The Role of Intermolecular Disulfide Bonding in Deposition of GP140 in the Extracellular Matrix

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ABSTRACT   Human WI-38 fibroblasts in cultures synthesized at least three molecular forms of
the major, extracellular matrix glycoprotein (GP), GP140: (a) cytoplasmic GP140 (1.2 ng of
GP140/μg of cell protein) was detergent-soluble, underglycosylated, and possessed detectable
levels of intermolecular disulfide bonding; (b) matrix GP140 (3.6 ng of GP140/μg of cell
protein) was detergent-insoluble, more highly glycosylated and polymerized by intermolecular
disulfide bonding, and co-distributed in the extracellular matrix with fibronectin; and (c)
released GP140 (2 ng of GP140/μg of cell protein per 24 h) was recovered in the conditioned
culture media and lacked intermolecular disulfide bonding. Cytoplasmic GP140 was the
immediate biosynthetic precursor of the matrix form of GP140. In addition, various human
adult and fetal tissues contained a form of GP140 that resembled the fibroblast matrix GP140
in the degree of intermolecular disulfide bonding, relative molecular mass, and immunological
reactivity. Analysis of the sequence of events in assembly of GP140 and fibronectin in the
extracellular matrix detected the following: (a) fibronectin was first to appear in the extracellular
matrix; (b) GP140 accumulated in the cytoplasm, then deposited in the extracellular matrix
and co-aligned with the established fibronectin; and (c) maturation of the extracellular matrix
proceeded by continued intermolecular disulfide bonding. To evaluate possible roles for
intermolecular disulfide bonding in cell interactions, a unique assay system was utilized based
on the ability of labeled cells to incorporate radioactive matrix components into a biotinylated
exogenous matrix. Precipitation of the biotinylated matrix from extracts of the cultures using
avidin indicated: (a) disulfide bonding of radioactive GP140 and fibronectin into the exogenous
biotinylated matrix required cell contact with the matrix. The newly deposited GP140 and
fibronectin derived from the cells and not from GP140 and fibronectin present in the
conditioned culture media. (b) Pro-α1 and Pro-α2 procollagens, present in the culture media,
bound to the exogenous matrix in a noncovalent manner and were independent of cell
contact. (c) SV40 transformed cells (WI-38 VA13) synthesized released form GP140 but did
not deposit GP140 into the biotinylated matrix.

The extracellular matrix of cultured human fibroblasts has been reported to contain fibronectin (1–10), procollagens, (2, 4, 7), chondroitin sulfate (5), and heparan sulfate (5). Two additional cell surface-labeled glycoproteins (GPs),[1] GP250 and GP140, have also been described as components of the

detergent-insoluble matrix of WI-38 fibroblasts (8–10). GP250 and GP140, like fibronectin, were absent from the extracellular matrix of SV40 transformants of WI-38 cells (WI-38 VA13 cells) and the human fibrosarcoma cell line HT-1080 (10). However, both normal and transformed cells accumulated these glycoproteins in their conditioned media (10).

The quantities of GP140 and fibronectin in the matrix of normal fibroblasts were elevated with increasing cell density and time in culture (8). The matrix fibronectin and GP140

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[1] Abbreviations used in this paper: BNS, 50 mM sodium borate buffer, pH 7.8, containing 25 mM NaCl and 1 mM EDTA; DME, Dulbecco's modified Eagle's medium; GP, glycoprotein; NEM, N-ethylmaleimide.

were both resistant to solubilization with nonionic and dipolar ionic detergents and urea. Similarly, both glycoproteins were readily solubilized with chaotropic agents but only in the presence of reducing agents.

