# Identification of Multiple Cell Adhesion Receptors for Collagen and Fibronectin in Human Fibrosarcoma Cells Possessing Unique α and Common β Subunits

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*Abstract.* Using monoclonal antibody technology and affinity chromatography we have identified four distinct classes of cell surface receptors for native collagen on a cultured human fibrosarcoma cell line, HT-1080. Two classes of monoclonal antibodies prepared against HT-1080 cells inhibited adhesion to extracellular matrix components. Class I antibodies inhibited cell adhesion to collagen, fibronectin, and laminin. These antibodies immunoprecipitated two noncovalently linked proteins (subunits) with molecular masses of 147 and 125 kD, termed α and β, respectively. Class II antibodies inhibited cell adhesion to native collagen only and not fibronectin or laminin. Class II antibodies immunoprecipitated a single cell surface protein containing two noncovalently linked subunits with molecular masses of 145 and 125 kD, termed α and β, respectively. The two classes of antibodies did not cross-react with the same cell surface protein and recognized epitopes present on the α subunits. Pulse-chase labeling studies with [$^{35}$S]methionine indicated that neither class I nor II antigen was a metabolic precursor of the other. Comparison of the α and β subunits of the class I and II antigens by peptide mapping indicated that the β subunits were identical while the α subunits were distinct. In affinity chromatography experiments HT-1080 cells were extracted with Triton X-100 or octylglucoside detergents and chromatographed on insoluble fibronectin or native type I or VI collagens. A single membrane protein with the biochemical characteristics of the class I antigen was isolated on fibronectin-Sepharose and could be immunoprecipitated with the class I monoclonal antibody. The class I antigen also specifically bound to type I and VI collagens, consistent with the observation that the class I antibodies inhibit cell adhesion to types VI and I collagen and fibronectin. The class II antigen, however, did not bind to collagen (or fibronectin) even though class II monoclonal antibodies completely inhibited adhesion of HT-1080 cells to types I and III–VI collagen. The class Iβ and IIβ subunits were structurally related to the β subunit of the fibronectin receptor described by others. However, none of these receptors shared the same α subunits. Additional membrane glycoprotein(s) with molecular mass ranges of 80–90 and 35–45 kD, termed the class III and IV receptors, respectively, bound to types I and VI collagen but not to fibronectin. Monoclonal antibodies prepared against the class III receptor had no consistent effect on cell attachment or spreading, suggesting that it is not directly involved in adhesion to collagen-coated substrates.

These results suggest that the class I and II receptors are two new members of a family of cell surface receptors for the extracellular matrix involved in mediating cell adhesion and shall be referred to as ECMRI and II. Each member of this family possesses a common β subunit and a unique α subunit. The class II receptor appears to be a primary mediator of specific cell adhesion to collagen. The promiscuous class I receptor also mediates cell adhesion to collagen but appears to interact with fibronectin and laminin as well. The class III receptor is not a member of this family of extracellular matrix adhesion receptors but does bind specifically to native collagens.

FIBRONECTIN has been shown to interact with a family or families of related cell surface proteins with molecular masses of ~140 kD in fibroblasts (Brown and Juliano, 1985, 1986; Akiyama et al., 1986; Wylie et al., 1979), osteosarcoma cells (Pytela et al., 1985), endothelial cells (Plow et al., 1986), lymphoid cells (Brown and Juliano, 1986), platelets (Pytela et al., 1986; Gardner and Hynes, 1985), and muscle cells (Horwitz et al., 1985; Dambsky et al., 1985; Chapman, 1984). The interaction of fibronectin with this cell surface receptor appears to mediate cell adhesion to fibronectin and to proceed via interaction of the receptor with the amino acid sequence RGDS located in the

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cell attachment domain of fibronectin (Pierschbacher and Ruoslahti, 1984*a*, *b*; Akiyama et al., 1985).

However, the extracellular matrix (ECM)[1] of untransformed cultured human fibroblasts, such as WI-38 cells, contains large quantities of at least two other transformation-sensitive adhesion proteins, GP250 and GP140 (Carter and Hakamori, 1981; Carter, 1982*a*, *b*), as well as fibronectin. GP140 is a hybrid protein with a major globular domain and a minor collagenous domain that corresponds to the α2 and/or α1 subunits of type VI collagen isolated from pepsin digests of human placenta (Heller-Harrison and Carter, 1984). Purified, nonreduced, and nonpepsinized type VI collagen, or GP140, as well as GP250, can induce stable cell adhesion and spreading; demonstrating that the ECM contains major adhesive proteins other than fibronectin. Since the ECM is therefore a complex organization of adhesive proteins (and other macromolecular substances such as glycosaminoglycans) it is reasonable to hypothesize the existence of cell surface receptors other than the fibronectin receptor for ECM adhesive components, such as type VI or other collagens. In fact, Hayman et al. (1985) have shown that cell adhesion to collagen is not inhibited by RGDS-containing peptides. Similarly, Nagata et al. (1985) concluded that collagen noncompetitively inhibited cell spreading but not cell attachment to fibronectin. These data strongly suggest that cell adhesion to collagen is mediated by a receptor distinct from the fibronectin receptor. Despite considerable effort, conclusive evidence implicating a particular cell surface receptor(s) in attachment to collagen has not been presented even though various cell surface proteins that do interact with collagen have been described in platelets (Chiang and Kang, 1982; Kotite and Cunningham, 1986; Santoro, 1986; Saito et al., 1986), chondrocytes (Mollenhauer and von der Mark, 1983; Mollenhauer et al., 1984), hepatocytes (Rubin et al., 1981), and other cells (Kurkinen et al., 1984).

Furthermore, since type VI collagen differs from collagen types I–V in various ways, there is also the possibility that the cell surface receptor involved in mediating cell adhesion or assembly of type VI collagen into the ECM may be different than the receptor(s) for other collagen types. The unique characteristics of type VI collagen relative to other collagen types include: (*a*) a relatively low affinity for the gelatin-binding domain of plasma fibronectin when compared with collagen types I–V (Carter, 1982*b*; Heller-Harrison, R. A. and W. G. Carter, manuscript in preparation), (*b*) extensive intermolecular disulfide bonding and complex-type glycosylation within the pepsin-resistant domains (Heller-Harrison and Carter, 1984; Carter, 1984), (*c*) resistance to digestion with bacterial collagenase in the nonreduced state (Heller-Harrison and Carter, 1984), and (*d*) preferential accumulation in the ECM as a detergent-insoluble complex (half-life of 73 h) in contrast to other collagens (half-life of 5 h) that accumulate as soluble components in cell culture media (Carter, 1982*a*).

In preliminary work we described three cell surface glycoproteins with molecular masses of 140, 80–90, and 45 kD that bound to type VI collagen in affinity chromatography experiments and considered to be potential cell surface receptors for type VI collagen involved in cell adhesion (Carter and Wayner-Carter, 1986). In the present paper we have extended the previous studies to include inhibition of cell attachment with monoclonal antibodies as well as affinity chromatography on insolubilized ECM components in order to define four cell surface proteins (classes I–IV) with affinity for type VI collagen (and/or fibronectin). We also present conclusive evidence directly implicating class I and II receptors in the collagen adhesion process. The role of these receptors in mediating cell adhesion to type VI and other collagen types as well as fibronectin is examined and discussed.

1. *Abbreviations used in this paper*: ECM, extracellular matrix; FNR, fibronectin receptor; 2-ME, 2-mercaptoethanol; NEM, *N*-ethylmaleimide.

## Materials and Methods

### Materials

Phenylmethylsulfonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), diisopropyl fluorophosphate, 2-mercaptoethanol (2-ME), BSA, Triton X-100, *n*-octyl-β-D-glucopyranoside, EDTA, thermolysin (protease type X), pepsin, protein A–agarose, gelatin (swine skin), tuftsin peptide, and V8 protease (from *Staphylococcus aureus*, strain V8, protease type XVII) were purchased from Sigma Chemical Co. (St. Louis, MO). Lactoperoxidase and glucose oxidase were from Calbiochem-Behring Corp. (San Diego, CA). Fluorescein-conjugated (goat) anti-mouse IgG and IgM (H and L chains) were obtained from Tago, Inc. (Burlingame, CA). Rabbit anti-mouse IgG (H and L) antiserum was obtained from Cappel (CooperBiomedical, Malvern, PA). [$^{51}$Cr]Sodium chromate was from New England Nuclear. [$^{35}$S]methionine (Trans $^{35}$S-label, 1010 Ci/mmol) was from ICN Radiochemicals (Irvine, CA). $^{125}$I was from Amersham Corp. (Arlington Heights, IL). The GRGDS peptide was obtained from Peninsula Laboratories Inc. (Belmont, CA). Rabbit polyclonal antibodies prepared against the fibronectin receptor as described by Pytela et al. (1985) were the generous gift of Dr. M. D. Pierschbacher (La Jolla Cancer Research Foundation, La Jolla, CA).

