A Cell Surface Receptor Complex for Collagen Type I Recognizes the Arg-Gly-Asp Sequence

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Abstract. To isolate collagen-binding cell surface proteins, detergent extracts of surface-iodinated MG-63 human osteosarcoma cells were chromatographed on affinity matrices of either type I collagen–Sepharose or Sepharose carrying a collagen-like triple-helical peptide. The peptide was designed to be triple helical and to contain the sequence Arg-Gly-Asp, which has been implicated as the cell attachment site of fibronectin, vitronectin, fibrinogen, and von Willebrand factor, and is also present in type I collagen. Three radioactive polypeptides having apparent molecular masses of 250 kD, 70 kD, and 30 kD were distinguishable in that they showed affinity toward the collagen and collagen-like peptide affinity columns, and could be specifically eluted from these columns with a solution of an Arg-Gly-Asp–containing peptide, Gly-Arg-Gly-Asp-Thr-Pro. These collagen-binding polypeptides associated with phosphatidylcholine liposomes, and the resulting liposomes bound specifically to type I collagen or the collagen-like peptide but not to fibronectin or vitronectin or heat-denatured collagen. The binding of these liposomes to type I collagen could be inhibited with the peptide Gly-Arg-Gly-Asp-Thr-Pro and with EDTA, but not with a variant peptide Gly-Arg-Gly-Glu-Ser-Pro. We conclude from these data that these three polypeptides are membrane molecules that behave as a cell surface receptor (or receptor complex) for type I collagen by interacting with it through the Arg-Gly-Asp tripeptide adhesion signal. The lack of binding to denatured collagen suggests that the conformation of the Arg-Gly-Asp sequence is important in the recognition of collagen by the receptor complex.

COLLAGEN is a major component of the extracellular matrix and can influence cell proliferation (Adamson, 1983; Gay et al., 1974; Liotta et al., 1978; Kleinman et al., 1981), differentiation (Hauschka and Konigsberg, 1966; Kosher and Church, 1975; Reddi and Anderson, 1976; Bunge and Bunge, 1978), migration (Bard and Hay, 1975; Couchman et al., 1982; Rovasio et al., 1983), and specific gene expression (Lee et al., 1984, 1985). Furthermore, type I collagen has an important function in mesenchymal–epithelial interactions during organogenesis (Hay, 1981), and appears to play a role in not only establishing the mechanical stability of the circulatory system, but also in mediating complex cell interactions involved in hematopoiesis (Lohler et al., 1984).

The direct interaction of the cell with collagen is required for collagen to exert these effects on cell behavior (Lash et al., 1977; Rubin et al., 1984). Early studies showed that fibronectin binds to collagen (Engvall and Ruoslahti, 1977; Engvall et al., 1982) and can mediate the attachment of cells to collagen (Klebe, 1974; Pearlstein, 1976; Vaheri and Mosher, 1978). However, a considerable amount of evidence suggests that various types of cells are also capable of attaching directly to native collagen (Schor and Court, 1979; Rubin et al., 1981; Briles and Haskew, 1982; Mollenhauer and von der Mark, 1983; Lesot et al., 1985; Pierschbacher et al., 1985), as well as to isolated collagen α chains (Rubin et al., 1978). These data, therefore, suggest the existence of specific cell surface receptors for collagen. Membrane proteins that bind various types of collagen have been described for a variety of cell types (Ocklind et al., 1980; Chiang and Kang, 1982; Mollenhauer and von der Mark, 1983; Koda and Bernfield, 1984; Kurkinen et al., 1984; Rubin et al., 1984; Saito et al., 1986).

The amino acid sequence Arg-Gly-Asp has been shown recently to play an important role in the adhesion of cells not only to fibronectin (Pierschbacher and Ruoslahti, 1984*a*), but also to vitronectin (Hayman et al., 1983, 1985; Silnutzer and Barnes, 1985; Suzuki et al., 1985). It has also been shown to play an important role in the binding of fibronectin, fibrinogen, and von Willebrand factor to platelets (Ill et al. 1984; Gardner and Hynes, 1985; Ginsberg et al., 1985; Haverstick et al., 1985; Plow et al., 1985; Pytela et al., 1986*a*). Each of these extracellular proteins contains the tripeptide sequence Arg-Gly-Asp, and the cell surface receptor(s) for each appears to interact specifically with this sequence. However, each receptor is distinct in its structure and specificity (Pytela et al., 1985*b*; Akiyama et al., 1986; Horwitz et al., 1985; Pytela et al., 1986*a*; Ruoslahti and Pierschbacher, 1986).

The Arg-Gly-Asp sequence appears in four different locations in the α2 chain and two locations in the α1 chain of the triple-helical region of type I collagen (Bernard et al., 1983). One of these sequences, Arg-Gly-Asp-Thr-Gly-Ala-Thr-Gly-Arg, has been shown to support cell attachment as a synthetic

peptide (Pierschbacher and Ruoslahti, 1984*b*). Because we have found affinity chromatography to be a good method for isolating low affinity adhesion receptors (Ruoslahti and Pierschbacher, 1986), we used immobilized, native collagen, as well as an affinity matrix made out of a synthetic collagen-like peptide to isolate a cell surface membrane protein(s) having affinity for the Arg-Gly-Asp sequence in collagen type I. We report here 250-, 70-, and 30-kD polypeptides that interact with type I collagen in an Arg-Gly-Asp–dependent manner and appear to constitute a cell surface receptor complex recognizing type I collagen. We also show that the specificity of this receptor complex for collagen depends on the presence of the Arg-Gly-Asp recognition unit apparently only in the triple-helical conformation unique to collagens and that this signal can be reproduced in a synthetic triple-helical peptide.

Materials and Methods

Type I collagen, bacterial collagenase, and egg yolk phosphatidylcholine were purchased from Sigma Chemical Co. (St. Louis, MO), octylglucoside and octylthioglucoside from Calbiochem-Behring Corp. (La Jolla, CA). ^{125}I-Sodium iodide was from Amersham Corp. (Arlington Heights, IL), and ^{3}H-phosphatidylcholine and ^{14}C-methylated molecular weight protein markers were from New England Nuclear (Boston, MA). Chemicals used for SDS PAGE were from Bio-Rad Laboratories (Richmond, CA). Fibronectin was prepared from human plasma according to Engvall and Ruoslahti (1977) and vitronectin according to Hayman et al. (1983).

Cells

MG-63 human osteosarcoma cells (Billiau et al., 1977) were grown on 175-cm^2 tissue culture dishes in DME supplemented with 10% FCS, glutamine, and penicillin/streptomycin.

Synthetic Peptides

Peptides were synthesized using a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) using the chemistry provided by the manufacturer, or were purchased from Bachem (Torrance, CA). The sequence of the "collagen-like" peptide was based on published results indicating that a peptide consisting of five to ten repeating units of Gly-Pro-Hyp adopts a stable triple-helical conformation (Sakakibara et al., 1973). Sequencing was performed on a gas phase sequencer (model 470A, Applied Biosystems, Inc.). Optical rotation measurements were kindly made by Dr. E. Miller (Birmingham, AL) as described (Rhodes and Miller, 1978).

