffer (containing 0.188 M Tris-HCl, pH 6.8, 30% glycerol, 6% SDS, and 0.15% bromphenol blue) was diluted 10-fold in the incubation mixture, and proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). A crude membrane fraction of fibroblasts was prepared by scraping the cells, homogenization with a Polytron (2 $\times$ 20 s), and centrifugation at 30,000*g* for 20 min. The supernatant was kept and the pellet resuspended in 0.05 M HEPES buffer, pH 7.4.

RGD Affinity Chromatography. A GRGDSP affinity column was prepared by coupling 25 mg of GRGDSP to 2.5 ml of Affi-Gel 10 matrix (Bio-Rad Laboratories) according to the manufacturer's instructions. Proteins (2 mg) of NP-40-solubilized fibroblasts were incubated for 2 h with the gel. After the sample settled in a 10-ml column, the gel was washed with 20 ml of 0.05 M HEPES, pH 7.4, 5 mM $MnCl_2$, and 0.1% NP-40. Proteins were eluted with 10 ml of 0.05 M HEPES, pH 7.4, 10 mM EDTA, and 0.1% NP-40. Fractions (1 ml) were collected and subsequently concentrated in Centricon-30 (Amicon, Bedford, MA). Each fraction was either analyzed for ^{125}I-echistatin binding as described previously or boiled and subjected to a 7.5% gel SDS-PAGE for the immunoblotting analysis of integrin subunits.

SDS-PAGE and Immunoblotting. Proteins samples were separated by SDS-PAGE according to the method of Laemmli (1970) in a Mini-Protean II cell system (Bio-Rad Laboratories). Prestained calibration protein standards were from Life Technologies (Burlington, ON, Canada; catalog no. 10748-010). Electrophoresis was run until the red protein marker (~60–65 kDa) reached the end of the gel. Proteins were then stained with Coomassie R-250. Dried gels were subjected to autoradiography for 1 to 3 h on X-OMAT AR5 film. Radioactive bands were quantitated by a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA) or by gamma counting after cutting out. In the case of immunoblotting, immediately after SDS-PAGE, proteins were transferred to Hybond ECL (Amersham Canada Ltd.) in Tris-glycine buffer containing 20% methanol. The nitrocellulose membrane was dried and exposed to a X-OMAT film. After exposure, the membrane was recovered and used for immunoblotting. It was saturated for 60 min in blotting buffer (0.05 M $NaPO_4$, pH 7.4, 0.154 M NaCl, 0.05% Tween 20, 0.1% polyvinylpyrrolidone 40) supplemented with 1% polyvinylpyrrolidone 40. The membrane was then incubated for 90 min in the presence of appropriate diluted antibodies. After the membrane was washed, antibody binding was visualized either by anti-mouse (or anti-rabbit) Ig antibody coupled to peroxidase or by streptavidine-peroxidase conjugate, depending on the primary antibody. Peroxidase activity was revealed by chemiluminescence with ECL (Amersham Canada Ltd.).

Immunoneutralization. Solubilized fibroblast proteins (0.5 μg) were incubated for 2 h at room temperature in the presence of 2 μl of integrin antiserum in a volume of 18 μl in 0.05 M HEPES, pH 7.4, 5 mM $MnCl_2$, and 1% NP-40. ^{125}I-Echistatin (25,000 cpm, 2 μl) was then added to every tube and, after a 90-min incubation, SDS sample buffer was added to obtain a final SDS concentration of 0.6%. Proteins, without heating and reducing agents, were separated on a 6% gel by SDS-PAGE. The gels were then stained, dried, and autoradiograms were obtained.

Results

Binding Properties of ^{125}I-Echistatin to Cardiac Fibroblasts. Fibroblasts in suspension were used to test whether ^{125}I-echistatin can bind to the cell surface in an RGD-dependent fashion. Initial experiments were designed

to optimize basal conditions resulting in a maximum signal. ^{125}I-Echistatin bound to fibroblasts demonstrated the presence of binding sites with several properties attributed to receptors. Specific binding was 1) proportional to cell number, 2) time dependent, reaching a plateau after 90 min of incubation, 3) partially reversible, because addition of 10^{-6} M echistatin after reaching equilibrium was able to displace the labeled tracer by 30% in 90 min, 4) saturable, and 5) RGD dependent, because peptides with an RGD motif (echistatin, cycloGRGDSPA, GRGDTP, and GRGDSP) were able to displace labeled echistatin, whereas peptides such as GRGESP with an RGE motif, or unrelated peptides such as atrial natriuretic peptide, angiotensin II, and endothelin-1, had no effect (results not shown). The saturability of fibroblasts with ^{125}I-echistatin is shown in Fig. 1. Transformation of the saturation curve by Scatchard analysis indicated that the equilibrium affinity constant of ^{125}I-echistatin binding to fibroblast integrins was 1.24 nM with a density of 160,000 sites/cell. A correlation coefficient of 0.98 suggested that ^{125}I-echistatin bound to a single site.

Formation of SDS-Stable ^{125}I-Echistatin-Protein Complexes. Although we attempted to chemically cross-link ^{125}I-echistatin to binding sites on the cell surface, the resulting complexes could not be clearly resolved by SDS-PAGE (results not shown). However, we observed that ^{125}I-echistatin, once bound to fibroblast proteins, formed complexes resistant to 0.6% SDS in the absence of heating. Figure 2a shows that incubation of ^{125}I-echistatin with fibroblasts resulted in the appearance of three radioactive bands (designated f1, f2, and f3). These bands were SDS-resistant unless the samples were heated at high temperatures or a reducing agent (β-mercaptoethanol) was added. The sensitivity to reduction was not unexpected because echistatin and integrins possess several disulfide bridges. These radioactive bands, compared with protein standards, have molecular masses of 220, 210, and 180 kDa. Their molecular masses are compatible with the binding of ^{125}I-echistatin to heterodimeric integrins rather than to their α or β-subunits individually. The molecular masses of α- and β-subunits of RGD-dependent integrins, under nonreduced conditions, range from 80 to 180 kDa (Hemler, 1990; Schnapp et al., 1995).