### Cells and Cell Culture

Normal embryonic human lung diploid fibroblasts, WI-38 cells, and SV-40 virus transformants of WI-38 cells, WI-38 VA13 cells, a human fibrosarcoma cell line, HT-1080 cells, and a human rhabdomyosarcoma cell line, A204 cells, were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 medium and 10% FBS in a 5% $CO_2$ atmosphere.

### Preparation of Monoclonal Antibodies

Monoclonal antibodies were produced by the methods of Oi and Herzenberg (1980) and Taggart and Samloff (1983). Briefly, either BALB/c or RBF/Dn mice (Jackson Laboratory, Bar Harbour, ME) were immunized with nontrypsinized WI-38 VA13 or HT-1080 cells. Immune spleens were removed and fused with either SP2/0, or the NS-1/FOX-NY myeloma cell lines. Viable heterokaryons were selected in RPMI 1640 supplemented with hypoxanthine/aminopterin/thymidine or adenine/aminopterine/thymidine. Heterokaryons were screened for the production of antibodies in a three-stage assay as described below. After screening, selected heterokaryons were cloned by limiting dilution with thioglycolate-induced irradiated BALB/c peritoneal macrophages or thymocytes as feeder cells.

### Screening of Heterokaryons

***Stage One: Solid-Phase Assay.*** Heterokaryons that produced antibodies that reacted with HT-1080 cells were identified in a solid-phase assay. HT-1080 cells were grown to confluence in 96-well culture plates (No. 3072; Falcon Labware, Oxnard, CA), washed with PBS, fixed with 4% wt/vol paraformaldehyde in PBS, blocked with 10% FBS, and then reacted with culture supernatant from the heterokaryons. The HT-1080 cells were washed, reacted with the secondary antibody rabbit anti-mouse IgG (H and L chains), followed by reaction with $^{125}$I-protein A and autoradiography of the culture plates.

***Stage Two: Immune Precipitation.*** Heterokaryons producing antibodies to cell surface proteins of HT-1080 cells were identified by immunoprecipitation. HT-1080 cells were surface-labeled with radioactive iodine using the lactoperoxidase/glucose oxidase method (Hynes, 1973), followed by extraction with 1% vol/vol Triton X-100 detergent in 25 mM Tris-HCl buffer (Tri-

ton/Tris), pH 7.5, containing 1 mM diisopropyl fluorophosphate or 1 mM PMSF plus 2 mM NEM as protease inhibitors. After centrifugation at 35,000 *g* for 20 min the soluble detergent extract was mixed with BSA coupled to Sepharose (BSA-Sepharose) and then recentrifuged to preclear the labeled extract. The extract was immunoprecipitated with cell culture supernatants from the heterokaryons. To reduce the number of samples to be precipitated, the culture supernatants were placed in a matrix organization and then pooled in columns and rows so every supernatant was present in two pools derived from one column and one row. The pooled supernatants were then used for immunoprecipitation. The precipitates from the columns and rows could then be used to identify supernatants with specific antigen reactivity. Immunoprecipitation proceeded as follows. (*a*) Rabbit anti-mouse IgG was bound to protein A-agarose by incubation in Triton/Tris containing 0.5% wt/vol BSA (Triton/Tris/BSA) and then washed by centrifugation. (*b*) The immobilized secondary antibodies were incubated with the pooled heterokaryon culture supernatants to immobilize the primary antibodies, then washed by centrifugation. (*c*) The immobilized primary antibodies were incubated with the labeled HT-1080 cell extract, washed in Triton/Tris containing 400 mM NaCl, and the bound antigens visualized on SDS-PAGE gels by autoradiography.

***Stage Three: Inhibition of Cell Adhesion.*** Antibodies that would alter cell adhesion to either or both fibronectin and type VI collagen were selected as follows. (*a*) 48-well virgin styrene plates were coated with pepsin-generated type VI collagen or human plasma fibronectin (200 μl protein solution/well, 5 μg protein/ml) as previously described (Rauvala et al., 1981; Carter, 1982*a*). The plates were blocked with 200 μl PBS supplemented with 10 mg/ml heat-denatured BSA (80°C for 3 min). The 48-well plates were obtained by special order from Costar (Van Nuys, CA) and consisted of a culture plate (No. 3548, Costar) that had not been treated to induce cell adhesion. In the absence of exogenous adhesive protein, HT-1080 cells would not adhere to these plates. (*b*) HT-1080 cells were labeled with $Na_2{}^{51}CrO_4$ (50 μCi/ml for 2–4 h; New England Nuclear, Boston, MA) and were suspended with trypsin (0.05% wt/vol) in PBS containing 5 mM EDTA as previously described (Carter, 1982*a*). The cells ($2 \times 10^5$/well) were incubated with the heterokaryon culture supernatants (1:2 dilution in PBS supplemented with 1 mg/ml heat-denatured BSA) or control SP2 cell culture supernatant for 1 h at 4°C with agitation, then the cells were allowed to adhere to the fibronectin- or collagen-coated surfaces in the presence of the hybridoma supernatants for 15–30 min at room temperature or 37°C. (*c*) Nonadherent cells were removed by washing with PBS. The adherent cells were dissolved in SDS/NaOH and quantitated in a gamma counter.

## Sequential Immune Precipitation

Detergent extracts from HT-1080 cells labeled with radioactive iodine were subjected to three cycles of immunoprecipitation using antibodies bound to protein A–agarose. Each cycle of precipitation involved the following steps: (*a*) incubation of the antibody-agarose with the labeled cell extract; (*b*) separation of the antigen antibody agarose from the unbound extract by centrifugation; and (*c*) incubation of the next antibody–agarose complex with the unbound extract from step *b*.

## Preparation of Adhesive Proteins

***Fibronectin.*** Human plasma fibronectin was purified from human plasma by affinity chromatography on gelatin-Sepharose according to the method of Engvall and Ruoslahti (1977).

***Cell Adhesion Domain of Fibronectin.*** Human plasma fibronectin was digested with thermolysin as described by Sekiguchi and Hakomori (1983) and the gelatin-binding domain was removed by affinity chromatography on gelatin-Sepharose. The 140-kD cell adhesion domain was isolated by chromatography on Sephacryl S-200.

***Nonpepsinized Type VI Collagen.*** Type VI collagen was isolated from human placenta without pepsin digestion or reduction of disulfide bonds by extraction of urea-insoluble residue with sodium trichloroacetate (NaTCA) as previously described (Carter, 1982*a*). Briefly, human placentae were washed in PBS to remove blood and debris, and were then homogenized in PBS in a blender (Waring Products, New Hartford, CT). All steps were carried out in the presence of 1 mM PMSF, 2 mM NEM, and 5 mM EDTA. The homogenate was centrifuged at 25,000 *g* for 15 min and pellets were sequentially extracted with 25 mM Tris-HCL, pH 7.8, containing 2% Empigen BB and 0.1% SDS; and twice with 25 mM Tris-HCl, pH 7.8, containing 8 M urea with 1 M NaCl. The resulting pellet was homogenized in 4 M NaTCA and incubated with stirring for 4 h. After centrifugation, the supernatant fraction, which contained most of the type VI collagen, was dialyzed extensively against water. During dialysis the material which precipitated was collected by centrifugation, redissolved in 8 M urea in 25 mM Tris-HCL, pH 7.8, and finally dialyzed against 0.5 M acetic acid. The dialysate was centrifuged, and the supernatant fraction containing the interstitial collagens and small quantities of types IV and V collagens was discarded. Trace quantities of other collagens were removed by digestion with bacterial collagenase under nonreducing conditions. In the absence of reducing agents type VI collagen is resistant to digestion with collagenase. The type VI collagen was further purified by molecular sieve chromatography in 8 M urea or SDS on Sephacryl S500.