Surface Labeling and Preparation of Cell Extracts

Cells were detached by incubating them with 1 mM EDTA for 15 min, washed twice with phosphate-buffered saline (PBS: 150 mM NaCl, 10 mM Na phosphate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.3), and resuspended in PBS containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The suspended cells were radioiodinated according to Lebien et al. (1982) using 2 mCi of ^{125}I-sodium iodide and 0.2 mg/ml lactoperoxidase per 10^8 cells. All subsequent operations were carried out at 4°C. Cells were lysed by adding 1 ml of 50 mM Tris-HCl, pH 7.5, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 mM octylglucoside, and 2 mM PMSF per 10^8 packed cells and incubating for 15 min with occasional shaking. Insoluble material was removed by centrifugation at 12,000 *g* for 15 min.

Chromatography on Type I Collagen and "Collagen-like" Synthetic Peptide Affinity Matrices

Type I collagen (90 mg) was dissolved in 40 ml 0.1 N acetic acid at 4°C, and dialyzed against 0.1 M $NaHCO_3$, pH 8.5, containing 0.5 M NaCl (coupling buffer) for 3 h at 4°C. After making the volume up to 100 ml with coupling buffer, the collagen was coupled to 3 g of cyanogen bromide–activated Sepharose (Sigma Chemical Co.) according to the manufacturer's instructions. The collagen–Sepharose was then washed with three cycles of cold 0.1 M acetate buffer, pH 4.0; 0.5 M NaCl followed by 0.1 M borate buffer, pH 8.0; 0.5 M NaCl.

The collagen-like peptide affinity matrix was prepared using a modification of the procedure described above. The peptide (35 mg) was allowed to dissolve overnight in the smallest possible volume of 0.05% acetic acid at 4°C at which point it appeared from polarimetry to have assumed a triple-helical conformation (Rhodes and Miller, 1978). The solution was then neutralized by dilution to 5 ml with cold coupling buffer. The pH of the solution was brought up to 7.5 by titration with 1 N NaOH. The peptide solution was then mixed with cyanogen bromide–activated Sepharose as described above. After washing the resin with cold coupling buffer to remove nonbound material, the remaining active groups were quenched with 0.1 M glycine in PBS for 2 h at 4°C. The peptide–Sepharose affinity matrix was then washed extensively with cold coupling buffer before application of the sample.

The octylglucoside extract of cells was applied to a column containing either 5 ml of type I collagen affinity matrix or 3 ml of collagen-like peptide affinity matrix, both of which had been equilibrated with column buffer (50 mM Tris-HCl, pH 7.5, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 mM octylglucoside, and 2 mM PMSF). The sample was allowed to enter the columns very slowly at 4°C and then transferred to a room maintained at 17°C. At this temperature the triple-helical conformation of collagen remains intact (Piez, 1983). The columns were then washed with 5 bed vol of column buffer containing 20 mM NaCl and 25 mM octylthioglucoside instead of octylglucoside. Elution of the columns were carried out with octylthioglucoside containing column buffer supplemented with GRGDTP peptide (1 mg/ml). Fractions (1 ml) were collected and the eluted proteins were analyzed by SDS PAGE.

Electrophoresis and Autoradiography

Samples for SDS PAGE were boiled for 3 min in the presence of 2% SDS, with or without 5% 2-mercaptoethanol, and electrophoresed on polyacrylamide gels according to Laemmli (1970). ^{14}C-Methylated protein standards were used as molecular mass markers. Autoradiography was carried out by placing Kodak XAR x-ray film between the dried gel and an intensifying screen (DuPont Co., Wilmington, DE) at −70°C.

Preparation of Liposomes

Liposomes were prepared by the method of Mimms et al. (1981) as described by Pytela et al. (1985*a*), except that 50 mM Tris-HCl, pH 7.5, containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$ was used for the removal of detergent by dialysis. The liposomes were purified by sucrose density centrifugation as described by Pytela et al. (1985*a*) except that centrifugation was carried out at 356,000 *g* in a Beckman TL-100 ultracentrifuge for 5 h at 4°C.

Liposome-binding Assay

Wells of a polystyrene microtiter plate (Linbro/Titertek, Inglewood, CA) were coated with protein solutions in PBS by incubating overnight at 4°C. Unoccupied sites were then saturated by incubation with 2.5 mg/ml BSA in 50 mM Tris-HCl, pH 7.5, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 3 h at 17°C. ^{3}H-labeled liposomes suspended in 50 mM Tris-HCl, pH 7.5, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 2.5 mg/ml BSA were added to the wells and incubated for 5 h or overnight at 4°C. The supernatants were then removed and the wells washed twice with PBS. Bound liposomes were dissolved in 1% SDS (100 μl/well) and quantitated by liquid scintillation counting.

Cell Attachment Assay

Attachment of MG-63 cells to protein coated microtiter wells was carried out as described by Ruoslahti et al. (1982).

Results

Arg-Gly-Asp–dependent Attachment of MG-63 Cells to Collagen

MG-63, human osteosarcoma cells were used as the source for the isolation of the collagen receptor because these cells are capable of attaching directly to type I collagen (Fig. 1) and also because large numbers of these cells can be readily

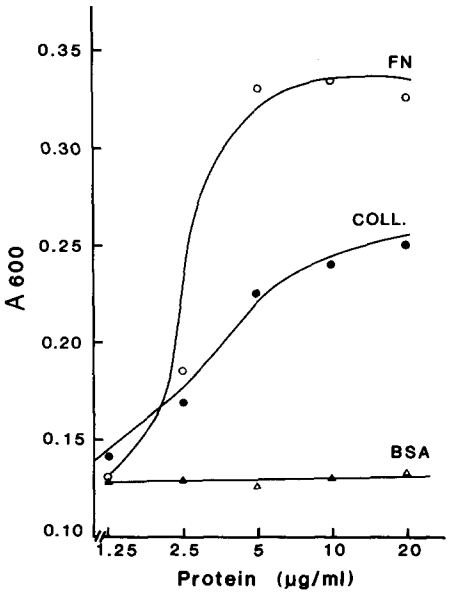

Figure 1. Attachment of MG-63 cells to type I collagen. MG-63 cells (3×10^4) were plated in wells coated with increasing concentrations of fibronectin (*open circles*), type I collagen (*solid circles*), or bovine serum albumin (*open triangles*). The plates were incubated at 17°C for 3 h in an atmosphere of 7% CO_2. Non-attached cells were washed away with PBS, and the attached cells were fixed with 3% paraformaldehyde and stained with 0.5% Toluidine blue in 3.7% formaldehyde. Cell attachment was measured as a function of the absorbance of destained cells at 600 nm.

obtained due to their rapid growth rate in culture. The synthetic peptide Arg-Gly-Asp-Ser mimics the cell attachment signal of fibronectin and inhibits attachment of cells to fibronectin (Pierschbacher and Ruoslahti, 1984*a*) but not to collagen (Hayman et al., 1985). In screening a number of peptides (in which the position following aspartic acid was substituted with different residues) for their ability to inhibit cell attachment, a hexapeptide glycyl-L-arginyl-glycyl-L-aspartyl-L-threonyl-L-proline (GRGDTP) was found to be active as an inhibitor of cell attachment to type I collagen (Fig. 2). We, therefore, made use of this threonine-containing peptide in the isolation of a collagen receptor from the MG-63 cells by affinity chromatography.