Formation of SDS-stable complexes required divalent cations: 5 mM Mn^{2+} gave an optimal signal when compared with Mg^{2+} or Ca^{2+} (Fig. 2b). Consequently, all experiments were performed in the presence of 5 mM Mn^{2+}. After the complexes reached equilibrium, 10 mM EDTA completely disrupted them, and 10^{-6} M echistatin displaced the radioligand in a time-dependent manner (results not shown).

We next examined whether binding level was dependent on the method used to harvest the cells and extract protein (results not shown). Cells were either collected by trypsin digestion or by scraping and dispersion through a 21 gauge needle. Cell proteins were solubilized with either 0.6% SDS or 1% NP-40. Trypsin digestion resulted in lower binding, probably because trypsin destroyed the proteins implicated in the bands. Cell scraping and dispersion improved the signal obtained by both filtration and SDS-PAGE. When compared to filtration, the SDS-PAGE signal was about 2-fold higher, indicating that the glass fiber filter may not retain all insoluble material of interest, unless ^{125}I-echistatin is bound to soluble cytosolic proteins. Lysis of dispersed or monolayered cells and protein membrane solubilization with a nonionic detergent, NP-40, resulted in the highest signal, even in the presence of SDS. However, initial solubilization of proteins with a buffer containing only 0.6% SDS as detergent considerably reduced (by 2-fold) the intensity and quality of the signal. Our results demonstrated that these proteins can withstand solubilization with a mild detergent without loss of their binding properties. Initial solubilization with SDS

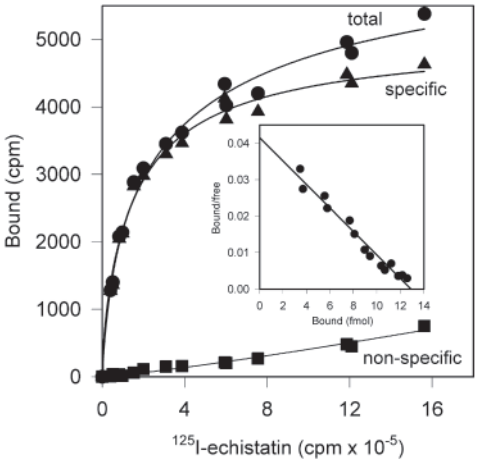


Fig. 1. Saturation analysis of ^{125}I-echistatin binding to cardiac fibroblasts in suspension as assessed by filtration. Twenty thousand cells were incubated for 90 min in the presence of increasing amounts of ^{125}I-echistatin. Nonspecific binding was determined in the presence of 10 mM EDTA. After incubation, samples were filtered and washed through glass fiber paper. The inset shows the Scatchard transformation of specific binding data.

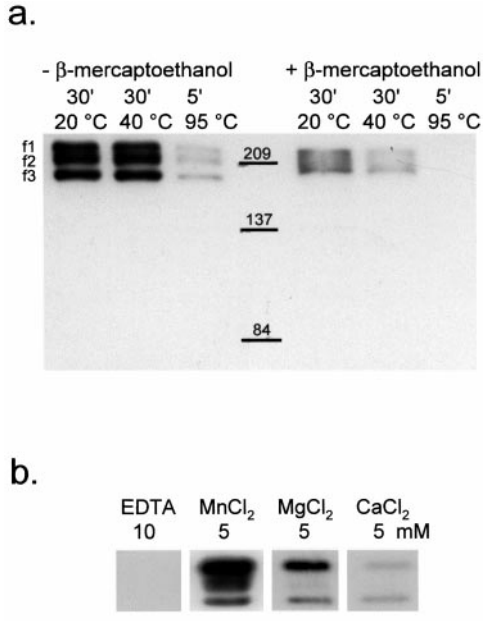


Fig. 2. a, analysis of ^{125}I-echistatin binding to cardiac fibroblasts by SDS-PAGE. Fifteen μg of protein from NP-40-solubilized cardiac fibroblasts were incubated with 12.5×10^6 cpm/ml ^{125}I-echistatin for 90 min. After incubation, SDS sample buffer (with or without 0.7 M β-mercaptoethanol) was added to each sample and heated as indicated. Samples were then separated on 8% SDS-PAGE, stained, dried and exposed to X-ray film. b, cation dependence of ^{125}I-echistatin binding. Ten μg of NP-40-solubilized fibroblasts were incubated for 90 min with 12.5×10^6 cpm/ml ^{125}I-echistatin in the presence of either EDTA, $MnCl_2$, $MgCl_2$ or $CaCl_2$. After incubation, proteins were separated on 6% SDS-PAGE and an autoradiogram was obtained.

seemed to considerably reduce their stability. However, when ^{125}I-echistatin was bound first to integrins, it had a protective effect against dissociation by SDS.

To determine whether the binding signal on cardiac fibroblasts was associated specifically with the cell membrane, a crude membrane fraction was prepared. ^{125}I-Echistatin binding to the crude membrane fraction was 12-fold higher than to the corresponding cytosolic fraction (3567 ± 128 versus 284 ± 96 cpm/μg protein), suggesting that these proteins were membrane associated.