Characterization of GP140 (8–10) indicated the following: (*a*) isolated multimeric GP140, but not reduced monomeric GP140, induced stable cell attachment. (*b*) Matrix GP140 contained both hydroxyproline and hydroxylysine residues, suggesting that it was a collagenlike glycoprotein. A comparison of GP140 with collagen types I–VI utilizing both immunological and biochemical techniques has indicated that GP140 is related to type VI collagen (11). In tissue, the native form of GP140 is a precursor to subunits of pepsin-generated type VI collagen. (*c*) In ligand-blot assays and affinity chromatography, pro-α1 and pro-α2 and GP140 were found to associate with fibronectin, suggesting interactions on the cell surface either directly or indirectly. (*d*) GP140 in the extracellular matrix, like fibronectin and GP250, possessed extensive intermolecular disulfide bonds, resulting in the formation of multimers of high molecular mass. Published results suggested that GP140 formed homomultimers that did not involve intermolecular disulfide bonding of GP140 with other matrix glycoproteins such as fibronectin (8, 9).

The study reported here describes: (*a*) multiple forms of GP140 present in the cytoplasm, matrix, and conditioned media of cultured cells and in tissue; (*b*) the localization and sequence of deposition of GP140 and fibronectin in the extracellular matrix; (*c*) the degree of intermolecular disulfide-dependent polymerization of the various forms of GP140 in normal and transformed cells; and (*d*) the possible role of intermolecular disulfide bonding in modulating the assembly of the extracellular matrix.

MATERIALS AND METHODS

Materials

Dithiothreitol, phenylmethylsulfonyl fluoride, paraformaldehyde, *N*-ethylmaleimide (NEM), rabbit γ-globulin, *Lens culinaris* lectin, BSA, Triton X-100, EDTA, avidin, and adenosine triphosphate were purchased from Sigma Chemical Co., (St. Louis, MO). SDS, nitrocellulose sheets, and molecular mass standards were from Bio-Rad Laboratories (Richmond, CA). Protein A and Sepharose were from Pharmacia Fine Chemicals (Piscataway, NJ). 2-Mercaptoethanol and *N*-hydroxysuccinimidobiotin were from Pierce Chemical Co. (Rockford, IL). Empigen BB, a dipolar ionic detergent, was a generous gift from Albright & Wilson (Whitehaven, England). Rhodamine-conjugated (goat) anti-rabbit IgG (heavy and light chains) and fluorescein-conjugated (goat) anti-mouse IgG (heavy and light chains) were from Bionetics Laboratory Products (Kensington, MD). IgG sorb (*Staphylococcus aureus*) was from The Enzyme Center (Boston, MA).

Cells and Cell Culture

Normal embryonic human lung diploid fibroblasts, WI-38, and SV-40 transformants of these cells, WI-38 VA13, were obtained from the American Type Culture Collection. A human rhabdomyosarcoma cell line, A204, and a human astrocytoma cell line, 251 MG, were obtained from Dr. Kari Alitalo, Department of Microbiology and Immunology, University of California, San Francisco, CA. Cells were grown in Dulbecco's modified Eagle's (DME) medium and 10% fetal bovine serum in a 5% CO_2 atmosphere.

Preparation of Matrix and Empigen BB-soluble Extract

Matrix was prepared from cultured cells grown on tissue culture plates as follows: the conditioned culture medium was removed and a minimal volume (~2 ml/15-cm plate of cells) of 2% vol/vol Empigen BB and 0.1% wt/vol SDS in 50 mM sodium borate buffer, pH 7.8, containing 25 mM NaCl, 1 mM

EDTA (referred to as BNS buffer), 1 mM phenylmethylsulfonyl fluoride, and 2 mM NEM were added. The adherent matrix and the detergent were scraped from the plate with a rubber policeman and the suspension was centrifuged at 35,000 *g* for 15 min. The pellet was termed "Empigen BB-insoluble matrix," and the supernate, "Empigen BB-soluble extract."

Preparation of Antisera

ANTISERA TO GP140 AND FIBRONECTIN: Antisera to GP140 and fibronectin were prepared in rabbits and affinity purified as previously described (9, 10).

MONOCLONAL ANTIBODY TO FIBRONECTIN: The monoclonal antibody (D-5 preparation, 12) was prepared and donated by Mark Patterson and Dr. Kiyo Sekiguchi.