***Pepsinized Collagen Types I and III–VI.*** All collagen types were isolated from fresh human placenta after digestion with pepsin as described by Miller and Rhodes (1982) for collagen types I and III–V, and as described by Jander et al. (1981) for collagen type VI.

***Laminin.*** Mouse laminin was isolated from Englebreth-Holm-Swarm sarcoma grown in BALB/c mice as described by Timpl et al. (1979).

## Pulse-Chase Experiments

HT-1080 cells were grown to confluence in 6.0-cm culture plates ($1 \times 10^6$ cells/plate), washed with methionine-free culture medium, and incubated in the same medium for 2 h. The methionine-starved cells were pulse-labeled with [$^{35}$S]methionine (75 μCi/ml, 1.5 ml/plate) for 1 h, and then washed and chased with RPMI medium containing 10% FBS. At the indicated time points the plates were washed with PBS, 1.0 ml of Triton/Tris buffer containing 1 mM PMSF was added to each plate, and the cell residue scraped with a rubber policeman. The detergent suspension was incubated on ice for 30 min, centrifuged at 35,000 *g* for 20 min and 100-μl aliquots of the supernatant quantitatively immunoprecipitated with the indicated antibodies as described above. The immunoprecipitates were visualized on SDS-PAGE gels by fluorography using Enlightning (Bio-Rad Laboratories, Richmond, CA).

## Peptide Analysis

Peptide analysis followed the basic procedure of Cleveland et al. (1977) with modifications as follow. (*a*) Class I and II antigens were immunoprecipitated from detergent extracts of iodine-labeled HT-1080 cells. (*b*) The precipitated antigens were fractionated on SDS-PAGE gels (7% acrylamide) in the absence of 2-ME in order to resolve the α and β subunits of both antigens. The gels were dried, without fixation, and then visualized by autoradiography in order to locate the labeled subunits. (*c*) The dried regions of the gels containing the α and β subunits of the class I and II antigens were excised and placed in the wells of a second SDS-PAGE gel (20% acrylamide) and allowed to rehydrate in the presence of sample buffer for 1 h. (*d*) V8 protease in sample buffer was added to each well (500 ng protease/sample) of the gel and incubated for 60 min at room temperature. The completed gels were visualized by autoradiography.

## Polyacrylamide Gel Electrophoresis

SDS-PAGE gels were prepared following the basic stacking gel system of Laemmli (1970). Samples prepared under reducing conditions were dissolved in a sample buffer containing 2% wt/vol SDS with 5% vol/vol 2-ME and heated in a boiling water bath for 5 min. Samples prepared under nonreducing conditions were treated as above except that 10 mM NEM was substituted for the 2-ME. Prestained protein standards for relative molecular mass estimation were obtained from Bethesda Research Laboratories (Bethesda, MD) and were as follows: lysozyme, 14,300; B-lactoglobulin, 18,400; A-chymotrypsin, 25,700; ovalbumin, 43,000; BSA, 68,000, phosphorylase B, 97,400; and myosin (H chain) 200,000. Protein was determined by the fluorescamine method (Udenfriend et al., 1972).

## Preparation of Affinity Resins

Human plasma fibronectin, purified collagen types I and VI, BSA, and gelatin were coupled to Sepharose CL-4B using the cyanogen bromide activation method (Parikh et al., 1974).

## Differential Cell Extracts

HT-1080 cells were surface labeled following the lactoperoxidase method and $^{125}$I (Hynes, 1973). Labeled cells were extracted sequentially in order to prepare subpopulations of labeled cell proteins. The sequential extraction protocol was basically as previously described (Carter, 1982*b*) and involved

homogenization of labeled cells with a glass Dounce homogenizer (type A) in 25 mM Tris-HCl buffer, pH 7.5, and 1 mM PMSF at 4°C plus the following in sequence: (*a*) 0.34 M sucrose and 1 mM EDTA, in the absence of detergent to solubilize cytoplasmic and peripherally associated cell surface components; (*b*) 1% vol/vol Triton X-100 detergent to solubilize components that required detergent for solubilization; and (*c*) 0.5% wt/vol SDS at 100°C for 5 min to solubilize residual material.

### *Affinity Chromatography*

Cell extracts were chromatographed on BSA-Sepharose in detergent/Tris buffer containing 0.5% wt/vol BSA (detergent/Tris/BSA) to preclear the extracts. Equal aliquots of the labeled proteins were chromatographed on various affinity columns (10 ml protein–Sepharose/column) in the detergent/Tris/BSA buffer. The columns were washed with 2 bed vol of detergent/Tris/BSA, 2 bed vol of detergent/Tris, and 5 bed vol of 0.1% vol/vol detergent/Tris to remove unbound proteins. The labeled proteins bound to the affinity columns were eluted with final wash buffer containing 200 mM NaCl and/or 6 M urea as indicated. The eluted components were dialyzed against water to remove NaCl and urea, lyophilized, and redissolved in water.

Fibronectin receptor was isolated from octylglucoside extracts basically as described by Pytela et al. (1985) and as follows. Labeled HT-1080 cells were extracted in 100 mM octylglucoside detergent in PBS at 4°C containing 1 mM PMSF as a protease inhibitor. The octyglucoside extracts were chromatographed on insoluble fibronectin at 4°C and washed with octylglucoside (25 mM) in PBS. Tuftsin peptide (thr-lys-pro-arg, 1 mg/ml) failed to elute the fibronectin receptor while the peptide gly-arg-gly-asp-ser (1 mg/ml) specifically eluted a 140-kD bound receptor.

## *Results*

Initially, we attempted to identify a human fibroblast cell line that could be used for the isolation of type VI and other collagen receptors. We needed a cell line that would demonstrate rapid and promiscuous attachment to ECM components, including types I and III–VI collagen and fibronectin, and it was critical that the cells not synthesize a stable ECM or significant quantities of disulfide-bonded type VI collagen. As seen in Table I, both normal and transformed human mesenchymal cells attach to pepsinized collagen types I, III, IV, V, and VI, native nonpepsinized type VI collagen (results not shown), fibronectin, and laminin. All cells examined did synthesize soluble forms of native type VI collagen to varying degrees, but only the normal WI-38 cells assembled a stable detergent-insoluble ECM containing disulfide-bonded type VI collagen (Carter, 1982*b*, 1984). The transformed cells, however, maintained the ability to attach and spread on type VI collagen and other ECM components, indicating that they still possessed the full range of receptors. HT-1080 fibrosarcoma cells exhibited the most rapid and extensive adhesion to ECM components at room temperature (data not shown) as well as 37°C (Table I) and were selected for use in isolation of cell surface receptors for type VI collagen. Curiously, A204 cells appeared to exhibit a preference for type VI collagen when compared with other collagen types, even though type V collagen is the major collagenous protein synthesized by these cells (Alitalo et al., 1982).

*Table I. Adhesion of Normal and Malignant Mesenchymal Cells to Protein-coated Surfaces*

| | Cell adhesion (percent of cells added)‡ | | | |
|---|---|---|---|---|
| Adhesion surface* | A204 | HT-1080 | WI-38 | WI-38 VA13 |
| BSA | 0.5 | 0.6 | 0.2 | 0.2 |
| Concanavalin A§ | 38.5 | 89.9 | 85.5 | 52.3 |
| Fibronectin | 36.3 | 73.5 | 63.6 | 64.1 |
| Laminin | 5.6 | 36.1 | 65.9 | 53.7 |
| Collagen | | | | |
| I | 6.8 | 53.4 | 68.4 | 57.6 |
| III | 9.0 | 33.0 | 64.1 | 57.9 |
| IV | 4.5 | 44.0 | 58.1 | 53.1 |
| V | 2.0 | 43.0 | 60.1 | 50.4 |
| VI | 29.4 | 52.8 | 65.8 | 62.7 |

* 48-well virgin styrene plates were coated with the indicated proteins (200 μl protein solution/well, 25 μg protein/ml for 2 h), then washed and blocked with 1% wt/vol heat-denatured BSA in PBS.

‡ Cells were labeled with $^{51}Cr$, suspended by trypsin digestion, washed, placed in the protein-coated wells, and incubated for 30 min. The nonadherent cells were removed by washing and the bound cells were dissolved in SDS/NaOH and counted in a gamma counter. Cell adhesion = cpm (cells bound)/cpm (total cells added).