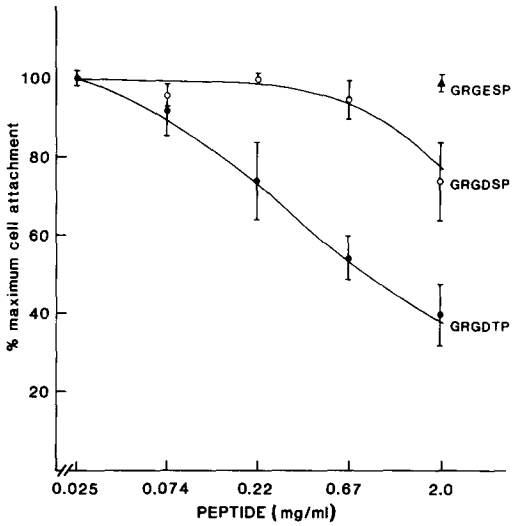

Figure 2. Inhibition of MG-63 cell attachment to type I collagen by synthetic peptides. Cells (2×10^4) were plated in microtiter wells coated with type I collagen (20 μg/ml) and containing the indicated concentrations of synthetic peptides dissolved in DME containing 2.5 mg/ml BSA. Cell attachment was determined as described in Fig. 1.

Affinity Chromatography of ^{125}I-Surface-labeled MG-63 Cell Extracts on Collagen

To isolate collagen-binding MG-63 cell surface proteins, living cells were labeled by lactoperoxidase-catalyzed surface iodination, extracted in buffer containing octylglucoside,

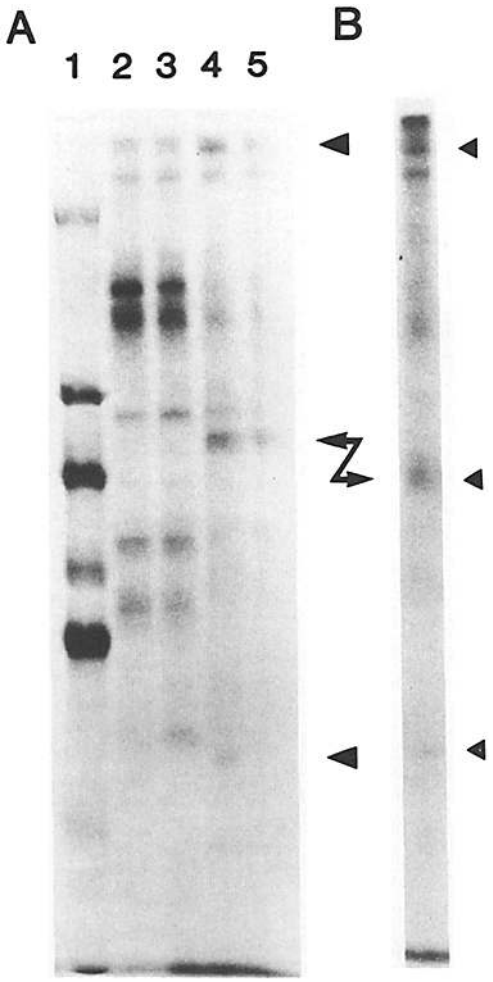

Figure 3. SDS PAGE analysis of fractions eluted from type I collagen–Sepharose affinity matrix. MG-63 human osteosarcoma cells (5×10^8) were surface labeled with ^{125}I as described in Materials and Methods. A octylglucoside extract (3 ml) of the labeled cells was chromatographed at 17°C on a column of Sepharose 4B (5.0 ml bed volume) carrying covalently bound type I collagen. Fractions of 1 ml were collected and aliquots (75 μl) were analyzed by SDS PAGE (7.5% acrylamide) under reducing (*A*) and nonreducing (*B*) conditions. (Lane *1*) ^{14}C-Labeled molecular mass standards. (Lanes *2–5*) ^{125}I-Labeled proteins present in fractions 2, 4, 6, and 8, respectively, after the beginning of elution with 1 mg/ml GRGDTP. *B* shows the same fraction as in lane *4* of *A* under nonreducing conditions. Molecular mass markers are myosin (200 kD), phosphorylase B (92.5 kD), albumin (67 kD), γ IgG (50 kD), and ovalbumin (43 kD). Arrowheads indicate the positions of the 250-, 70-, and 30-kD proteins.

and loaded onto a collagen–Sepharose affinity matrix. After washing the column, it was eluted with buffer containing octylthioglucoside and the synthetic peptide GRGDTP. Three radiolabeled polypeptides, the elution of which was dependent upon the presence of the synthetic peptide, were identified by SDS PAGE analysis of the fractions. These polypeptides had apparent molecular masses of 250, 70, and 30 kD under reducing conditions (Fig. 3 *A*), and do not appear to be covalently linked to one another, since the same pattern was seen under nonreducing conditions (Fig. 3 *B*). The 70-kD polypeptide, however, migrated more slowly under reducing conditions, thus suggesting that this protein has intramolecular disulfide bonds the reduction of which results in a more open conformation of the molecule. Although other proteins were present in the fractions eluted from the column, the 250-, 70-, and 30-kD polypeptides were the only ones affected specifically by elution with the GRGDTP peptide (lane *4* in Fig. 3 *A*). These three proteins were not present in the initial fractions but became visible only after 1.5 column volumes of elution buffer had gone through the column, as expected of a specific elution. In contrast, the other proteins were present in the early fractions (fractions 2–4) which contain the wash buffer, and diminished in the fractions containing the elution buffer (fractions 6–8). The proteins present in the early fractions represent major components of the cell extract being washed slowly from the columns and not proteins specifically eluted by the GRGDTP-containing buffer.

In a separate experiment, the collagen–Sepharose column was eluted with column buffer containing increasing amounts of NaCl in 50 mM Tris–HCl, pH 7.5. All three proteins remained bound to the column at less than 100 mM salt, but were eluted at between 100 and 150 mM salt (not shown).

When an MG-63 cell extract was applied to a gelatin (denatured collagen)–Sepharose column and the column subsequently eluted with GRGDTP peptide, the 250-, 70-, 30-kD polypeptides were not seen in the eluate. In addition, when the flow-through from the gelatin column was then applied to a collagen–Sepharose column, these three proteins bound to the column and were eluted specifically by the GRGDTP peptide showing that they had failed to bind to the gelatin matrix. To study further the specificity requirements for the binding of the 250-, 70-, and 30-kD polypeptides, we synthesized a collagen-like peptide and used that in affinity chromatography.

Affinity Chromatography of Surface ^{125}I-labeled Cell Extracts on a Synthetic "Collagen-like" Matrix

A 33-amino acid peptide consisting of a collagen-like sequence $(Gly\text{-}Pro\text{-}Hyp)_n$ and including the Arg-Gly-Asp-Thr sequence (Fig. 4 *A*) was synthesized. The peptide existed as a triple helix at low temperatures as judged by the high degree of negative optical rotation which underwent a sharp change as the temperature was increased producing a "melting" curve having an inflection point (second derivative equal to 0; Tm) at 30°C (Fig. 4 *B*).