Polyacrylamide Gel Electrophoresis

Polyacrylamide slab gels containing 0.1% SDS were prepared following the basic stacking SDS-gel technique of Laemmli (13). Samples were dissolved in a sample buffer containing 2% wt/vol SDS, 5% vol/vol 2-mercaptoethanol and heated in a boiling water bath for 5 min. Slab gels were stained with Coomassie Blue R-250 or silver stain (from Bio-Rad Laboratories). Fluorography of slab gels followed the procedure of Bonner and Laskey (14). Protein standards for relative molecular mass estimation in polyacrylamide gels were as follows: ovalbumin, 45,000; BSA, 66,200; phosphorylase *b*, 92,500; β-galactosidase, 116,250; myosin, 200,000. Standards were from Bio-Rad Laboratories. Protein was determined by the fluorescamine method (15).

Electrophoretic Blotting and Reaction with Antibodies and Lectins

After completion of the electrophoresis, the proteins were transferred to sheets of nitrocellulose using a modified version of the procedure described by Towbin et al. (16). Reaction with antibodies and ^{125}I-protein A and autoradiography were as previously described (11). Protein standards were labeled with ^{125}I for relative molecular mass estimation on the autoradiograms and for making comparisons of multiple autoradiograms. Reaction with [^{125}I]-labeled lentil lectin or avidin after electrophoretic transfer, followed the previously described lectin blotting procedure (11). Protein A, lectins, and molecular mass standards were iodinated utilizing the chloromine T method (17). Avidin was labeled with ^{125}I-Bolten-Hunter reagent (18).

In the results presented in Fig. 6 (q.v.), the transfer of the polymeric forms of GP140 to nitrocellulose for immunoblotting was inefficient under nonreducing conditions. In order to increase the efficiency of transfer, samples to be analyzed under nonreducing conditions were: first, run under nonreducing conditions then reducing sample buffer was applied to the top of the gels 30 min before termination of the electrophoresis.

Cell Density-dependent Alterations in GP140 and Fibronectin

The quantities of GP140 and fibronectin present in the Empigen BB soluble and insoluble extracts and conditioned culture media were assayed as follows: WI-38 cells were passaged with trypsin/EDTA/phosphate-buffered saline (500 μg/ml, 10 mM at 37° for 15 min) under conditions known to remove cell surface fibronectin and GP140. The cells were washed two times with DME medium containing 10% vol/vol fetal bovine serum that had been depleted of fibronectin by passage over gelatin-Sepharose (19). The cells were then plated on 15-cm culture dishes (2 × 10⁵ cells/plate) and cultured for the indicated time periods (see Fig. 2). Media was changed on all plates every 3 d. 24 h before each time point, two plates were washed three times with DME and were continued in culture with 15 ml of DME. The next day, the conditioned DME was pooled, adjusted to a final concentration of 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, and 2 mM NEM, and centrifuged at 35,000 *g* for 15 min. The supernatants were saved for analysis of released-form matrix components.

One plate of cells from each time point was utilized for quantitation of cell number, after suspension with trypsin, by counting with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). The remaining plate was extracted as described under the subsection "Preparation of Matrix and Empigen BB-soluble Extract."

Aliquots of each extract, derived from 25 μg of total cell protein, were fractionated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and reacted with antibody and ^{125}I-protein A as described below.

Quantitation of Antibody Binding to GP140 and Fibronectin

After autoradiography of the labeled nitrocellulose sheets to locate radioactive spots, the spots were excised along with negative control spots of the same size, and counted in a γ-counter. Standard samples of purified GP140 (9) and WI38 released form fibronectin (protein range of 0.01–1 μg) were utilized as protein standards for quantitation of radioactivity bound to unknowns. Standards and unknowns were always analyzed on the same gel to avoid variation in transfer efficiency to nitrocellulose.