Identification of the Components of SDS-Stable Complexes as Integrins. RGD peptides like echistatin have been documented to bind to a subfamily of integrins. To verify the identity of these proteins as RGD-dependent integrins, the following strategy was used. RGD-dependent integrins were purified on a GRGDSP affinity column. and the EDTA-eluted fractions were analyzed for ^{125}I-echistatin binding and for integrin subunit immunoreactivity. As illustrated in Fig. 3, the three radioactive protein bands were still observed in the eluted fractions when incubated with ^{125}I-echistatin, although EDTA caused a differential elution of these bands. This elution pattern probably reflects the affinity of the proteins for GRGDSP peptide. Immunoblotting of eluted fractions, which were heated before separation by SDS-PAGE, revealed the presence of β_1-, α_5-, α_3-, α_v-, and α_8-subunits of expected molecular mass under nonreducing conditions. The α_1-subunit, which may form $\alpha_1\beta_1$-integrin, a non-RGD-dependent integrin that binds collagen, could not be detected in the eluted fractions, although its presence in fibroblast extract was well detected as a 190-kDa band. The anti-β_3 antibody (F11) failed to recognize the 90-kDa denatured β_3-subunit and bound only the native β_3-heterodimer (see also Fig. 4b). These results suggest that ^{125}I-echistatin bind only to RGD-dependent integrins.

We attempted to identify the f1, f2, and f3 bands by combining autoradiography and immunoblotting on the same nitrocellulose sheet. NP-40-solubilized fibroblast proteins were incubated with ^{125}I-echistatin, separated by SDS-PAGE, and transferred to nitrocellulose paper. The sheet was then exposed to X-ray film to obtain an autoradiogram and subsequently incubated with a specific anti-integrin antibody detected by chemiluminescence to examine the immunoreactivity of the radioactive bands. By superposing the autoradiogram and the film of the immunoblot obtained after exposure to chemiluminescence, it was possible to identify the radioactive proteins (Fig. 4). To confirm the specificity of the immunoreaction, some fibroblast extracts were incubated with increasing quantities of ^{125}I-echistatin (Fig. 4, a, c, and g), different fibroblast extracts were used (Fig. 4, b and e) or analyzed in duplicate (Fig. 4, d and f). With an anti-β_1 antibody (no. 130), only the f1 and f2 bands were positive (Fig. 4a). Similar results were obtained with the anti-β_1 antibody Ha2/5 (results not shown). An anti-β_3 antibody (F11) revealed that the f3 band contained a β_3-integrin (Fig. 4b). The f2 band showed immunostaining with an anti-α_3 antibody (no. 8-4; Fig. 4c) as well as with an anti-α_5 antibody (MAB1928; Fig. 4d). An anti-α_8 antibody recognized the f1 band (Fig. 4f). Finally, the f3 band was positive with an anti-α_v antibody (MAB1930), and the f2 band also showed some cross-reactivity (Fig. 4e). Anti-α1 antiserum (AB1934) recognized some protein bands at molecular masses larger than 180 kDa, but their intensity was constant whatever the amount of ^{125}I-echistatin in the extract, suggesting that this subunit was not present in the radioactive bands. Other antibodies, against rat α_2-, α_4-, or β_5-subunits, failed to recognize any radioactive bands and subunits.

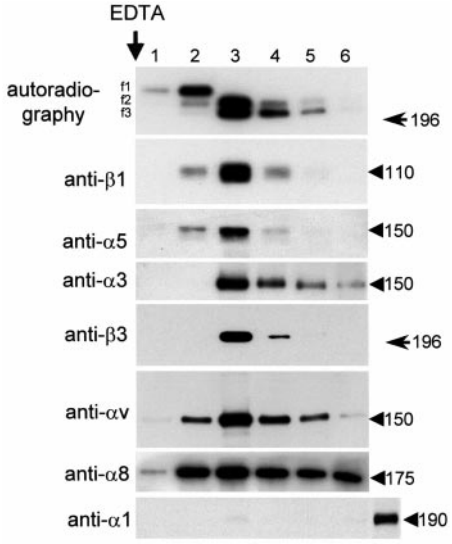


Fig. 3. Analysis of ^{125}I-echistatin binding and identification of integrin subunits following affinity chromatography purification of integrins. NP-40-solubilized fibroblasts were applied to a GRGDSP affinity matrix, and proteins were eluted with 10 mM EDTA. Samples from each EDTA-eluted fraction were analyzed for ^{125}I-echistatin binding (autoradiography) or were heated in SDS sample buffer and western blotted with anti-integrin subunit antibodies. Arrow heads indicate the position of integrin subunits, and arrows the protein standard. At the right end of the anti-α1 lane a fibroblast extract is shown as a positive control.

Because there was some uncertainty in identifying f2 and f3 as positive for α_5-, α_3-, or α_v-subunits, samples were fractionated by two-dimensional SDS-PAGE (Fig. 5). A fibroblast extract was first separated on 6% SDS-PAGE under mild conditions (no heating and no β-mercaptoethanol), and the f1, f2, and f3 bands were excised by gel slicing (0.5 mm). These gel slices were then heated at 100°C for 5 min to dissociate integrins into their subunits and reapplied in individual wells for second-dimension 7.5% SDS-PAGE in the absence of reducing agents. The proteins were blotted onto nitrocellulose and incubated with antisera. Positive bands, with a molecular mass of ~150 kDa, were detected in the total fibroblast extract (as a positive control) and for the f2 band, compatible with the presence of nonreduced α_3-, α_5-, and α_v-subunits. The f3 band was also positive for the α_v-subunit. These data demonstrate that the α_3-, α_5-, and α_v-subunits are effectively present in the f2 band.