When an octylglucoside extract of the ^{125}I-surface-labeled MG-63 cells was fractionated on a column prepared by coupling this peptide onto Sepharose, the same 250-, 70-, and 30-kD polypeptides could be eluted with the GRGDTP peptide (Fig. 5) as were eluted from the collagen–Sepharose (Fig. 3). As with the collagen column, it appears that less 30-kD polypeptide than 70-kD polypeptide was eluted from this affinity column. This is probably due to unequal labeling of these two polypeptides because of the smaller size of the 30-kD polypeptide. There could also be fewer accessible tyrosine residues in this polypeptide than in the 70-kD polypeptide. A band at around 220-kD was also present but in a number of experiments its presence was inconsistent and appeared to be nonspecific. Moreover, it did not become incorporated into liposomes (see below). Modification of the arginine residues on the peptide matrix with cyclohexane dione (Patthy and Smith, 1975) abrogated its capacity to bind the 250-, 70-, and 30-kD polypeptides, and this capacity could be regained by removing the blocking agent (not shown). Thus it appears probable that the 250-, 70-, and 30-kD polypeptides interact with the Arg-Gly-Asp sequences in this peptide and also in type I collagen.

Characterization of Liposomes Prepared with 250-, 70-, and 30-kD Polypeptides

The fact that the 250-, 70-, and 30-kD polypeptides became labeled by cell surface iodination suggested that they are cell

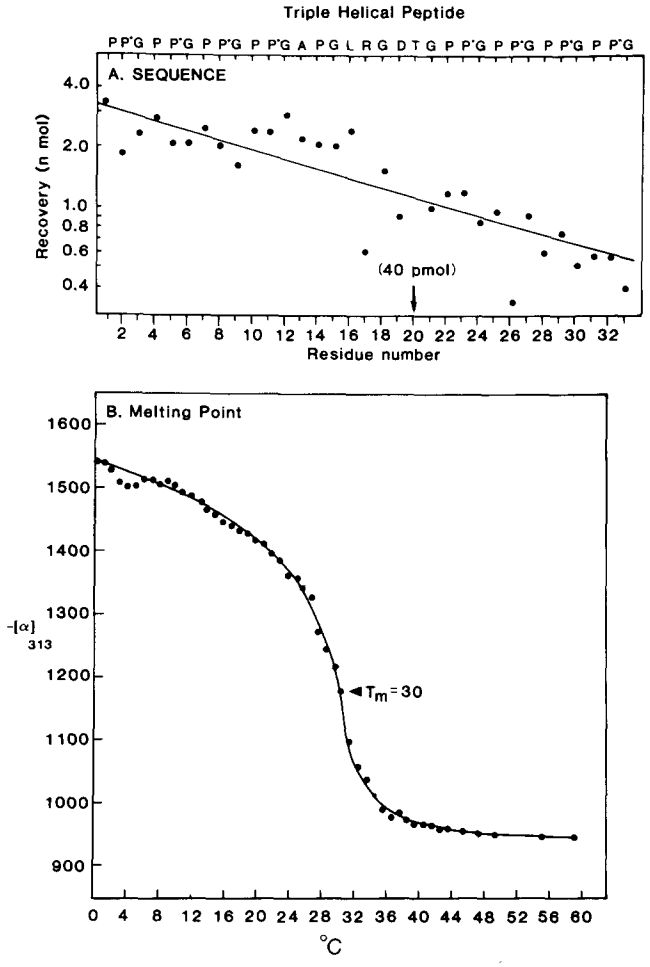

Figure 4. Characterization of the collagen-like synthetic peptide. The sequence of the collagen-like, triple-helical peptide was confirmed by automated Edman degradation, and the sequence along with the recovery is shown in *A*. *P** indicates 4-hydroxyproline and the single letter code used is as follows: *P*, proline; *G*, glycine; *A*, alanine; *L*, leucine; *R*, arginine; *D*, aspartic acid; and *T*, threonine. *B* records the change in optical rotation caused by a solution of the peptide as a function of increasing temperature.

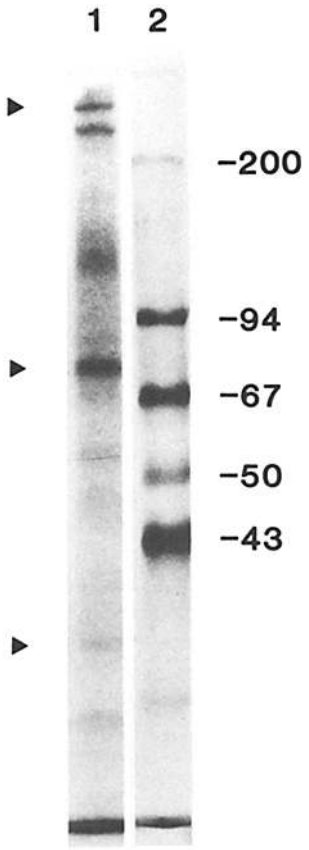

Figure 5. SDS PAGE analysis of material eluted from collagen-like peptide–Sepharose. An extract prepared from ^{125}I-surface labeled MG-63 human osteosarcoma cells was applied to a column of the collagen-like peptide–Sepharose affinity matrix. After washing the column with column buffer containing 20 mM NaCl, the column was eluted with 1 mg/ml of synthetic peptide GRGDTP. The eluted fractions were pooled, dialyzed against 0.05% SDS, lyophilized, and analyzed by SDS PAGE under reducing conditions followed by autoradiography. Lane *1* represents GRGDTP eluted material. (Lane *2*) Molecular mass markers. Arrowheads indicate the 250-, 70-, and 30-kD polypeptides.

surface proteins. To determine whether these polypeptides could function as would be expected of cell surface receptors for type I collagen, an eluate from collagen affinity matrix was mixed with ^{3}H-phosphatidylcholine, and liposomes were prepared by dialyzing out the octylthioglucoside detergent, as well as the peptide used for the elution. The resulting liposomes were purified by ultracentrifugation during which the bulk of the ^{125}I-label floated from the bottom of the tube to the top of the gradient with the ^{3}H-labeled lipid vesicles (Fig. 6 *A*) suggesting that proteins had become associated with the liposomes. Contaminating proteins were recovered in the fractions at the bottom of the gradient and were not seen in the liposome fraction. The 250-, 70-, and 30-kD polypeptides were the only proteins detectable in the liposome fraction (results not shown). In contrast, when the same preparation was treated as described above, but in the absence of any lipids, most of the ^{125}I-labeled protein remained at the bottom of the tube (Fig. 6 *B*). We next examined the ability of liposomes containing the 250-, 70-, and 30-kD polypeptides to bind to polystyrene surfaces coated with various adhesive molecules.