Pulse-Chase Experiments and Immune Precipitation

Identical plates (6.0-cm diam) of W138 cells were grown to confluence and then metabolically labeled with radioactive methionine (100 μCi [^{35}S]methionine for 2 h). At time 0, the labeling medium was removed and the plates were washed three times with complete DME medium containing 10% vol/vol fetal bovine serum followed by incubation in the same media. At 2, 4, 18, 46, and 72 h after time 0, the media was removed from a plate, the plate was washed three times with phosphate-buffered saline and the cells were suspended in 1 ml of Empigen BB in BNS buffer as described above in "Preparation of Matrix." The Empigen BB-soluble extract was collected by centrifugation and the Empigen BB-insoluble matrix was suspended in 1 ml of 0.5% wt/vol SDS in 25 mM sodium borate, pH 7.8, plus 1 mM EDTA + 2 mM NEM and 1 mM phenylmethylsulfonyl fluoride and heated to 100°C for 5 min.

Before immune precipitation, aliquots (100 μl) of each extract were incubated at 100°C for 5 min with and without 0.05% vol/vol 2-mercaptoethanol. Immune precipitation was as previously described (8). In control experiments the amount of antibody required to precipitate all GP140 had been determined.

Where indicated, as in Figs. 4 and 7 the immune precipitated proteins were: (a) detected on gels by fluorography, (b) quantitated by excision from the gel and dissolution in H$_2$O$_2$/perchloric acid (20) and counted in a liquid scintillation counter or (c) transferred to nitrocellulose and examined by the immunoblot technique.

Disulfide Bonding of GP140 to Biotinylated Exogenous Matrix

WI-38 cells were grown to confluence on two cell culture plates of 15-cm diam (See Fig. 8). The culture plate had a glass divider (1.5 mm high) fixed to the bottom of the culture surface forming two halves on each plate. Both plates were then extracted with Empigen BB to prepare adherent monolayers of Empigen BB-insoluble matrix. Each divider plate received 5 ml of phosphate-buffered saline followed by 50 μl of dimethyl sulfoxide or 50 μl of dimethyl-sulfoxide containing 5 mg of N-hydroxysuccinimidobiotin. The plates were incubated for 15 min at room temperature with occasional agitation, then washed four times with phosphate-buffered saline.

In parallel, WI-38 cells were metabolically labeled with [^{3}H]glycine, washed, trypsinized for suspension, washed again twice with culture media containing 10% vol/vol fetal bovine serum, then plated on one side only of the biotinylated and nonbiotinylated divider plates.

After 3 h of incubation to permit cell attachment, additional culture media was added to allow free access of conditioned culture media over the barrier to both sides of the plate. The plates were continued in culture for 2 d, then re-extracted with Empigen BB, followed by 1 M urea in 1 M NaCl to lyse nuclei and release nucleic acids. The presence of nucleic acids interfered with the subsequent steps. The remaining insoluble residue on each side of the divider was dissolved in 2.0 ml of 0.5% wt/vol SDS in 25 mM sodium borate pH 7.8 containing 2 mM NEM by heating at 100° for 5 min. Duplicate aliquots (250 μl) of each extract were incubated with or without 0.5% 2-mercaptoethanol at 100° for 5 min, followed by cooling and mixing with 500 μl of BNS buffer containing 1% vol/vol Triton X-100 and 1% wt/vol BSA. Duplicate samples were incubated with or without 100 μg of avidin for 18 h on ice. The precipitates were collected by centrifugation at 12,000 g in a microfuge and washed twice with BNS containing 1% Triton X-100.