§ In most cases, cell attachment to concanavalin A represented the maximal number of cells that could bind to the adhesion surface.

### *Cell Adhesion to Collagen and Fibronectin Is Mediated by Independent Receptors*

Previous results (Carter, 1982*a*) have shown that polyclonal antibodies that react specifically with fibronectin will inhibit cell adhesion to fibronectin but not type VI collagen. Thus, cell adhesion to type VI collagen does not proceed via interaction with fibronectin on the cell surface. The possibility that cell adhesion to type VI collagen and fibronectin might

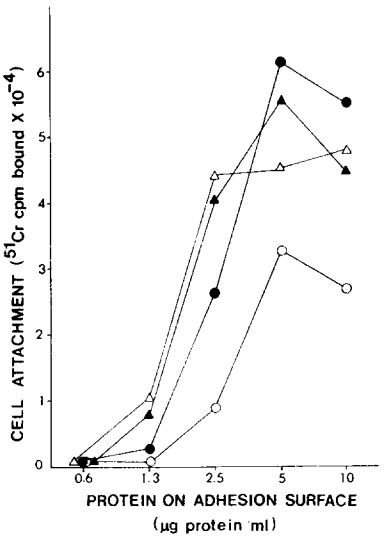


*Figure 1.* Inhibition of cell attachment to fibronectin and type VI collagen with soluble cell attachment domain of fibronectin. Fibronectin and type VI collagen were coated on plastic surfaces as described in Materials and Methods (0.6–10.0 μg protein/ml per well). The soluble cell attachment domain of fibronectin was isolated from thermolysin digests and added to the BSA-blocked adhesion surfaces as a competitive inhibitor of cell adhesion (final concentration of 1 mg/ml). HT-1080 cells were labeled with $^{51}Cr$ and added to the attachment assay, incubated for 30 min, and then washed to remove nonadherent cells. The adherent cells were dissolved in SDS/NaOH and counted in a gamma counter. (*Open triangles*) VI plus cell adhesion domain of fibronectin; (*solid triangles*) VI minus cell adhesion domain of fibronectin; (*open circles*) fibronectin plus cell adhesion domain of fibronectin; (*solid circles*) fibronectin minus cell adhesion domain of fibronectin.

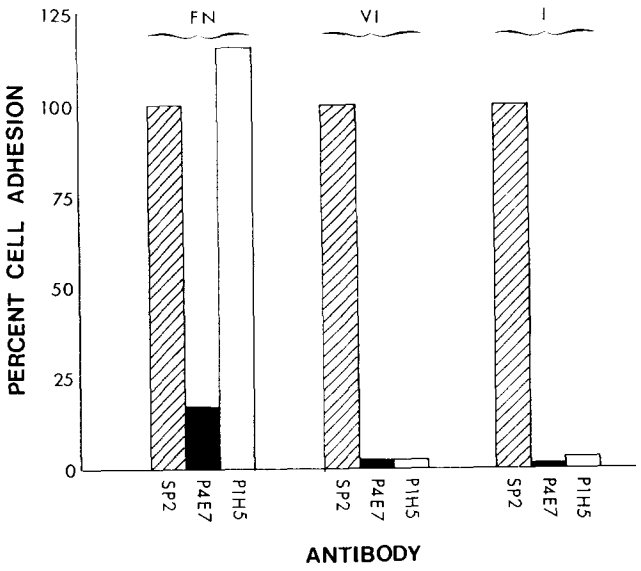


*Figure 2.* Inhibition of HT-1080 cell attachment to fibronectin, type I, and type VI collagens with class I (P4E7) and class II (P1H5) monoclonal antibodies. Cells were labeled with $^{51}Cr$ and incubated on plastic surfaces coated with fibronectin (FN), type VI collagen (VI), and type I collagen (I) in the presence of the indicated antibodies (100 μg antibody/ml final concentration) as described in Materials and Methods. After 30 min incubation the nonadherent cells were removed by washing and the adherent cells dissolved in SDS/NaOH and counted in a gamma counter. Total cells bound to each adhesion surface in the presence of SP2 cell culture supernatant alone, control, are indicated as 100%. The inhibitory effect of the class I (P4E7) and class II (P1H5) antibodies is calculated as a percentage of cell adhesion in the presence of SP2 cell culture supernatant alone.

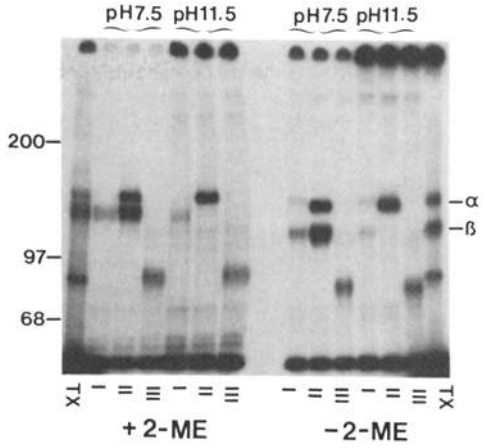


*Figure 3.* The effect of reducing agents and basic pH on the migration and association of the subunits of class I–III antigens. HT-1080 cells were surface labeled with radioactive iodine, then extracted with Triton X-100 detergent (*TX*). Aliquots of the soluble extract were immunoprecipitated with class I (P4E7), class II (P1H5), and class III (P1G12) monoclonal antibodies. Before immunoprecipitation, the extracts were either untreated or subject to temporary elevation of the pH to 11.5 followed by neutralization in order to dissociate the subunits. The immune precipitates were developed on SDS-PAGE gels (7% acrylamide) in the presence and absence of 2-ME (*+ or − 2-ME*) and visualized by autoradiography. Migration of the α and β subunits of the class I and II antigens under nonreducing conditions are indicated at right.

involve distinct receptors was investigated by means of a competitive inhibition assay (Fig. 1). The data depicted in Fig. 1 show clearly that HT-1080 cell attachment to type VI collagen (or type I and III collagen, results not shown) was not inhibited by a proteolytic fragment of plasma fibronectin containing the cell attachment domain (140 kD, Fig. 1). In contrast, the cell attachment domain of plasma fibronectin (1 mg/ml) significantly inhibited HT-1080 cell attachment to fibronectin (54%). In addition, HT-1080 cell attachment to types I, III, and VI collagen were also unaffected by the arg-gly-asp-ser (RGDS)-containing peptides (data not shown). Similar inhibition results for cell adhesion to interstitial collagens with RGDS-containing peptides have been reported by Hayman et al. (1985). On the basis of these data it can be concluded that types I, III, and VI collagen and fibronectin do not compete for the same receptor binding site and that the collagen receptor may be RGDS independent.

### *Preparation of Monoclonal Antibodies that Specifically Inhibit the Interaction of Human Fibroblasts with Type VI Collagen*

We attempted to identify the cell surface receptor(s) involved in attachment of mesenchymal cells to type VI collagen by preparing monoclonal antibodies to HT-1080 cells that would alter cell adhesion to ECM components. Monoclonal antibodies were successfully screened via a three-stage process. (*a*) Hybridomas producing antibodies that would react with whole HT-1080 cells in a solid-phase assay were identified. (*b*) Antibodies from HT-1080–positive hybridomas were screened for their ability to immunoprecipitate cell surface proteins from Triton X-100 detergent extracts of HT-1080 cells surface-labeled with radioactive iodine by the lactoperoxidase method. (*c*) Antibodies that would immunoprecipitate cell surface proteins were subsequently screened for their ability to inhibit or increase cell adhesion to type VI collagen. Since we were interested in obtaining antibodies to receptors specific for type VI collagen we also screened hybridoma supernatants for the ability to inhibit type I collagen, fibronectin, and laminin-mediated attachment as well. All positive hybridomas were subsequently cloned by limiting dilution to ensure monoclonality.