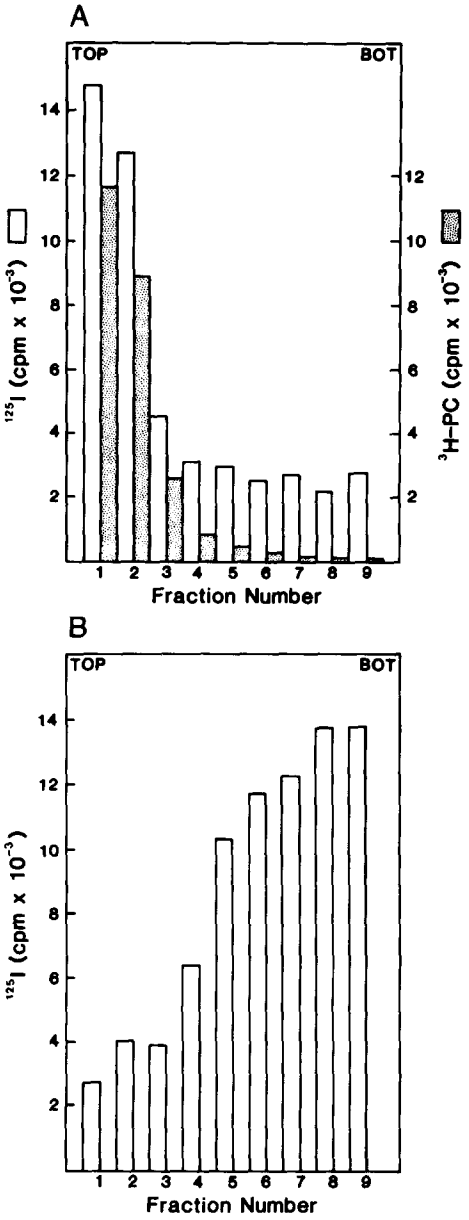

Figure 6. Purification of liposomes prepared with the 250-, 70-, and 30-kD polypeptides. A ^{125}I-labeled fraction containing the 250-kD, 70-kD, and 30-kD proteins obtained as described in Fig. 3 was split into two equal parts. Liposomes were prepared from one of these and fractionated by sucrose density gradient centrifugation as described by Pytela et al. (1985*a*) and 200 μl fractions were analyzed for ^{3}H-labeled lipid (*stippled bars*) and for ^{125}I-labeled proteins (*open bars*). (*A*) Sucrose density gradient centrifugation of liposomes prepared from a fraction containing the 250-, 70-, and 30-kD proteins. (*B*) Sucrose density gradient centrifugation of a similar fraction taken through the liposome preparation steps in the absence of added lipid.

Binding of Liposomes Containing the Collagen-binding Polypeptides to Collagen and Other Substrates

Liposomes prepared from fractions 5 to 8 eluted from the type I collagen–Sepharose column (Fig. 3) and containing the collagen-binding polypeptides bound to type I collagen-coated microtiter wells in a dose-dependent manner (Fig. 7). Liposomes prepared from the wash fractions or from fractions 1 to 4 eluted from the same column (Fig. 3) and not containing the 250-, 70-, and 30-kD proteins, did not bind to the collagen-coated wells. The binding of the liposomes to type I collagen was inhibited by the synthetic peptide GRGDTP (1 mg/ml) (Fig. 8), a finding which is in agreement with the ability of this synthetic peptide to dissociate the radiolabeled polypeptides from the affinity matrices (Fig. 3). The binding of the liposomes to collagen was also inhibited

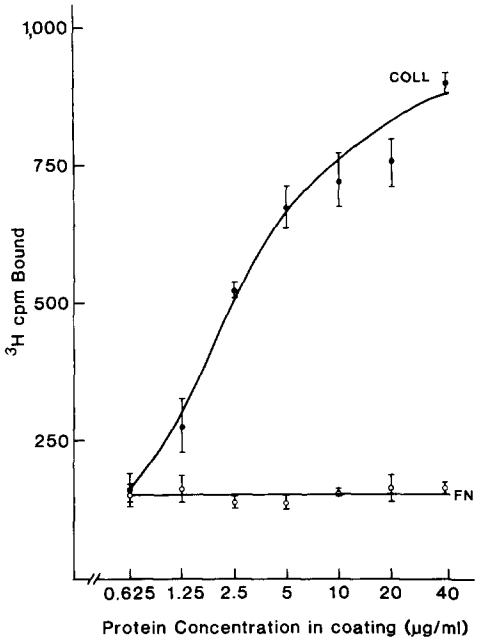

Figure 7. Binding of liposomes prepared from 250-, 70-, and 30-kD protein-containing fractions to type I collagen-coated substrate. A fraction (1 ml) containing the three proteins specifically eluted from the type I collagen affinity matrix by the GRGDTP peptide was added to 200 μg phosphatidylcholine and 2.5 × 10^6 cpm of ^{3}H-phosphatidylcholine and dialyzed against 50 mM Tris-HCl, pH 7.5, containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$ for 24 h at 4°C. 100 μl of the liposome suspension supplemented with 2.5 mg/ml BSA (total ^{3}H cpm of 20,000) were added to microtiter plates coated with various concentrations of type I collagen (*solid circle*) or fibronectin (*open circle*). The binding assay was carried out as described in Materials and Methods and the radioactivity bound was determined by scintillation counting.

by EDTA which is known to interfere with the function of other adhesion receptors (Oppenheimer-Marks and Grinnell, 1984; Pytela et al. 1986*b*). In contrast, the synthetic peptide GRGESP, which inhibits cell attachment to neither fibronectin (Pierschbacher and Ruoslahti, 1984*a*) nor collagen (data not shown), had no effect on the binding of these liposomes to type I collagen. In addition, these liposomes did not bind to collagen which had previously been boiled (gelatin) or treated with bacterial collagenase. The liposomes did not show any significant binding to either fibronectin or vitronectin (Figs. 7 and 8), indicating that the 250-, 70-, and 30-kD polypeptides do not have any affinity for these two extracellular matrix molecules, despite the fact that both have an Arg-Gly-Asp cell attachment-promoting sequence. Because of the finding described above that the collagen-binding polypeptides were eluted from the collagen matrices at relatively low salt concentrations, we included sodium chloride at various concentrations in some of the liposome assays. As shown in Fig. 8 *B*, the liposomes bound to collagen-coated substrate even at salt concentrations above physiological.

These liposomes were also tested for their ability to bind to the triple-helical synthetic peptides shown in Fig. 4. The liposomes bound almost as efficiently to this peptide as they did to type I collagen (Fig. 8 *A*). Liposomes prepared from the 250-, 70-, and 30-kD-containing fractions that eluted from the collagen-like peptide-affinity matrix behaved in a manner identical to those obtained from the polypeptides eluted from the collagen column (results not shown).

Discussion

We present here several lines of evidence that converge to demonstrate that three polypeptides having molecular masses of 250, 70, and 30 kD behave as one would expect of a cell surface receptor for type I collagen, and, more specifically, that the recognition of collagen by these polypeptides involves the Arg-Gly-Asp sequences within the triple-helical region of the collagen molecule. First, the attachment of MG-63 cells to type I collagen can be inhibited by a synthetic peptide containing the Arg-Gly-Asp sequence. It is of interest to note that the hexapeptide with the sequence Gly-Arg-Gly-Asp-Thr-Pro is considerably more effective in inhibiting the attachment of MG-63 cells to type I collagen than a peptide with sequence Gly-Arg-Gly-Asp-Ser-Pro. This is in agreement with unpublished observations that, whereas the Arg-Gly-Asp tripeptide is absolutely essential for the cell attachment activity, the serine residue can be substituted with other amino acids, and that such substitutions result in increased or decreased cell attachment-promoting activity (Pierschbacher and Ruoslahti, unpublished observa-