RESULTS

Empigen BB-soluble and Matrix Forms of GP140

GP140 was first described as a cell surface glycoprotein present in the Empigen BB-insoluble matrix of cultured human fibroblasts (8–10). However, as seen in Figs. 1 and 2, the
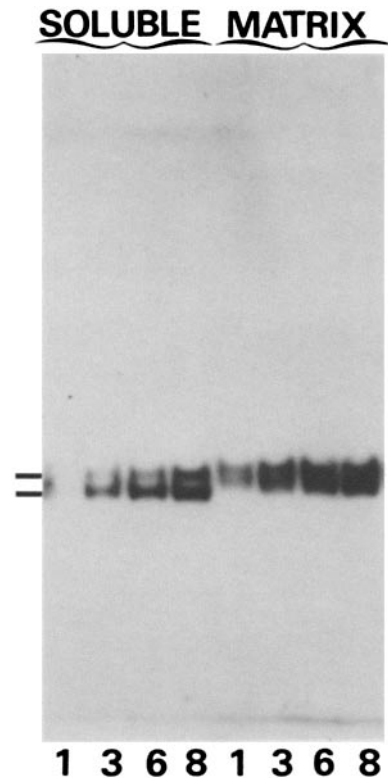

SOLUBLE MATRIX

1 3 6 8 1 3 6 8

FIGURE 1   Accumulation of GP140 in cultured W138 cells. WI-38 cells were suspended by trypsin digestion and replated at high cell density (5 × 10^6 cells/15-cm plate). At the indicated time points (1, 3, 6, and 8 d) after replating, the cells were washed 3 times with PBS, then extracted with Empigen BB to prepare Empigen BB Soluble and insoluble Matrix preparations. Extracts from 75 μg of cell protein were analyzed by polyacrylamide gel electrophoresis in the presence of SDS (5% acrylamide) followed by electrophoretic transfer to nitrocellulose and reaction with specific antiserum to GP140. GP140 in the Empigen-soluble and matrix extract migrated as two closely spaced bands indicated by lines in the left margin.

accumulation of the insoluble form of GP140 coincided with the accumulation of an Empigen BB-soluble GP140. The soluble GP140 was recovered in the supernatant after centrifugation at 100,000 g for 60 min and migrated as two closely spaced bands with slightly higher mobility than the matrix form after electrophoresis on polyacrylamide gels (Fig. 1).

The accumulation of the soluble and insoluble forms of GP140 was apparent with increased cell density and prolonged time in culture after confluence (Fig. 2A and B), reaching levels of 1.2 and 3.6 ng of GP140 per μg of cell protein, respectively. In contrast, only relatively small quantities of soluble fibronectin (0.2–0.4 ng/μg of cell protein) were detected at any cell density, in agreement with a report (21) that no large pools of detergent-soluble (cytoplasmic) fibronectin accumulated in cultured fibroblasts. The majority of cell-associated fibronectin was detected as an Empigen BB-insoluble matrix (Fig. 2).

The relative quantities of the soluble and insoluble GP140 were not affected to any appreciable degree by the addition of sodium ascorbate (50 μg/ml) to the culture media, a modulator of collagen hydroxylation (22). Also, neither daily changes of the culture media nor the addition of conditioned culture media from old confluent cells to new confluent cells had any effect on the accumulation of the insoluble GP140.