To further confirm the identity of integrins, we attempted to perform immunoneutralization experiments (Fig. 6). We reasoned that preincubation of specific anti-subunit antibody with fibroblast membrane proteins, before the addition of ^{125}I-echistatin, should be able either to prevent the binding of ^{125}I-echistatin to integrin, resulting in the disappearance of bands, or to cause a shift (supershift) in the mobility of ^{125}I-echistatin-integrin complexes. Fibroblast extracts were thus first incubated with antibody and then with ^{125}I-echistatin. Because the samples were not heated before SDS-PAGE, antibodies against β_1-, α_5-, and α_8-subunits shifted to a higher molecular mass the f1 and f2, the f2, and f1 bands, respectively. Other antibodies (β_3, α_v, and α_3) prevented par-

tially or totally the formation of the f3 and f2 bands, respectively.

The results from immunoblotting, immunoneutralization. and RGD affinity purification thus indicate that the f1 band corresponds to $\alpha_8\beta_1$- integrin, f3 corresponds to $\alpha_v\beta_3$-integrin, and f2 appears to be heterogeneous, consisting of $\alpha_3\beta_1$-, $\alpha_5\beta_1$-, and $\alpha_v\beta_1$-integrins. The molecular masses of the f1 and f3 bands (220 and 180 kDa) are compatible with the formation of disintegrin-integrin complexes. Indeed, the calculated $\alpha_8\beta_1$- and $\alpha_v\beta_3$-integrin molecular masses are 290 and 240 kDa, respectively, based on the reported nonreduced molecular mass of each human subunit (β_1, 110 kDa; β_3, 90 kDa; α_v, 150 kDa, and α_8, 180 kDa; Hemler, 1990; Schnapp et al., 1995) and of echistatin (5.4 kDa). The α_3-, α_5-, and α_v-subunits, possessing similar molecular masses (α_3, 150 kDa; α_5, 155 kDa, and α_v 150 kDa) and associated with the β_1-subunit, would migrate as one band with an intermediate molecular mass between f1 and f3: the f2 band probably corresponds to $\alpha_3\beta_1$-, $\alpha_5\beta_1$-, and $\alpha_v\beta_1$-integrins. The difference between the apparent and calculated molecular masses may be attributed to the close association between α- and β-subunits, resulting in a more compact protein than the sum of their subunits, to slight differences between the molecular masses of rat integrin subunits and their human counterparts, and, finally, to the lack of precision in assessing by SDS-PAGE the molecular mass of proteins larger than 200 kDa.

Analysis of the Binding Properties of RGD Peptides to Fibroblast RGD-Dependent Integrins. To evaluate whether or not other RGD-containing disintegrins and peptides are potential ligands of fibroblast RGD-dependent integrins, we examined, by radioligand-binding filtration assay, the ability of four different disintegrins (echistatin, elegantin, flavostatin, and flavoridin) and of three synthetic RGD peptides (acPenRGDC, cycloRGDdFV, and GRGDdSP) to compete with ^{125}I-echistatin from fibroblasts. As shown in Fig. 7, disintegrins were about 1000 times more potent than synthetic RGD peptides in displacing ^{125}I-echistatin, because the most potent RGD peptide (acPenRGDC) presented an IC_{50} of 1280 nM, whereas disintegrins have IC_{50} values ranging from 0.044 to 1.1 nM. Analysis of the competition curves demonstrated that the slope factor of each curve, with the exception of echistatin (slope factor of 0.93), was below unity, suggesting that these peptides may interact with different