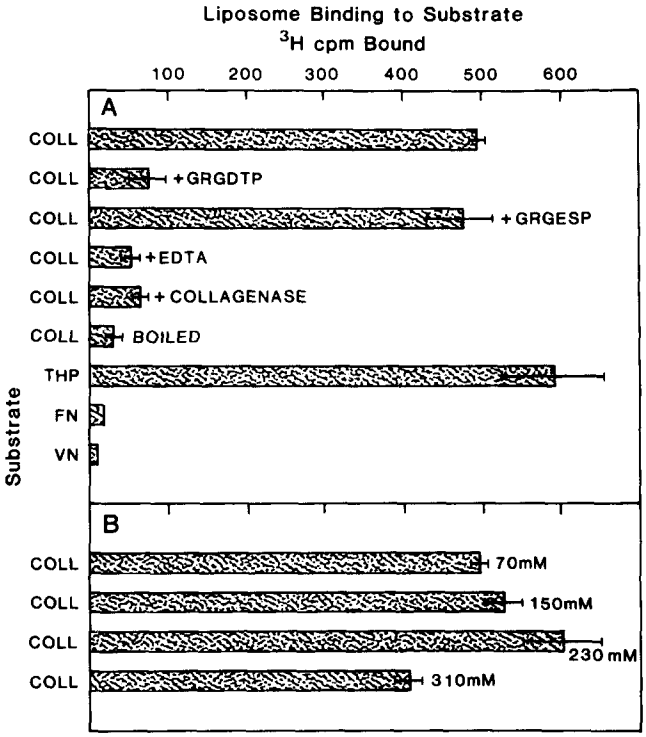

Figure 8. Specificity of the binding of 250-, 70-, and 30-kD polypeptide-containing liposomes. The liposome-binding assay was carried out as described in the legend of Fig. 7. The microtiter wells were coated with 20 μg/ml of collagen (*COLL*), fibronectin (*FN*), vitronectin (*VN*), or the collagen-like, triple-helical peptide (*THP*). (*A*) Liposomes were incubated in these wells alone or in the presence of EDTA (1 mM), GRGDTP peptide (1 mg/ml), or GRGESP peptide (1 mg/ml). Some of the type I collagen-coated wells were pretreated with bacterial collagenase (100 μg/ml) before the liposome binding. (*B*) Sodium chloride was included in some of the collagen-coated wells at the concentration indicated.

tions). This difference probably reflects differences in the conformations favored by the two peptides as discussed below. The incomplete inhibition of attachment of these cells to type I collagen by the Thr-containing peptide may be due to the contribution of other non-Arg-Gly-Asp–dependent collagen-binding proteins in this process. On the other hand, it may indicate that even the GRGDTP peptide (since it is almost surely not triple helical) has a relatively poor fit into the collagen receptor compared with the Arg-Gly-Asp sequences in the collagen molecule. Secondly, the 250-, 70-, and 30-kD polypeptides can be specifically eluted from a type I collagen–Sepharose affinity matrix by the Gly-Arg-Gly-Asp-Thr-Pro peptide, but not by a control peptide containing the sequence Arg-Gly-Glu. Specificity of the binding is also indicated by the fact that the 250-, 70-, and 30-kD polypeptides do not bind to a gelatin–Sepharose affinity column. Moreover, these same polypeptides bind to an affinity matrix prepared with a collagen-like peptide containing the Arg-Gly-Asp sequence in a triple-helical conformation and can also be specifically eluted from this matrix with the inhibitory peptide. Modifying the arginine on this latter matrix nullifies its binding capacity in a reversible manner. Finally, liposomes containing the 250-, 70-, and 30-kD polypeptides bind to immobilized type I collagen or the collagen-like peptide and this binding can be inhibited by the GRGDTP peptide, whereas liposomes prepared from fractions not containing these three polypeptides do not bind to collagen. Because each of the putative receptor polypeptides is accessible to lactoperoxidase-catalyzed iodination at the cell surface, interacts specifically with type I collagen, and exhibits properties expected of integral membrane proteins as judged by an ability to associate with lipid bilayers, we conclude that they act as a receptor complex for collagen at the cell surface.

At the present time we do not know what relationship, if any, our type I collagen-binding cell surface polypeptides have to the other collagen-binding proteins reported in the literature. That the 250-, 70-, and 30-kD–containing liposomes can be inhibited from binding to type I collagen by EDTA agrees with the findings of Rubin et al. (1984) who showed that Mg^{2+} is required for the attachment of hepatocytes to native collagen. However, the protein they assigned this function to has a molecular mass of ~100 kD, clearly different than the sizes of any of our polypeptides. Apparently, none of our polypeptides is fibronectin-related since they did not bind to gelatin–Sepharose. In addition, the 140–150-kD polypeptides present in the early elution fractions (Fig. 3, lanes *2* and *3*) are not related to the fibronectin or vitronectin receptors since antibodies to these receptors failed to immunoprecipitate these polypeptides from these fractions (unpublished data). Johansson and Smedsrod (1986) have recently described a gelatinase which has a molecular mass of 72,000 D and which like fibronectin also binds to gelatin. However, the 70-kD collagen-binding polypeptide described in this report did not possess any gelatinase activity as determined by zymography of the 250-, 70-, and 30-kD complex carried out according to Johansson and Smedsrod (1986). The MG-63 cells did yield a 43-kD polypeptide that was eluted from a gelatin–Sepharose column and possessed gelatinase activity, but this was not detected in the fractions eluted from the collagen column with peptide (our unpublished results).

Mollenhauer and von der Mark (1983) have isolated a 31-kD chick chondrocyte cell surface protein that binds to several types of collagen. The size of this protein suggests that it could be related to our 30-kD polypeptide. Moreover, similar to the findings of these authors, the 250-, 70-, and 30-kD polypeptides dissociated from the collagen matrix at an ionic strength of 100 mM in the presence of detergents, whereas when incorporated into lipid vesicles these polypeptides effected the binding of the liposomes to collagen at salt concentrations of up to 300 mM. Perhaps it is an inherent property of these collagen-binding proteins that they are stable when associated with lipids. Alternatively, the apparent increase in the affinity of the lipid-associated receptor for collagen may reflect the multivalency of the receptor-containing liposomes.

The 70-kD protein among our collagen-binding polypeptides resembles a type I collagen-binding protein isolated from the surfaces of platelets (Chiang and Kang, 1982; Kotite and Cunningham, 1986). Since the latter authors also detected a collagen-binding 30-kD polypeptide in extracts of ^{125}I-surface-labeled platelets these polypeptides may well be related to ours. However, the 70-kD polypeptide seems to be different from the "a" chain of platelet factor XIII which can also bind to collagen and has a molecular mass of ~70-kD (Saito et al., 1986), because the binding of the factor XIII to collagen was found to be independent of divalent cations.

The 70- and 30-kD molecules could be degradation products of the 250-kD component, although this is unlikely because the ratios of the three polypeptides remain constant, and the characteristic behavior of the 70-kD polypeptide in electrophoresis is not shared by the other two polypeptides. Whether the 250-, 70-, and 30-kD polypeptides interact with collagen individually or as a complex still needs to be determined. Other Arg-Gly-Asp receptors have been isolated from MG-63 cells; e.g., for fibronectin and vitronectin. These other receptors have also been shown to have three polypeptide chains (Pytela et al., 1985*a*, *b*; Argraves et al., 1986; Suzuki et al., 1986). However, the Arg-Gly-Asp–dependent receptor for discoidin I in the slime mold *Dictyostelium discoideum* appears to be a single polypeptide of M_r 67,000 (Gabius et al., 1985; Springer et al., 1984) suggesting that the 250-, 70-, and 30-kD polypeptides might also be active individually. Perhaps the discoidin I receptor represents a primordial collagen receptor.