Using this screening process, we identified two classes of monoclonal antibodies that could alter HT-1080 cell attachment to type VI collagen. Class I monoclonal antibodies, represented here by P1B5, P2E6, and P4E7, all inhibited HT-1080 cell attachment to type VI and I collagens and, surprisingly, partially inhibited attachment to fibronectin (Fig. 2) and laminin as well (results not shown). Class I antibodies all precipitated the same cell surface protein. By SDS-PAGE, the class I antigens migrated as two bands with relative molecular masses of 147 and 125 kD under nonreducing conditions and were termed α and β, respectively (Fig. 3). In the presence of 2-ME, the α and β subunits reversed their relative order of migration but were poorly resolved and migrated with molecular masses of 130 and 135 kD, respectively. The fact that the α subunit of the class I antigen was found to migrate with lower relative molecular mass than the β subunit under reducing conditions was determined as fol-

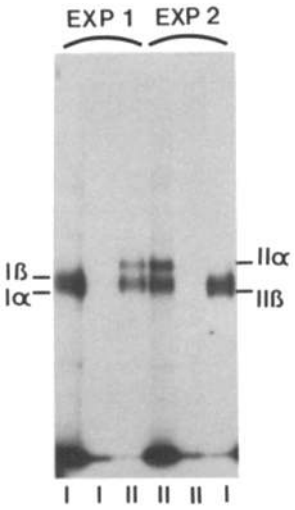


*Figure 4.* Sequential immunoprecipitation of class I and class II antigens. Experiment 1 (*EXP 1*): Triton X-100 detergent extracts were prepared from HT-1080 cells after surface labeling with radioactive iodine. An aliquot of the labeled extract was subject to three cycles of immunoprecipitation as follows. (*a*) Class I (P1B5) antibody was used to precipitate antigen from the extract. (*b*) The extract was then reprecipitated with class I antibody to ensure that the class I antigen was quantitatively removed. (*c*) The class I–free extract was then immunoprecipitated with class II (P1H5) antibody. The antigens from the three sequential immunoprecipitation steps were analyzed on SDS-PAGE gels (7%) in order (labeled I, I, and II, from left to right) in the presence of 2-ME followed by autoradiography. Migration of the α and β subunits of the class II (*right*) and class I (*left*) are indicated. Experiment 2 (*EXP 2*): the procedure was repeated as described above except that the sequence of precipitation steps was class II, class II, and class I (labeled II, II, and I from left to right).

lows. The α and β subunits were purified on a preparative SDS-PAGE gel under nonreducing conditions, where the subunits are resolved. The isolated subunits were again subjected to electrophoresis under reducing conditions (both side by side and remixed), where the α subunit migrated with a lower molecular mass than the β subunit (results not shown).

Class II monoclonal antibodies, represented by clone P1H5, inhibited cell adhesion to type VI and I collagens (and type III, IV, and V collagens, data not shown) only and not to fibronectin (Fig. 2) or laminin (data not shown). The class II antibodies precipitated a single cell surface protein with two subunits with molecular masses of 145 and 125 kD for the α and β subunits, respectively, under nonreducing conditions. In the presence of 2-ME, the α and β subunits migrated with molecular masses of 140 and 135 kD, respectively. The data obtained from inhibition of cell attachment studies with class II antibodies strongly suggested that attachment to collagen was mediated by this class II receptor.

A third class of monoclonal antibody, termed class III, and represented by clones P1G12 and P3H9, either increased cell adhesion to collagen (under conditions of minimal cell attachment) or had no effect (similar to the SP2 control in Fig. 2, when attachment approached maximum), and immunoprecipitated an antigen that migrated as a diffuse single band on SDS-PAGE gels with a relative molecular mass of 90 kD under reducing conditions and 80 kD under nonreducing conditions (Fig. 3). The class III antigen appeared to be similar to an 80–90-kD cell surface glycoprotein we previously reported as binding to type VI collagen (Carter and Wayner-Carter, 1986). Therefore, antibodies reactive with class III antigen were selected for further study.

As previously indicated (Fig. 3), class I–III antigens could be labeled by lactoperoxidase-catalyzed iodination, indicating that they are present on the cell surface. These proteins could also be metabolically labeled with radioactive amino acids (Fig. 6) and glucosamine (data not shown), indicating that they are cell-synthesized glycoproteins. The membrane localization of all three classes of antigen was further established by immunofluorescence microscopy (data not shown).

These results indicated that HT-1080 cell adhesion to fibronectin and collagen could be differentiated on the basis of inhibition with the class I and class II monoclonal antibodies. They further suggested that cell adhesion to collagen was mediated by both class I and II antigens and that adhesion to fibronectin was mediated, at least in part, by the class I antigen. Results to be presented below and elsewhere (Wayner, E. A., and W. G. Carter, work in progress) suggested that there are at least two distinct fibronectin receptors. The relationship of the class I antigen to the fibronectin receptor described by others (Pytela et al., 1985) will be discussed in detail (see below).

### *Comparison of Class I and II Antigens*

Sequential immunoprecipitation of the class I and II antigens with the appropriate antibodies indicated that neither antibody class recognized antigen from the other class (Fig. 4). Similar comparisons were also made between class III and class I or II antigens using sequential immunoprecipitation, with no indication of cross-reactivity between the antibody classes. Immunoprecipitation of the class II antigen after temporary adjustment of the pH from 7.5 to 11.5 and back again in order to dissociate the subunits resulted in precipitation of only the α subunit (Fig. 3). Thus, the class IIβ subunit is noncovalently associated with the α subunit and is not recognized by the class II antibody. Subunit-dissociating conditions (pH 11.5, see Fig. 3, SDS or SDS plus 2-ME) either failed to dissociate the α and β subunits of the class I antigens or denatured the epitope recognized by the antibody. Thus, the α and β subunits of the class I antigens appeared to be more tightly associated than the subunits of the class II antigen or the class I antibody (P1B5) reacted with both subunits. Regardless, this method did not enable us to conclusively identify which subunit contained the epitope recognized by the class I antibody. However, since anti-class IIα did not cross-react with class I (Fig. 4) and since the class Iβ and IIβ subunits appeared to be identical (see below) it can be inferred that P1B5 anti-class I probably reacts with either an α-specific or an α–β complex. This possibility was confirmed by the pulse-chase labeling described below.

The β subunits of the class I and II antigens co-migrated under both reducing and nonreducing conditions (Fig. 3) in contrast to the α subunits, suggesting that the β subunits may be structurally related proteins. This possibility was confirmed by proteolytic digestion of the α and β subunits from both classes of antigen using V8 protease followed by comparison of the peptides by the method of Cleveland et al. (1977). As seen in Fig. 5, the peptide maps of the β subunits were virtually identical, suggesting a high degree of amino acid sequence homology. In contrast, the peptide maps of the α subunits were clearly distinguishable. Curiously, the class IIα subunit did exhibit some degree of homology to both β subunits. The structural similarities of the class I and II antigens suggested that one antigen may be a metabolic precursor of the other. This possibility was further analyzed by following the kinetics of synthesis of the two antigens by pulse-chase labeling studies with radioactive methionine

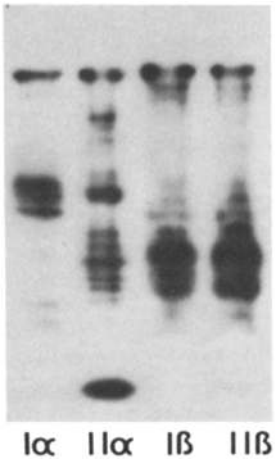


*Figure 5.* Comparison of the peptide maps of the α and β subunits of class I and II antigens after digestion with V8 protease. The α and β subunits of class I and II antigens were isolated from Triton X-100 extracts of surface-labeled HT-1080 cells by immunoprecipitation and preparative SDS-PAGE under nonreducing conditions. Each isolated subunit was digested with *S. aureus* V8 protease, followed by comparison of the peptide maps on SDS-PAGE gels (20% acrylamide), and autoradiography.

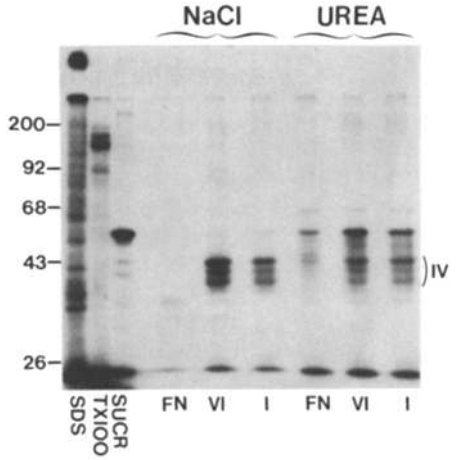


*Figure 7.* Identification of the peripheral cell surface receptors for type I and type VI collagen by affinity chromatography of HT-1080 cell extracts. Surface-labeled cells were differentially extracted as described in Materials and Methods. The three extracts, peripheral (*SUCR*), membrane (*TX*), and SDS-soluble (*SDS*) were compared. The extract containing the labeled peripheral components that did not require detergent for solubilization was divided into equal aliquots and chromatographed on type I collagen-Sepharose (*I*), type VI collagen-Sepharose (*VI*), and fibronectin-Sepharose (*FN*). Unbound protein was removed by washing and bound protein eluted first with 200 mM NaCl (*NaCl*) and second with 6 M urea (*UREA*). The eluted material was analyzed on SDS-PAGE gels (12% acrylamide) by autoradiography. Migration of standard proteins is indicated at left.