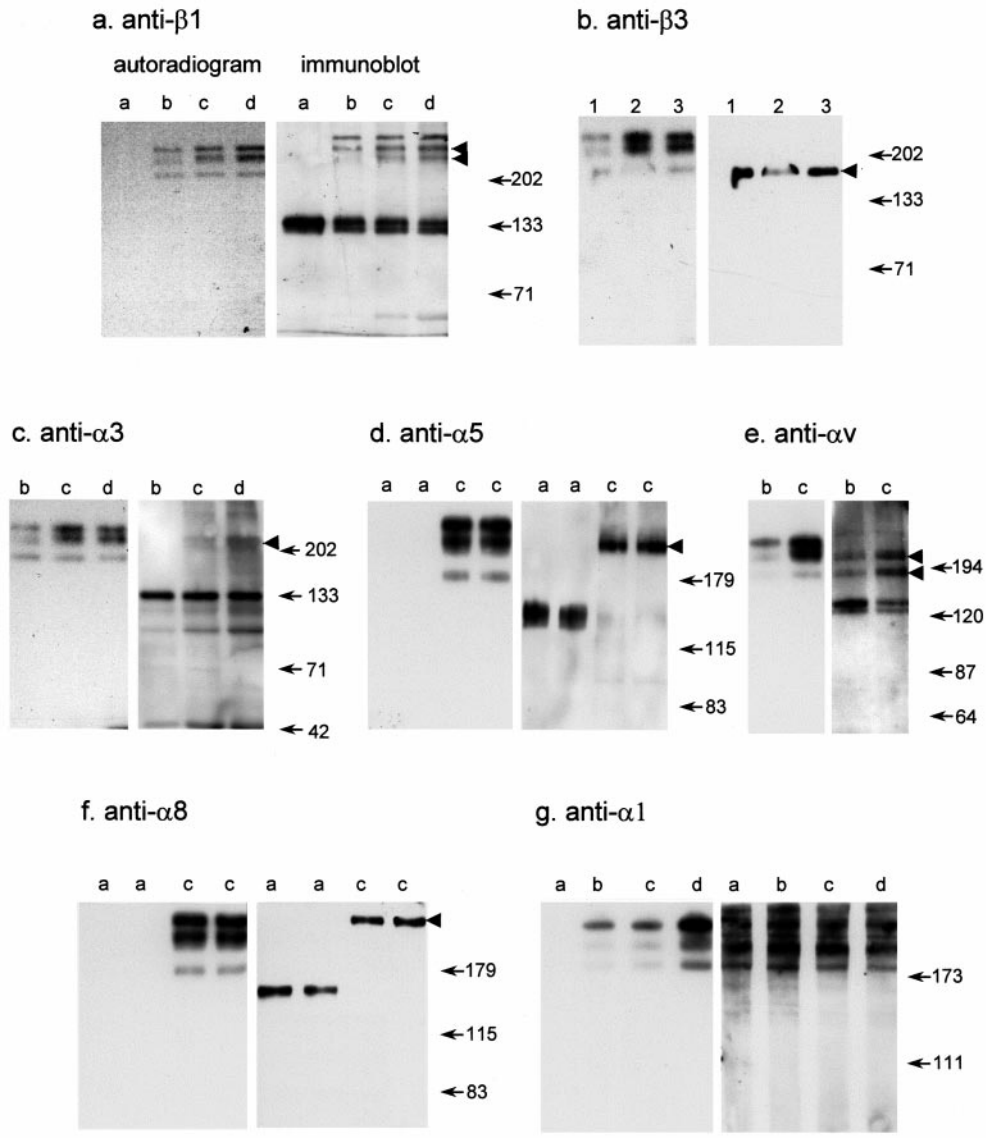


Fig. 4. Identification of ^{125}I-echistatin-integrin complexes by western blotting. NP-40-solubilized fibroblasts (15 μg) were incubated with increasing concentrations of ^{125}I-echistatin (1.5 (a), 3 (b), 6.2 (c), 12.5 (d) x 10^6 cpm/ml, as in a, c, e and g). Alternatively, different NP-40-solubilized fibroblasts (1, 2 and 3) (as in b) or samples in duplicate (as in d and f) were used and incubated with 12.5 $\times$ 10^6 cpm/ml ^{125}I-echistatin. Samples were separated on 6% SDS-PAGE and transferred to nitrocellulose, from which autoradiograms were obtained (on the left panels), and incubated with the antibody at the appropriate dilution. Horseradish-coupled second antibodies were revealed by chemiluminescence (right panels).

affinities to more than one site. Consequently, competition curves were also analyzed by SDS-PAGE, and each band was quantitated by PhosphorImager (Fig. 8 and Table 1). As suggested by the previous analysis, RGD peptides behave differently with each integrin, the strongest interaction being with $\alpha_v\beta_3$, although the affinities ranged from 10 to 50,000 nM. GRGDdSP had a very low affinity toward $\alpha_8\beta_1$ and a higher affinity for $\alpha_v\beta_3$. AcPenRGDC had a stronger interaction with $\alpha_v\beta_3$ than cycloRGDdFV. Among the disintegrins, elegantin and flavostatin had the highest affinities toward fibroblast integrins, and flavoridin showed a greater selectivity toward $\alpha_v\beta_3$ than for other integrins. As expected from Scatchard analysis (Fig. 1) and from the competition curve (Fig. 7), echistatin presented similar affinities for all integrins.

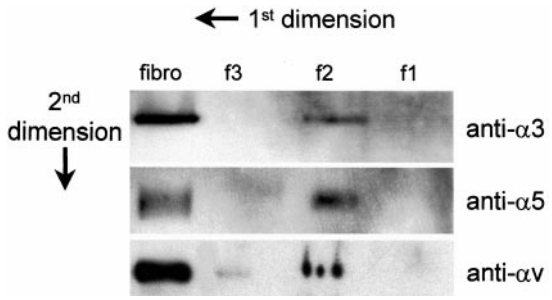


Fig. 5. Analysis of α_3, α_5 and α_v-subunit immunoreactivity by 2-dimensional SDS-PAGE. NP-40-solubilized fibroblasts, incubated with ^{125}I-echistatin, were separated on 6% SDS-PAGE. Three different gel lanes were frozen, cut in 0.5 mm slices and counted in a gamma counter. Slices corresponding to each band were pooled, heated at 100°C, and reapplied to 7.5% SDS-PAGE. After separation and transfer, the blots were incubated with specific antisera. The molecular mass of the immunoreactive bands was ≈150 kDa.

Discussion

Because they possess an RGD sequence, disintegrins mimic the binding motif of some ECM matrix proteins and can thus interact with several integrins for which this RGD sequence motif is a key binding element. ^{125}I-Echistatin has been used to evaluate the affinity of activated $\alpha_{IIb}\beta_3$-integrin on platelets by conventional radioligand experiments (McLane et al., 1994). More recently, it has been recognized that radioiodinated echistatin can interact with $\alpha_v\beta_3$- and $\alpha_5\beta_1$-integrins (McLane et al., 1994, 1998; Pfaff et al., 1994; Marcinkiewicz et al., 1996). In the present study, the binding of ^{125}I-echistatin to rat cardiac fibroblasts was investigated as a tool to detect the presence of individual RGD-dependent integrins. By radioligand-binding assay, it was observed that ^{125}I-echistatin binding presented all the characteristics of a ligand to a receptor, including specificity, reversibility, high affinity (in the nanomolar range), and limited density (saturability). It was also evident that RGD peptides displaced ^{125}I-echistatin from more than one site with relatively similar affinities, because the competition curves could not be resolved into their individual components, suggesting the presence of several integrins. SDS-PAGE analysis of unheated samples in the absence of reducing agents resulted in the detection of three distinct radioactive bands.