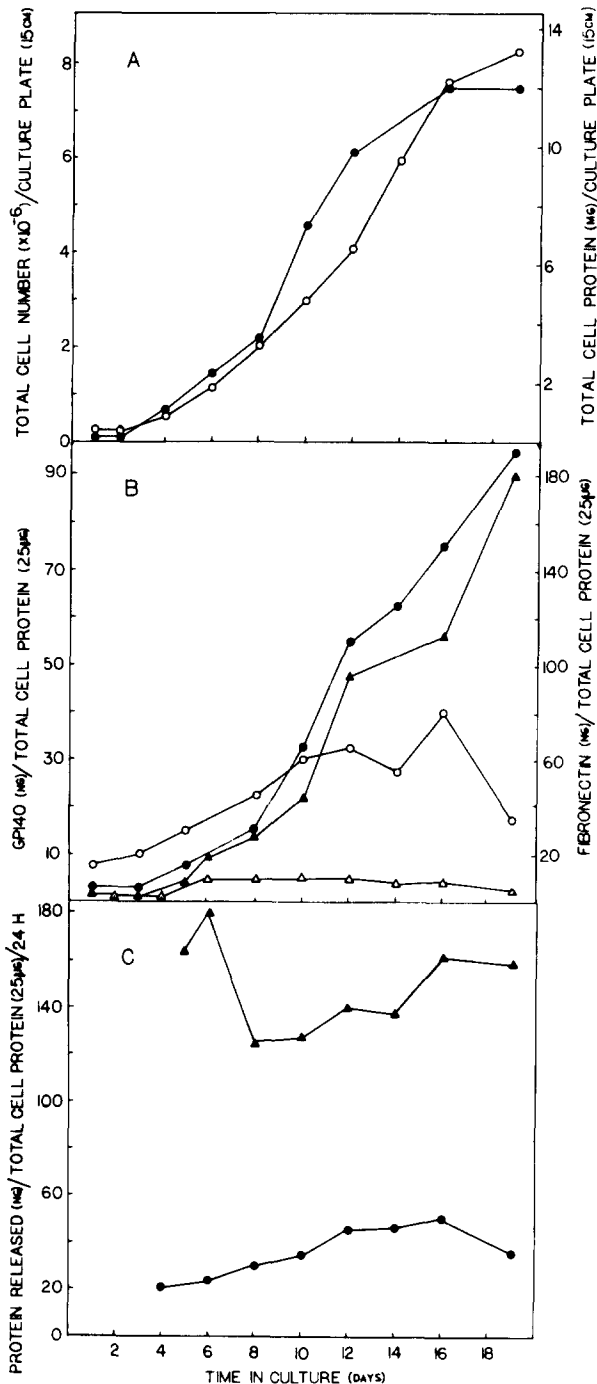RELEASED FORM: Normal fibroblasts also produced a

FIGURE 2 Quantitation of Empigen BB-soluble matrix and released forms of GP140 in WI-38 cells. After the indicated time in culture (days), duplicate plates were assayed for total cell number and total cell protein. Empigen BB-soluble extracts, Empigen BB-insoluble matrix, and the conditioned, serum-free culture media were assayed for their content of GP140 and fibronectin utilizing the immunoblot technique as described in Materials and Methods. (A) ●, cell number and ○, total cell protein; (B) ○, cytoplasmic GP140, △, fibronectin, ●, matrix GP140, and ▲, fibronectin; (C) ●, released form GP140 and ▲, fibronectin.

released form of GP140 that was recovered in the conditioned culture medium. The quantity of GP140 detected in the conditioned culture media after 24 h of growth (Fig. 2C), slowly increased with time in culture reaching a maximum of

~2 ng of GP140/μg of total cell protein per 24 h. The quantity of released fibronectin recovered during the same time periods was two to four times higher. WI-38 VA13 and HT-1080 fibrosarcoma cells synthesized the released GP140, although in reduced quantities (10), but failed to accumulate GP140 in the matrix. Similarly, A204 cells, a human rhabdosarcoma, and 251 MG cells, a human astrocytoma cell line, also failed to deposit GP140 into the matrix (results not shown), but did synthesize and release GP140 into the conditioned medium.

TISSUE FORM: Application of the differential extraction protocol, originally designed for isolation of GP140 from WI-38 cells (9), to fetal human lung tissue or adult liver, lung, or muscle tissue also partially purified a similar glycoprotein in the urea–DTT extract. The migration of the tissue form of GP140 was similar to WI-38 cell matrix GP140 on polyacryl-amide gels and reaction with antibodies prepared against GP140 from WI-38 cell matrix (results not shown). In contrast, GP140 was not detected in human demineralized bone collagenous matrix or human plasma.