(Fig. 6). A number of specific points were made clear by these studies. (*a*) Throughout the duration of the study (0–30 h), both the α and β subunits of class I and II antigens exhibited similar kinetics for methionine incorporation, as observed by immunoprecipitation. Thus, there was no indication that either class I or II antigen was a precursor to the other. (*b*) A metabolic precursor to the class I antigen, labeled preI in Fig. 6, was observed during the first 2 h of the chase period. The preI form did not co-migrate with any of the common β subunits in either class I or II and was not precipitated by the α-specific class II antibody. Therefore, preI probably corresponded to the precursor to the Iα subunit before association with the common β subunit. Thus, the PIB5 class I monoclonal antibody must react with the Iα subunit, not the Iβ or α–β combination. (*c*) We did not observe that the class IIα subunit was a precursor to the class IIβ subunit. Even after a prolonged chase period (30 h), there was no observed decrease of label in IIα corresponding to an increase in IIβ. The epitope for the class II antibody was localized to the α subunit (Fig. 3). Thus, the observed 30–60-min delay in precipitation of the IIβ subunit probably reflects the posttranslational association of the IIβ subunit with the IIα subunit. This delay in posttranslational association was also observed for the association of the Iα and Iβ subunits.

### *Identification of Multiple Cell Surface Receptors for Type VI Collagen and Fibronectin by Affinity Chromatography*

Surface-labeled HT-1080 cells were sequentially extracted with buffer containing: (*a*) isotonic sucrose, with no detergent, to solubilize peripheral components that were not intrinsic to cellular membranes; (*b*) Triton X-100 detergent, to solubilize membrane components; and (*c*) SDS to dissolve the residue (see Fig. 7). Greater than 70% of the total cell protein was soluble in the absence of detergent, including labeled proteins in the molecular mass range of 30–60 kD (Fig. 7). Extraction with Triton X-100 detergent released multiple

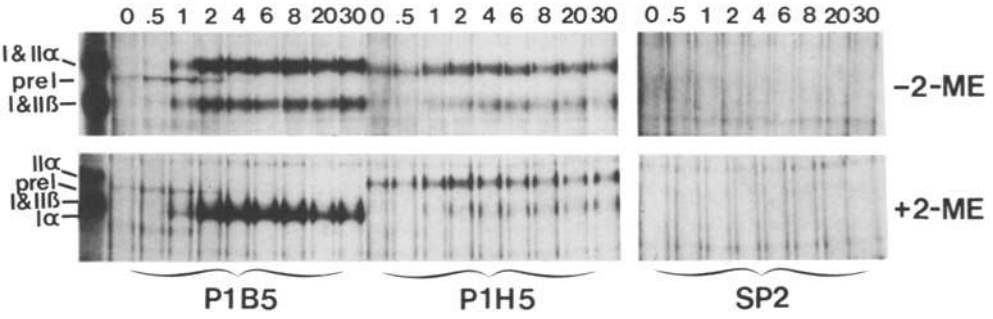


*Figure 6.* Analysis of the kinetics of synthesis of class I and II antigens by pulse-chase metabolic labeling. HT-1080 cells were pulse labeled with radioactive methionine for 1 h and then chased with nonradioactive methionine for the indicated time periods (0, 0.5 h, etc.). Time point 0 corresponds to the end of the pulse-labeling period. Triton X-100 detergent-soluble extracts were prepared from the cells and immunoprecipitated with class I (*PIB5*) and class II (*PIH5*) monoclonal antibodies or control culture supernatant (*SP2*) and then analyzed on SDS-PAGE gels (7% acrylamide) in the presence and absence of 2-ME (*+ or − 2-ME*) followed by fluorography. Only relevant regions of the fluorographs are shown. The 30-h SP2 control (*+ 2-ME*) time point is not shown. Migration of the α and β subunits of the class I and II subunits are indicated at left. Migration of precursor forms of the class I antigens are labeled *preI*. Migration of $^{125}I$ surface-labeled class I and II antigens from HT-1080 cells are shown in the gel lanes at far left.

labeled proteins that required detergent for solubilization and co-migrated with the antigens precipitated with the class I and III monoclonal antibodies (Fig. 7). The two extracts containing the peripheral and membrane components solubilized with Triton X-100 detergent were each diluted in buffer containing BSA (0.5% wt/vol) and Triton X-100 detergent and then passed over BSA-Sepharose to remove proteins that might bind nonspecifically to subsequent affinity columns. Gelatin-Sepharose, or denatured collagen, was also included as a further control to identify nonspecifically bound proteins and gave results similar to BSA-Sepharose (results not shown). Aliquots of the extracts were chromatographed on fibronectin-, type I collagen-, and type VI collagen-Sepharose. Each column was washed with buffer containing Triton X-100 to remove unbound components. Bound proteins were eluted with wash buffer containing, first, 200 mM NaCl, and second, 6 M urea. A two-stage elution protocol was used because we assumed that the various components, particularly the hydrophobic membrane proteins, might exhibit weak interactions with the affinity columns after solubilization with detergents and would be denatured by an ionic environment. Physiological salt concentrations have previously been reported to disrupt receptor–collagen interactions (Mollenhauer and von der Mark, 1983).

Three proteins with molecular masses of 38, 42, and 45 kD from the peripheral cell extracts (Fig. 7) bound to both type I and VI collagen but not fibronectin and were termed class IV receptors. These proteins could be partially eluted with NaCl. A protein of 56 kD bound to all the affinity columns, exhibiting no binding specificity, and required urea for elution. These results suggest that the class IV receptors bind preferentially to both type I and VI collagens and that these proteins are peripherally associated cell surface components and not intercalated into the plasma membrane.

The Triton X-100 detergent extract of HT-1080 cells contained two labeled cell surface proteins or groups of proteins that bound reproducibly to both type I and type VI collagen with molecular masses of approximately 140 kD (termed class I receptor) and 80–90 kD (termed class III receptor) when subjected to electrophoresis under reducing conditions (Fig. 8). In contrast, a single labeled protein of 140 kD bound to fibronectin. These results indicated that the class III and IV receptors bond only to collagen, while class I–like receptors, possibly identical, bond to both collagen and fibronectin. The failure of any of these receptors to bind to gelatin indicated that fibronectin, possibly present in the cell extract, did not link the receptors to the native collagen-Sepharose. It also indicated that the receptors preferentially bound to native collagen conformations.

### *Identification of the Collagen and Fibronectin Receptors as Class I and III Antigens*

The possibility that any of the class I–III antigens defined by the monoclonal antibodies may be identical to the cell surface proteins bound to the affinity columns was examined by (*a*) comparing the mobility of the various components on SDS-PAGE gels under both reducing and nonreducing conditions (Fig. 9) and (*b*) immunoprecipitation of the affinity-purified receptors with the monoclonal antibodies (Fig. 10). The class I antigen co-migrated with the class I receptor that bound to both fibronectin and collagens. The class I receptor exhibited the characteristic alterations in migration of the α and β subunits of the class I antigen under reducing and nonreducing conditions in contrast to the distinct migration of the class II antigen as previously described (Fig. 3). In addition, the class I antibody, but not the class II antibody, immunoprecipitated the class I receptors bound to both fibronectin

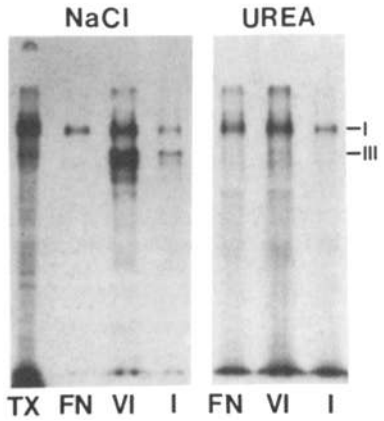


*Figure 8.* Identification of membrane receptors for type I and type VI collagen and fibronectin by affinity chromatography of HT-1080 cell extracts. Surface-labeled cells were differentially extracted as described in Fig. 7 and Materials and Methods. The labeled membrane components that required Triton X-100 detergent for solubilization (*TX*) were extracted, chromatographed, and analyzed as described in Fig. 7. The quantity of radioactive material eluted with urea was minimal compared with material eluted with NaCl, and was therefore given five times the exposure period of the NaCl-eluted material.