Based on the cross-reactivity of these bands with selective anti-α- and β-subunit antibodies, their dependence on divalent cations, in particular Mn^{2+}, their displacement by RGD peptides, and their molecular masses, it is suggested that

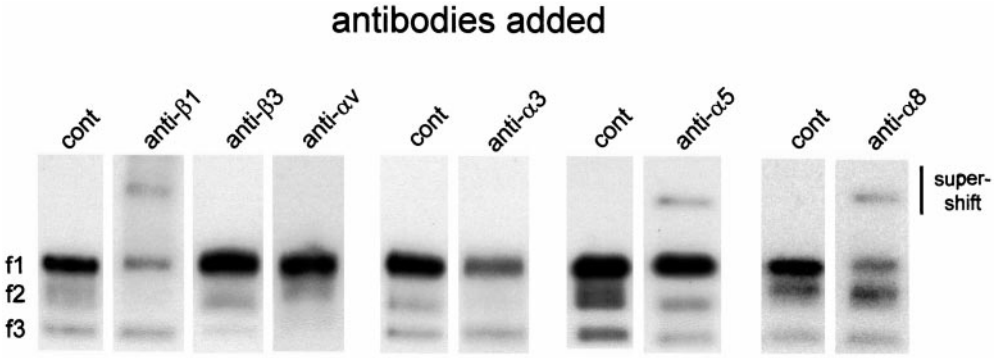
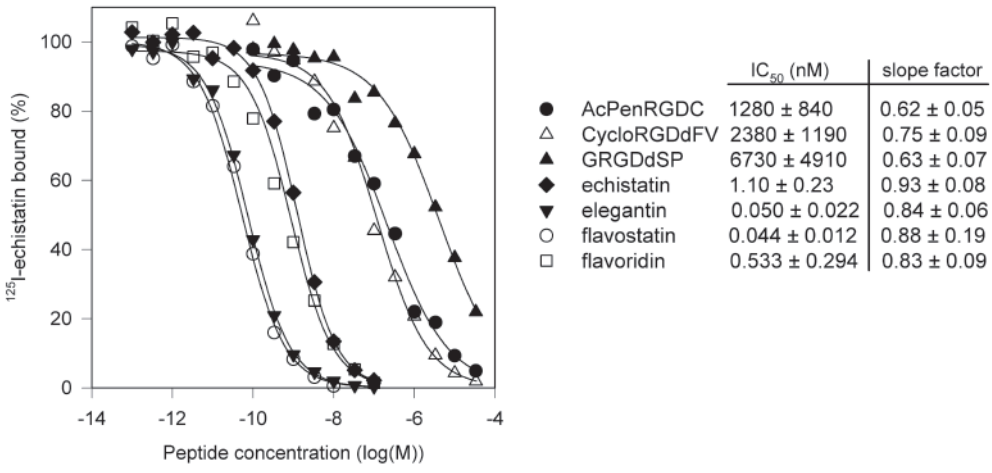


Fig. 6. NP-40-solubilized fibroblasts (0.5 μg) were first incubated with 2 μl of antiserum for 120 min, after which 2 μl of ^{125}I-echistatin (25,000 cpm) was added for another 90 min. Proteins were separated on a 6% gel SDS-PAGE and autoradiograms were obtained. Binding of the antibodies to integrins can either block ^{125}I-echistatin binding or, under mild SDS-PAGE conditions, cause a shift of the ^{125}I-echistatin-echistatin complexes to a higher molecular mass (supershift). cont stands for control.



	IC$_{50}$ (nM)	slope factor
● AcPenRGDC	1280 ± 840	0.62 ± 0.05
△ CycloRGDdFV	2380 ± 1190	0.75 ± 0.09
▲ GRGDdSP	6730 ± 4910	0.63 ± 0.07
◆ echistatin	1.10 ± 0.23	0.93 ± 0.08
▼ elegantin	0.050 ± 0.022	0.84 ± 0.06
○ flavostatin	0.044 ± 0.012	0.88 ± 0.19
□ flavoridin	0.533 ± 0.294	0.83 ± 0.09

Fig. 7. Analysis of RGD peptide affinities by radioligand-binding filtration assay. Fibroblasts in suspension (25,000) were incubated with 12.5 × 10^6 cpm/ml ^{125}I-echistatin for 90 min in the presence of increasing concentrations of disintegrins and RGD peptides. The reaction was stopped by filtration through glass fiber paper and washing. Competition curves were fitted according to Hill analysis. Slope factors below 1 indicated that the ligand may bind with different affinities to more than 1 site. Data are the means ± S. D. of 3 different experiments.

^{125}I-echistatin binds to $\alpha_8\beta_1$, $\alpha_5\beta_1$, $\alpha_3\beta_1$, $\alpha_v\beta_1$, and $\alpha_v\beta_3$ and forms a complex, in the presence of Mn^{2+}, with each of these integrins resistant to SDS under mild conditions. Therefore, ^{125}I-echistatin-integrin complexes can allow identification of individual RGD-dependent integrins. Because of the presence of Mn^{2+}, these complexes may not reflect the actual binding state of integrins in vivo. The present experiments also show that ^{125}I-echistatin does not bind $\alpha_1\beta_1$-integrin, a non-RGD-dependent integrin, but they do not rule out that ^{125}I-echistatin failed to recognize some RGD-dependent integrins or that some ^{125}I-echistatin-RGD-dependent integrin complexes were unstable in SDS. Further experiments with cell types harboring other RGD-dependent integrins are required to verify these possibilities.