Rubin et al. (1981) have suggested that native collagen contains several cell (hepatocyte) binding sites, as judged from the fact that several peptides generated after cleavage of collagen with cyanogen bromide serve as effective attachment substrates. This observation is consistent with our findings that the Arg-Gly-Asp tripeptide is involved in the attachment of cells to type I collagen because this tripeptide appears a number of times in the molecule (Bernard et al., 1983). Since other types of collagen also contain Arg-Gly-Asp sequences, it will be interesting to see how the 250-, 70-, and 30-kD polypeptides might interact with other collagen types. This will be the subject of future studies.

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References

Adamson, E. D. 1983. The effect of collagen on cell division, cellular differentiation and embryonic development. *In* Collagen In Health and Disease. M. Jayson and J. Weiss, editors. Churchill-Livingston, London. 218–243.

Akiyama, S. K., S. S. Yamada, and K. M. Yamada. 1986. Characterization of a 140-kD avian cell surface antigen as a fibronectin-binding molecule. *J. Cell Biol.* 102:442–448.

Argraves, W. S., R. Pytela, S. Suzuki, J. L. Millan, M. D. Pierschbacher, and E. Ruoslahti. 1986. cDNA sequences from the α subunit of the fibronectin receptor predict a transmembrane domain and a short cytoplasmic peptide. *J. Biol. Chem.* 261:12922–12924.

Bard, J. B. L., and E. D. Hay. 1975. The behavior of fibroblasts from the developing avian cornea. *J. Cell Biol.* 67:400–418.

Bernard, M. P., J. C. Myers, M. L. Chu, F. Ramirez, E. F. Eikenberry, and D. J. Prockop. 1983. Structure of a cDNA for the Pro α2 chain of human type I procollagen. Comparison with chick cDNA for pro α2(I) identifies structurally conserved features of the protein and the gene. *Biochemistry.* 22:1139–1145.

Billiau, A., V. G. Edy, H. Heremans, J. Van Damme, J. Desmyter, J. A. Georgiades, and P. DeSomer. 1977. Human interferon: mass production in a newly established cell line, MG-63. *Antimicrob. Agents Chemother.* 12:11–15.

Briles, E. B., and N. B. Haskew. 1982. Isolation of cloned variants of a rat hepatoma cell line with altered attachment to collagen, but normal attachment to fibronectin. *Exp. Cell Res.* 138:436–441.

Bunge, R. P., and M. B. Bunge. 1978. Evidence that contact with connective tissue matrix is required for normal interaction between Schwann cells and nerve fibers. *J. Cell Biol.* 78:943–950.

Chiang, T. M., and A. H. Kang. 1982. Isolation and purification of collagen α1(I) receptor from human platelet membrane. *J. Biol. Chem.* 257:7581–7586.

Couchman, J. R., D. A. Rees, M. R. Green, and C. G. Smith. 1982. Fibronectin has a dual role in locomotion and anchorage of primary chick fibroblasts and can promote entry in the division cycle. *J. Cell Biol.* 93:402–410.

Engvall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer.* 20:1–5.

Engvall, E., M. L. Bell, R. N. K. Carlsson, E. J. Miller, and E. Ruoslahti. 1982. Nonhelical, fibronectin-binding basement-membrane collagen from endodermal cell culture. *Cell.* 29:475–482.

Gabius, H.-J., W. R. Springer, and S. H. Barondes. 1985. Receptor for the cell binding site of Discoidin I. *Cell.* 42:449–456.

Gardner, J. M., and R. O. Hynes. 1985. Interaction of fibronectin with its receptor on platelets. *Cell* 42:439–448.

Gay, G. D., M. Svotelis, M. Foard, and F. B. Bang. 1974. Long-term growth of chicken fibroblasts on a collagen substrata. *Exp. Cell Res.* 84:63–71.

Ginsberg, M., M. D. Pierschbacher, E. Ruoslahti, G. Marguerie, and E. Plow. 1985. Inhibition of fibronectin binding to platelets by proteolytic fragments and synthetic peptides which support fibroblast adhesion. *J. Biol. Chem.* 260:3931–3936.

Hauschka, D. S., and I. R. Konigsberg. 1966. The influence of collagen on the development of muscle colonies. *Proc. Natl. Acad. Sci. USA.* 55:119–126.

Haverstick, D. M., J. F. Cowan, K. M. Yamada, and S. A. Santoro. 1985. Inhibition of platelet adhesion to fibronectin, fibrinogen, and von Willebrand factor substrates by a synthetic tetrapeptide derived from the cell-binding domain of fibronectin. *Blood.* 66:946–952.

Hay, E. 1981. Collagen and embryonic development. *In* Cell Biology of the Extracellular Matrix. E. Hay, editor. Plenum Publishing Corp., New York. 379–409.

Hayman, E. G., M. D. Pierschbacher, Y. Ohgren, and E. Ruoslahti. 1983. Serum spreading factor (vitronectin) is present at the cell surface and in tissues. *Proc. Natl. Acad. Sci. USA.* 80:4003–4007.

Hayman, E. G., M. D. Pierschbacher, and E. Ruoslahti. 1985. Detachment of cells from culture substrate by soluble fibronectin peptides. *J. Cell Biol.* 100:1948–1954.

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

Ill, C. R., E. Engvall, and E. Ruoslahti. 1984. Adhesion of platelets to laminin in the absence of activation. *J. Cell Biol.* 99:2140–2145.

Johansson, S., and B. Smedsrod. 1986. Identification of a plasma gelatinase in preparations of fibronectin. *J. Biol. Chem.* 261:4364–4366.

Klebe, R. J. 1974. Isolation of a collagen-dependent cell attachment factor. *Nature (Lond.).* 250:248–251.

Kleinman, H. K., R. J. Klebe, and G. R. Martin. 1981. Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol.* 88:473–485.

Koda, J. E., and M. Bernfield. 1984. Heparan sulfate proteoglycans from mouse mammary epithelial cells. Basal extracellular proteoglycan binds specifically to native type I collagen fibrils. *J. Biol. Chem.* 259:11763–11770.

Kosher, R. A., and R. L. Church. 1975. Stimulation of in vitro chondrogenesis by procollagen and collagen. *Nature (Lond.).* 258:327–330.

Kotite, N. J., and L. W. Cunningham. 1986. Specific adsorption of a platelet membrane glycoprotein by human insoluble collagen. *J. Biol. Chem.* 261: 8342–8347.

Kurkinen, M., A. Taylor, J. I. Garrels, and B. L. M. Hogan. 1984. Cell surface-associated proteins which bind native type IV collagen or gelatin. *J. Biol. Chem.* 259:5915–5922.

Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lash, J. W., E. Belsky, and N. S. Vasan. 1977. Stimulation of chondrogenic differentiation with extracellular matrix components: an analysis using scanning electron microscopy. *In* Cell Interactions in Differentiation. M. Karkinen-Jääskeläinen, L. Saxen, and L. Wein, editors. Academic Press, New York. 263–272.

Lebien, T. E., D. R. Boue, J. C. Bradley, and J. H. Kersey. 1982. Antibody affinity may influence antigenic modulation of the common acute lymphoblastic leukemia antigen *in vitro*. *J. Immunol.* 129:2287–2292.