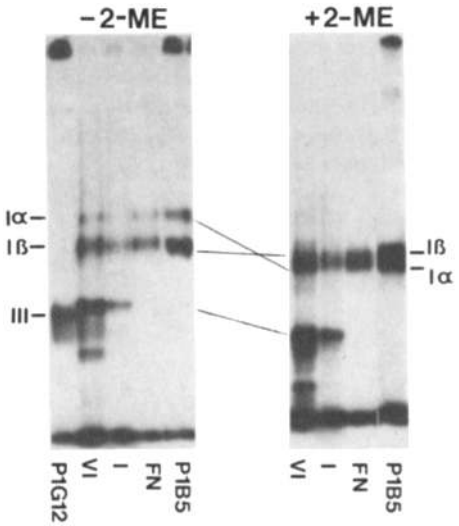


*Figure 9.* Comparison of the class I and III antigens to the affinity-purified receptors for fibronectin, type I, and type VI collagen. Surface-labeled HT-1080 cells were differentially extracted as described in Fig. 7 and Materials and Methods. The Triton X-100 detergent extract was used for affinity purification of receptors for fibronectin (*FN*), type I collagen (*I*), and type VI collagen (*VI*) as described in Fig. 8, using salt elution. The detergent extract was also used for immunoprecipitation with class I (P1B5) and class III (P1G12) antibodies. The relative migration of the isolated receptors and antigens were compared on SDS-PAGE gels (7% acrylamide) in the presence and absence of 2-ME (*+ or − 2-ME*) after autoradiography.

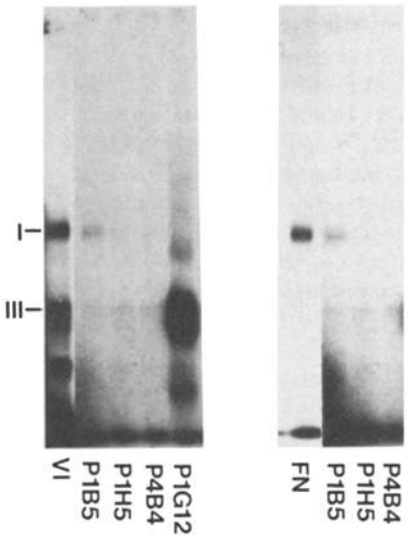


*Figure 10.* Immunoprecipitation of affinity-purified receptors for fibronectin, type I, and type VI collagen with class I and III monoclonal antibodies. Receptors for type VI collagen (*VI*) and fibronectin (*FN*) were affinity purified from Triton X-100 extracts of surface-labeled HT-1080 cells as described in Fig. 5. The receptors were then immunoprecipitated with the following antibodies: class I, P1B5; class II, P1H5 and P4B4; and class III, P1G12. The immunoprecipitates and the receptors were compared on SDS-PAGE gels (7% acrylamide) in the absence of 2-ME by autoradiography.

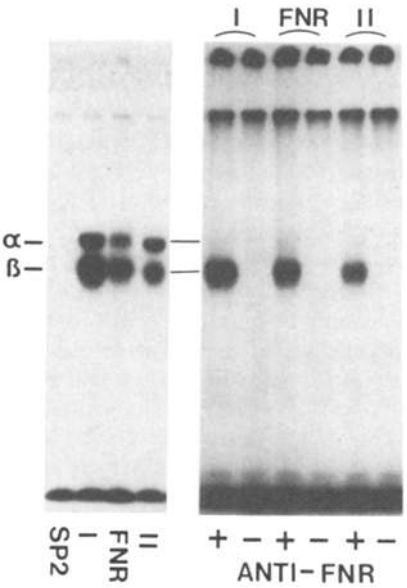


*Figure 11.* Relation of the class I and II receptors to the FNR. (*Left*) Triton X-100 detergent-soluble extracts were prepared from surface-labeled HT-1080 cells and immunoprecipitated with the class I, II, and anti-FNR antibodies or control SP2 culture supernatant. The antigens were fractionated on SDS-PAGE gels (8%) in the absence of 2-ME in order to resolve the α and β subunits. The migration of the subunits are indicated at left. (*Right*) The class I, II, and fibronectin receptors were purified by immunoprecipitation as above, and then the α and β subunits and antigen–antibody complexes were dissociated in SDS at 100°C. The dissociated subunits from each receptor were then reprecipitated with anti-FNR and examined on SDS-PAGE gels in the absence of 2-ME. The anti-FNR immunoprecipitated the β subunit from all three receptors.

and collagen (Fig. 10). These results indicated that the class I receptor that bound to fibronectin and collagen contained detectable levels of the class I antigen. The fact that the class I receptor could be immunoprecipitated from proteins affinity purified on type VI collagen and fibronectin confirmed the cell attachment data (Fig. 2) and strongly suggested the existence of fibroblast cell surface receptors capable of interacting with multiple ECM components. We are currently investigating whether the class I receptor can interact with RGD-containing peptides. In the presence of Triton X-100 or zwitterionic detergents such as Empigen BB, the class II receptor did not appear to bind native collagen (See Discussion).

As seen in Fig. 9, the class III antigen was found to co-migrate with the class III collagen receptor and this receptor was immunoprecipitated with the class III antibody (Fig. 10). A protein band migrating at ~90 kD was not immunoprecipitated with the class III antibody and may represent an additional receptor unrelated to the class III antigen. This possibility is currently under further investigation. We also found that the isolated class III receptor degraded on storage, with generation of lower molecular mass degradation products, primarily in the 60-kD range. These degradation products could not be immunoprecipitated with the class III antibodies.

### *The Relation of Class I Antigen to the Previously Described Fibronectin Receptor*

Both the class I and II receptors bore remarkable structural similarities to the fibronectin receptor (FNR) isolated from osteosarcoma cells by Pytela et al. (1985). However, both the class I and II receptors exhibit different binding specificities, based on inhibition of cell adhesion, then ascribed to the FNR. To facilitate a comparison of the class I and II receptors to the FNR, we obtained rabbit polyclonal antibodies prepared against the FNR, termed anti-FNR. The relationships of the class I and II antigens to the FNR were examined in the following series of experiments. (*a*) Immunoprecipitation of Triton X-100 detergent extracts prepared from surface-labeled HT-1080 cells was performed with class I and II monoclonal antibodies and anti-FNR. On SDS-PAGE gels, under reducing (Fig. 11) and nonreducing conditions (results not shown), the migration of the α and β subunits of the class I and FNR antigens were virtually identical (Fig. 11). As expected, under nonreducing conditions, only the β subunit of the class II antigens co-migrated with the other β subunits (data not shown). Thus, on the basis of subunit migration, the class IIα appeared to be distinct from the FNRα. In contrast, the class Iα subunit was similar to the α subunit of the FNR. (*b*) Sequential immunoprecipitation experiments revealed that the β subunits of all three antigens (I, II, and FNR) were identical. The class I and II antigens were purified by immunoprecipitation, denatured to separate the α and β subunits, and then reprecipitated with anti-FNR. As seen in Fig. 11, anti-FNR immunoprecipitated the β subunits of the class I and II antigens, suggesting that all three antigens bear the same β subunit in support of our peptide-

mapping studies (Fig. 5). Further, we compared the β subunits of the class I and II receptors and FNR by peptide analysis using the method of Cleveland et al. (1977) and detected extensive homology in all three β subunits (results not shown). Anti-FNR did not immunoprecipitate the Iα subunit, suggesting that the class Iα and FNRα antigens were not identical (Fig. 11). However, in control experiments, anti-FNR reacted only weakly with its own denatured FNRα subunit (Fig. 11), suggesting that anti-FNR was primarily β subunit specific. The possibility that anti-FNR might react more specifically with nondenatured FNRα or class Iα was evaluated by preclearing experiments. However, without prior dissociation of the α and β subunits, anti-FNR was found to partially immunoprecipitate both class I and II antigens as well as additional proteins containing similar α–β subunit structures, as would be expected due to the common β subunit specificity (results not shown). Because the anti-FNR antibodies reacted primarily with the common β subunit, further comparison of the class Iα and IIα subunits with the FNRα were not feasible using this antibody. (*c*) To compare the α subunits of the class I and II receptors to FNRα, we purified FNR from octylglucoside extracts of surface-labeled HT-1080 cells by chromatography of the extracts on insoluble fibronectin. The bound FNR was not eluted with a nonadhesive peptide, thr-lys-pro-arg (Tuftsin), but was specifically eluted with the adhesion-active peptide, gly-arg-gly-asp-ser, as described by Pytella et al. (1985). This affinity-purified FNR co-migrated with the class I receptor on SDS-PAGE gels under both reducing and nonreducing conditions. However, the RGDS peptide–eluted FNR did not react with the class I monoclonal antibody but did react with the β subunit–specific anti-FNR antibodies (results not shown).