The presence of these integrins, as revealed by SDS-PAGE, on cardiac fibroblasts is in partial agreement with the literature. Indeed, the presence of $\alpha_3\beta_1$-, $\alpha_5\beta_1$-, and $\alpha_v\beta_3$-integrins has already been repoted (Gullberg et al., 1990; Wilke et al., 1996; MacKenna et al., 1998). The finding of $\alpha_8\beta_1$-integrin on cardiac fibroblasts is novel and unexpected. Schnapp et al. (1995) could not detect its presence by immunohistochemistry in the rat cardiac ventricle, but found it on lung alveolar myofibroblasts. Whether or not culturing of cardiac fibroblasts amplifies the signal or augments the expression of this integrin remains to be determined.

Using SDS-PAGE to resolve echistatin-integrin complexes, affinities of RGD-containing peptides were determined simultaneously on $\alpha_8\beta_1$- and $\alpha_v\beta_3$-integrins. However, because the f2 band consists of $\alpha_3\beta_1$-, $\alpha_5\beta_1$-, and $\alpha_v\beta_1$-integrins, affinity can hardly be resolved on these individual integrins. The results demonstrate that disintegrins bind with high affinity to RGD-dependent integrins. Elegantin and flavostatin, with an affinity lower than 1 nM, appear to be the most potent disintegrins, and interact with the same potency to $\alpha_v\beta_3$ and $\alpha_8\beta_1$. These 2 peptides possess a similar sequence around the RGD motif with only 3 amino acid differences (Table 1) (Gould et al., 1990). Flavoridin had a 10- to 20-fold lower avidity for $\alpha_8\beta_1$- and $\alpha_{3/5/v}\beta_1$-integrin mixture and a comparable affinity for $\alpha_v\beta_3$. This implies that other residues beyond the immediate vicinity of the RGD motif may influence the 3-dimensional structures of the binding site.

Potency of short RGD peptides was also analyzed in competition experiments. These peptides were selected according to their ability to interact selectively with RGD-dependent integrins. The cyclic peptide acPenRGDC preferentially binds $\alpha_{IIb}\beta_3$ (Bugosky et al., 1993), whereas cycloRGDdFV may interact more specifically with $\alpha_v\beta_3$ (Hammes et al., 1996). GRGDdSP may inhibit cell adhesion to fibronectin and platelet aggregation (Leven and Tablin, 1992). The present results demonstrate that all 3 peptides preferentially bind to $\alpha_v\beta_3$-integrin, and acPenRGDC presents the highest selectivity toward $\alpha_v\beta_3$, with a K_i of 8 nM.

The existence of SDS-stable integrin-echistatin complexes thus offers a powerful means to detect, visualize and quan-