Lee, E. Y. H., G. Parry, and M. J. Bissell. 1984. Modulation of secreted proteins of mouse mammary epithelial cells by the collagenous substrata. *J. Cell Biol.* 98:146–155.

Lee, E. Y. H., W.-H. Lee, C. S. Kaetzel, G. Parry, and M. J. Bissell. 1985. Interaction of mouse mammary epithelial cells with collagen substrata: regulation of casein gene expression and secretion. *Proc. Natl. Acad. Sci. USA.* 82:1419–1423.

Lesot, H., V. Karcher-Djuricic, M. Mark, J.-M. Meyer, and J.-V. Ruch. 1985. Dental cell interaction with extracellular matrix constituents: type I collagen and fibronectin. *Differentiation.* 29:176–181.

Liotta, L. A., D. Vembu, H. K. Kleinman, G. R. Martin, and C. Boone. 1978. Collagen is required for proliferation of cultured connective tissue cells but not their transformed counterparts. *Nature (Lond.).* 272:622–624.

Lohler, J., R. Timpl, and R. Jaenisch. 1984. Embryonic lethal mutation in mouse collagen I gene causes rupture of blood vessels and is associated with erythropoietic and mesenchymal cell death. *Cell.* 38:597–607.

Mimms, L. T., G. Zampighi, Y. Nozaki, C. Tanford, and J. A. Reynolds. 1981. Phospholipid vesicle formation and transmembrane protein incorporation using octylglucoside. *Biochemistry.* 20:833–840.

Mollenhauer, J., and K. von der Mark. 1983. Isolation and characterization of a collagen-binding glycoprotein from chondrocyte membranes. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:45–50.

Ocklind, C., K. Rubin, and B. Öbrink. 1980. Different cell surface glycoproteins are involved in cell–cell and cell–collagen adhesion of rat hepatocytes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 121:47–50.

Oppenheimer-Marks, N., and F. Grinnell. 1984. Calcium ions protect cell–substratum adhesion receptors against proteolysis. Evidence from immunoabsorption and electroblotting studies. *Exp. Cell Res.* 152:467–475.

Patthy, L., and E. L. Smith. 1975. Reversible modification of arginine residues. Application to sequence studies by restriction of tryptic hydrolysis to lysine. *J. Biol. Chem.* 250:557–564.

Pearlstein, E. 1976. Plasma membrane glycoprotein which mediates adhesion of fibroblasts to collagen. *Nature (Lond.).* 262:497–500.

Pierschbacher, M. D., and E. Ruoslahti. 1984*a*. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature (Lond.).* 309:30–33.

Pierschbacher, M. D., and E. Ruoslahti. 1984*b*. Variants of the cell recognition site of fibronectin that retain attachment-promoting activity. *Proc. Natl. Acad. Sci. USA.* 81:5985–5988.

Pierschbacher, M. D., E. G. Hayman, and E. Ruoslahti. 1985. The cell attachment determinant in fibronectin. *J. Cell. Biochem.* 28:115–126.

Piez, K. A. 1983. Molecular and aggregate structures of the collagens. *In* Extracellular Matrix Biochemistry. K. A. Piez and A. H. Reddi, editors. Elsevier Science Publishers, New York. 1–13.

Plow, E. F., M. D. Pierschbacher, E. Ruoslahti, G. A. Marguerie, and M. H. Ginsberg. 1985. The effect of Arg-Gly-Asp–containing peptides on fibrinogen and von Willebrand factor binding to platelets. *Proc. Natl. Acad. Sci. USA.* 82:8057–8061.

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

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

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

Pytela, R., M. D. Pierschbacher, W. S. Argraves, S. Suzuki, and E. Ruoslahti. 1986*b*. ARG-GLY-ASP adhesion receptors. *Methods Enzymol.* In press.

Reddi, A. H., and W. A. Anderson. 1976. Collagenous bond matrix-induced endochondrial ossification and hemopoiesis. *J. Cell Biol.* 69:557–572.

Rhodes, R. K., and E. J. Miller. 1978. Physicochemical characterization and molecular organization of the collagen A and B chains. *Biochemistry.* 17:3442–3448.

Rovasio, R. A., A. Delouvee, K. M. Yamada, R. Timpl, and J. P. Thiery. 1983. Neural crest cell migration: requirements for exogenous fibronectin and high cell density. *J. Cell Biol.* 96:462–473.

Rubin, K., T. K. Borg, R. Holmdahl, L. Oareskog, and B. Öbrink. 1984. Interactions of mammalian cells with collagen. *In* Basement Membranes and Cell Movement. *CIBA Found. Symp.* 108:93–116.

Rubin, K., M. Höök, B. Öbrink, and R. Timpl. 1981. Substrate adhesion of rat hepatocytes: mechanism of attachment to collagen substrates. *Cell.* 24: 463–470.

Rubin, K., Å. Oldberg, M. Höök, and B. Öbrink. 1978. Adhesion of rat hepatocytes to collagen. *Exp. Cell Res.* 117:165–177.

Ruoslahti, E., E. G. Hayman, M. D. Pierschbacher, and E. Engvall. 1982. Fibronectin: purification, immunochemical properties and biological activities. *Methods Enzymol.* 82:803–831.

Ruoslahti, E., and M. D. Pierschbacher. 1986. Arg-Gly-Asp: a versatile cell recognition signal. *Cell.* 44:517–518.

Saito, Y., T. Imada, J. Takagi, T. Kikuchi, and Y. Inada. 1986. Platelet factor XIII. The collagen receptor? *J. Biol. Chem.* 261:1355–1358.

Sakakibara, S., K. Inouye, K. Shudo, Y. Kishida, Y. Kobayashi, and D. J. Prockop. 1973. Synthesis of (Pro-Hyp-Gly)$_n$ of defined molecular weights. Evidence for the stabilization of collagen triple helix by hydroxypyroline. *Biochim. Biophys. Acta.* 303:198–202.

Schor, S. L., and J. Court. 1979. Different mechanisms in the attachment of cells to native and denatured collagen. *J. Cell Sci.* 38:267–281.

Silnutzer, J. E., and D. W. Barnes. 1985. Effects of fibronectin-related peptides on cell spreading. *In Vitro.* 21:73–78.

Springer, W. R., D. N. W. Cooper, and S. H. Barondes. 1984. Discoidin I is implicated in cell–substratum attachment and ordered cell migration of *Dictyostelium discoideum* and resembles fibronectin. *Cell.* 39:557–564.

Suzuki, S., Å. Oldberg, E. G. Hayman, M. D. Pierschbacher, and E. Ruoslahti. 1985. Complete amino acid sequence of human vitronectin deduced from cDNA. Similarity of cell attachment sites in vitronectin and fibronectin. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2519–2524.

Suzuki, S., R. Pytela, H. Arai, W. S. Argraves, T. Krusius, M. D. Pierschbacher, and E. Ruoslahti. 1986. cDNA and amino acid sequences of the cell adhesion receptor recognizing vitronectin reveal a transmembrane domain and homologies with other adhesion receptors. *Proc. Natl. Acad. Sci. USA.* In press.

Vaheri, A., and D. F. Mosher. 1978. High molecular weight, cell surface-associated glycoprotein (fibronectin) lost in malignant transformation. *Biochim. Biophys. Acta.* 516:1–25.