In summary, based on the inhibition of cell adhesion data (Fig. 2) and the results with the β subunit–specific anti-FNR (Fig. 11) and affinity-purified FNR prepared as described by Pytela et al. (1985), we conclude that: (*a*) the class I, II, and FNR antigens are all members of a related family of cell surface ECM receptors that possess common β subunits; and (*b*) the α subunits of the class I, II, and FNR antigens are immunologically and functionally unique. Thus, class I and II represent two new members of the ECM receptor family that possess α subunits which are functionally distinct from the previously described FNR.

## Discussion

Many normal human mesenchymal cells synthesize a complex ECM which contains multiple detergent-insoluble adhesive glycoproteins, including fibronectin, type VI collagen, and GP250 (Carter, 1982*a*, *b*). We have previously proposed that these proteins may function cooperatively to mediate cell adhesion (Carter, 1982*a*). To further clarify the mechanisms of adhesion to and assembly of the ECM, we have identified four classes of cell surface protein receptors, classes I–IV, that interact with collagen and/or fibronectin as determined by: (*a*) inhibition of cell adhesion with monoclonal antibodies to receptors and/or (*b*) affinity chromatography on immobilized ECM proteins.

### *Class II Antigen: A Collagen-specific Receptor Involved in Cell Adhesion*

Although we initiated these studies to identify the receptor for type VI collagen, we did not obtain any antibodies that specifically inhibited cell attachment to type VI. All the class II antibodies that inhibited attachment to type VI collagen also inhibited attachment to other collagens, but not to fibronectin, laminin, or lectin-coated surfaces. However, very little class II antigen bound to native collagen columns under the present affinity chromatography conditions. In contrast, the promiscuous class I receptor exhibited a much more stable interaction with ligands and was shown to bind to both collagen and fibronectin in support of the monoclonal antibody inhibition data. At the present time we have no explanation for the difference in the stability of class I and II receptor–ligand interaction. One possible explanation might simply be that the association of the class II α and β subunits is unstable. For example, high pH dissociated the class II α and β subunits, but not the class I α and β subunits. We also observed that the α subunit–specific epitope recognized by the class II antibodies was denatured by Empigen BB, a zwitterionic detergent. This detergent had no effect on the epitopes recognized by the class I antibodies. Taken together, these results suggest that the class II receptor is unstable and may not retain its collagen-binding conformation after solubilization with most detergents. Alternatively, the naturally occuring receptor for the class II antigen may be a complex of collagen with some other component of the ECM, such as fibronectin or glycosaminoglycan. Both possibilities are currently being investigated. The class II antigen appears to be a new member of the family of adhesion receptors with common β subunits and unique α subunits, which confer binding specificity for multiple components of the ECM involved in cell adhesion.

### *Class I Receptor: A Promiscuous Cell Adhesion Receptor for Multiple Components of the ECM*

The class I receptor bound to fibronectin in affinity chromatography experiments and reacted with the class I monoclonal antibodies which inhibited cell adhesion to fibronectin. These results indicate that HT-1080 cell adhesion to fibronectin is mediated, at least in part, by the class I receptor. However, the class I receptor also bound to type I and VI collagens and cell adhesion to collagen and laminin were also inhibited by class I monoclonal antibodies. Because of their obvious biochemical similarities, it was necessary to investigate the relationship of the class I and II antigens to the FNR described by Pytela et al. (1985). Polyclonal antibodies specific to the FNR were found to cross-react with the β subunits of both the class I and II receptors, indicating that they are all member of the same ECM receptor family. However, no cross-reaction of the anti-FNR antibodies were observed with the α subunits of either the class I or II receptors after the α subunits were dissociated from the common β subunits. In addition, neither the class I nor II antibodies reacted with the FNR isolated by RGDS peptide elution from fibronectin-Sepharose. Thus, both the class I and II receptors are new members of the ECM receptor family and shall be referred to as ECMRI and II in the future. In support of the observed differences between the class I and II receptors and FNR, we have recently isolated a new class of monoclonal antibody, referred to as class VI. The class VI monoclonal antibodies specifically inhibit cell adhesion to fibronectin and immunoprecipitate an antigen containing the common β subunit and a third unique α subunit (Wayner, E. A., and W. G. Carter, work in progress). The relationship of the class VI receptor to the FNR is being investigated. However,

it seems clear from the present findings that attachment of cells to fibronectin may involve multiple independent receptors (class I, VI, and/or the FNR). Heterogeneity in the binding specificity of the FNR has already been suggested by Horwitz et al. (1985), who reported that the CSAT antibody inhibited cell adhesion to both laminin and fibronectin. It should be interesting to determine the amino acid sequences present in collagen and fibronectin recognized by the class I receptor. It is possible that this receptor either recognizes a sequence common to collagen and fibronectin, or is capable of interacting with multiple distinct adhesion-promoting sequences. Conceivably, such promiscuity in the binding specificity of the class I receptor from human fibrosarcoma HT-1080 cells may not be exhibited by this receptor in all cells. Further, differential expression of the three receptors, class I, II, and VI, by normal versus transformed mesenchymal cells, epithelial cells, and platelets have been observed (Wayner, E. A. and W. G. Carter, manuscript in preparation). Altered specificity and/or inappropriate expression of specific receptors for ECM components are attractive possible explanations for variation in the metastatic potential of malignant cells or tissue-homing specificity.

Recently, various groups have identified single or even families of related proteins that probably mediate cell adhesion to fibronectin, vitronectin, and laminin in human, rodent, and chicken cells. It seems clear that the collagen receptor, class II, and the promiscuous class I ECM receptor are new members of the family of ECM receptor proteins. Although we were not successful in isolating a specific receptor involved in adhesion to type VI collagen, we are still assuming that interaction of mesenchymal cells with different collagen types occurs at the level of a specific membrane receptor. Conceivably, interaction of a cell with a particular collagen type may depend on additional interactions with globular domains residing outside of the pepsin-resistant, collagenous domains.

### *Class III and IV Receptors*

In contrast to the inhibitory effect of class I and II antibodies on cell adhesion, class III antibodies stimulated attachment or had no effect. The class III antibodies immunoprecipitated a cell surface glycoprotein of 80–90 kD that could be metabolically or cell surface–labeled and was distinct from either class I or II antigens. We have previously described a similar cell surface glycoprotein which is resistant to extraction with detergent, suggesting a possible interaction with the ECM or cytoskeleton (Carter, 1982*a*), and which specifically binds to type VI collagen in affinity chromatography experiments (Carter and Wayner-Carter, 1986). We have also recently obtained evidence that class III shows a preferential association with cytoskeletal components (Carter, 1985; Carter, W. G., and E. A. Wayner, manuscript in preparation). These results suggest that the class III antigen may associate with type VI collagen in the ECM and perhaps with the cytoskeleton on the cytoplasmic side of the membrane. We have also purified three cell surface proteins, termed the class IV receptors, with subunit molecular masses of 38, 42, and 45 kD which bind to both type I and VI collagen. The class IV receptors differ from I–III in that they do not require detergent for solubilization from the membranes and are therefore probably peripherally associated with the cell surface. Kurkinen et al. (1984) have described similar proteins, and a major 47-kD protein, termed "colligin," which binds gelatin as well as native type IV collagen. The relation of the class IV receptors to these and other related proteins will be described elsewhere (Carter, W. G., and E. A. Wayner, manuscript in preparation).

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