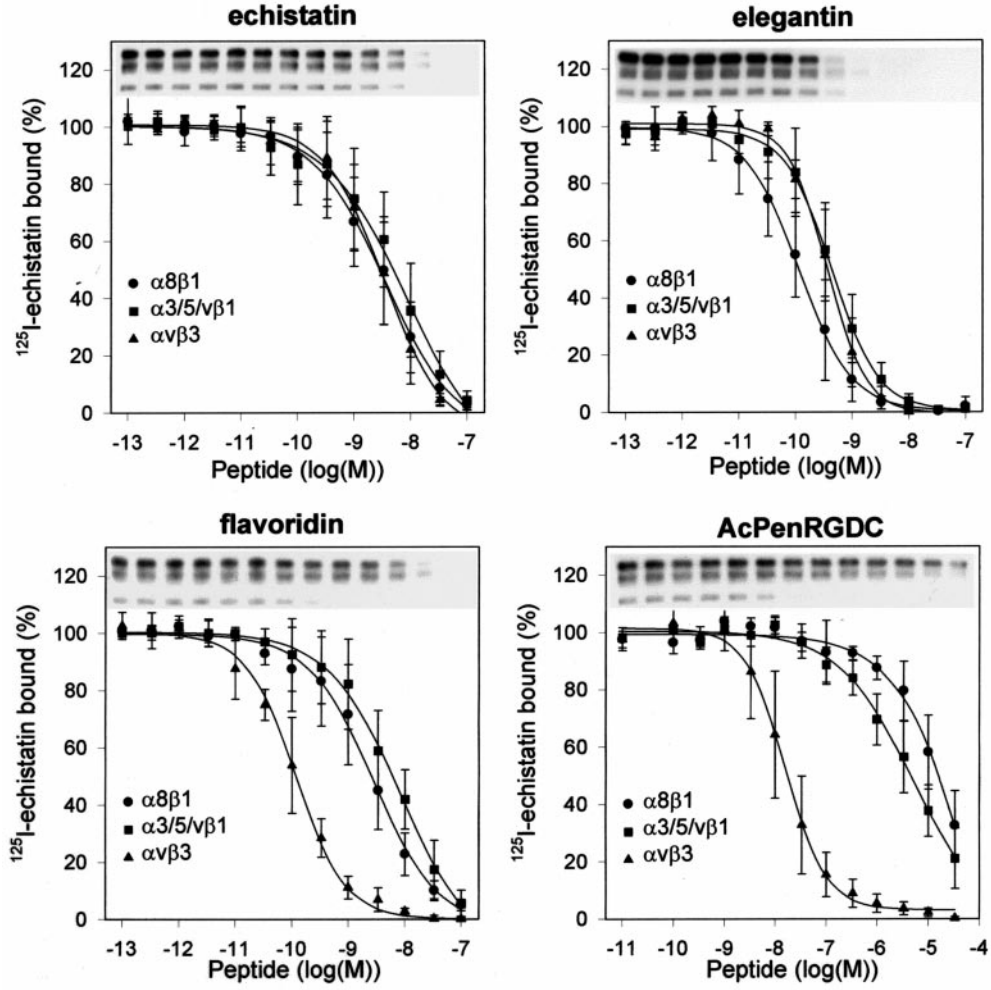


Fig. 8. Analysis of RGD peptide affinities by SDS-PAGE. NP-40-solubilized fibroblasts (15 μg) were incubated with 12.5×10^6 cpm/ml ^{125}I-echistatin for 90 min and increasing concentrations of disintegrins or RGD peptides. After separation on 6% SDS-PAGE, an autoradiogram was obtained and each radioactive band was subsequently quantitated by PhosphorImager. Each competition curve was fitted by the Hill equation, assuming the presence of a single site for each band. Only the results for echistatin, elegantin, flavoridin and acPenRGDC are illustrated. Each competition curve represents the means ± S. D. of 3 experiments. A representative autoradiogram of a competition experiment for each peptide is shown in the inset.

titate the presence of different RGD-dependent integrins on the cell surface. Radioligand-binding filtration assay allows quantitation of the total amount of RGD-dependent integrins on fibroblasts, but does not discriminate between them because echistatin binds tightly to fibroblast integrins. To date, there is no ligand reported to be specific for each of these integrins. In the absence of selective agonists or antagonists, conventional radioligand-binding assay cannot discriminate between the different RGD-dependent integrins. By using SDS-PAGE and autoradiography, the present study demonstrates, firstly, that each integrin has a high affinity state, but with a limited density, and, secondly, that some RGD-containing peptides possess greater or lesser avidity for each of these integrins. The first finding will allow examination of the regulation of expression of RGD-dependent integrins on cardiac fibroblasts and other cell types. Indeed, we also observed SDS-resistant echistatin-integrin complexes on rat cardiomyocytes, vascular smooth muscle cells, platelets, vascular tissue, human monocytes, and human skin fibroblasts (unpublished observations). Regarding the second finding, the potency of RGD peptides interacting with integrins has usually been evaluated by their efficacy in inhibiting cell adhesion or by their ability to bind to purified integrin in a solid phase assay. The alternative approach presented here offers the possibility of an assay to evaluate and to rapidly and directly compare the binding properties of RGD peptides on several integrins.

Another interesting observation relates to the signal of several integrin subunits (see Fig. 4, a, c, d, e, and f) that was detected by western blotting. The intensity of the signal related to the integrin subunits before and after addition of ^{125}I-echistatin indicated that a variable fraction of each integrin subunit appears to be involved in the formation of disintegrin-integrin complexes. This may suggest that each subunit may also be involved in the formation of other integrins, like $\alpha_1\beta_1$ and $\alpha_2\beta_1$, and therefore not recognized by echistatin. Alternatively, only a variable percentage of each integrin heterodimer may be in an active, high affinity state and able to bind echistatin. Inside-out signals have been recognized as a mechanism of activating integrins (Humphries, 1996). Disintegrins may detect changes in the affinity of activated platelet $\alpha_{IIb}\beta_3$-integrins (McLane et al., 1994). Whether or not they can be used to investigate the activation of other RGD-dependent integrins deserves further study.

In summary, the present experiment demonstrate for the first time that ^{125}I-echistatin forms SDS-stable complexes with molecular mass of 180–220 kDa when incubated with solubilized or intact fibroblast membranes. Because these complexes can be recognized by antibodies specific to integrin subunits, are divalent cation-dependent, and are displaceable by RGD peptides and disintegrins, they reflect the interaction of ^{125}I-echistatin with $\alpha_8\beta_1$-, $\alpha_5\beta_1$-, $\alpha_3\beta_1$-, $\alpha_v\beta_1$-, and $\alpha_v\beta_3$-integrins. This novel approach can be applied to identify the presence and regulation of RGD-dependent integrins on the cell surface, to investigate the potency of newly-designed RGD peptides and mimetics for interaction with RGD-dependent integrins, and to evaluate the functional state of RGD-dependent integrins.

TABLE 1

Binding affinities (K_i) of disintegrins and RGD peptides on integrins as assessed by SDS-PAGE and autoradiography

Dissociation constants were calculated by the Cheng-Prusoff equation from the EC_{50} estimated by Hill analysis assuming that ^{125}I-echistatin has a concentration of 1.25 nM and binds with an affinity of 1.24 nM (Fig. 1).

	$\alpha_8\beta_1$	$\alpha_{3/5/v}\beta_1$	$\alpha_v\beta_3$
Echistatin[a]	1.8 ± 0.5	4.2 ± 2.7	1.7 ± 0.5
Elegantin[a]	0.05 ± 0.12	0.22 ± 0.16	0.18 ± 0.21
Flavostatin[a]	0.05 ± 0.19	0.22 ± 0.46	0.18 ± 0.22
Flavoridin[a]	1.4 ± 0.6	4.2 ± 1.4	0.06 ± 0.22
acPenRGDC[b]	47 ± 136	2.6 ± 1.3	0.008 ± 0.001
cycloRGDdFV[b]	4.3 ± 0.8	4.1 ± 0.2	0.06 ± 0.01
GRGDdSP[b]	480 ± 483	270 ± 353	4.3 ± 1.9

Partial amino acid sequence (one-letter symbol) of the peptides used:

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Echistatin:.....KEGTICKRARGDDMDDYCNGK......
Elegantin:.......-KR---R-----NP--R-T-Q......
Flavostatin:.....-KR---RI----FP--R-T-L......
Flavoridin:......-KR---R-----NP--R-T--......
                       acPen---C
                       cyclo---dFV
                           G---dSP
```

[a] Results in nM.
[b] Results in μM.

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