CYTOPLASMIC PRECURSOR OF MATRIX FORM: The Empigen BB-soluble and insoluble matrix GP140 were both resistant to digestion with trypsin (10 μg/ml for 20 min) in the presence or absence of EDTA (5 mM). In contrast, fibronectin was readily digested under either condition. Pronase digestion (100 μg/ml for 30 min) or trypsin digestion at the same concentration with or without EDTA removed all the Empigen BB-insoluble GP140 and fibronectin in the matrix, but only degraded some of the soluble GP140 in whole cells (results not shown). These results suggested that the insoluble form of GP140 was located primarily at the cell surface along with fibronectin, probably as an extracellular matrix component as previously described (8–10). In contrast, the majority of the Empigen BB-soluble GP140 was resistant even to pronase, suggesting that it was cryptic, with a possible cyto-plasmic, non-cell surface location.

The soluble and insoluble forms of GP140, exhibited small but reproducible differences in their migration on polyacryl-amide gels (Fig. 3). The possibility that the apparent differences in relative molecular mass may be due to differences in the glycosylation of the two forms was examined utilizing the lectin blotting procedure (10). Similar quantities of soluble and insoluble GP140, as determined by quantitative immu-noblotting, bound strikingly different quantities of labeled lentil lectin (Fig. 3). Only the insoluble GP140 bound lentil lectin and this binding was specifically inhibited in the presence of α-methyl mannoside.

Pulse-chase experiments clarified the precursor-product relation of the soluble and insoluble GP140. As can be seen in Fig. 4, pulse-labeled GP140 accumulated in the Empigen BB insoluble matrix with increasing chase time up to 18 h. The increase in label in matrix GP140 was paralleled by a corresponding decrease in label associated with the Empigen BB-soluble GP140. The soluble and insoluble GP140 were identified by both immune precipitation and immunoblotting with antibodies to matrix GP140. In contrast, label in various other matrix components, including pro-α1 and pro-α2 (previously termed GP190 and GP170, respectively [10]), exhibited continuous decreases during the chase period (results not shown). Labeled fibronectin was also maximally recovered in the matrix at 0 time and decreased during the chase period (Fig. 4). These results indicated that Empigen BB-soluble GP140 is a cytoplasmic, under-glycosylated precursor to the matrix GP140.
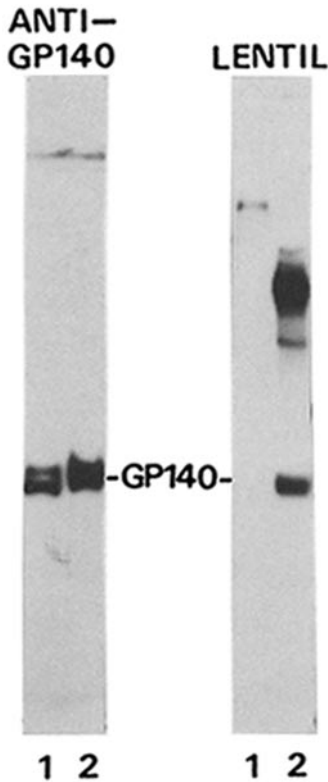
ANTI—
GP140   LENTIL

FIGURE 3 Binding of lentil lectin to GP140. The Empigen BB-soluble, and insoluble matrix of WI-38 cells was prepared and fractionated by polyacrylamide gel (5%) electrophoresis in the presence of SDS followed by electrophoretic transfer to nitrocellulose. The GP140 in the two extracts was detected by immunoblotting with antiserum to GP140 and differences in glycosylation were detected by reaction with [^{125}I]-lentil lectin. 1, Empigen BB-soluble extract; 2) Empigen BB-insoluble matrix.

siderably less than that of the matrix form to permit solubilization by extraction with Empigen BB.

Multimers of Disulfide-bonded GP140

Immune precipitation of SDS extracts of the Empigen BB insoluble matrix, under nonreducing conditions, using antibodies to GP140 precipitated a major band that comigrated with GP140 and was also reactive with anti-GP140 antibodies via immunoblot assay. In addition, a relatively minor labeled component that migrated in the region of GP250 or fibronectin was also immune precipitated. This higher molecular weight component did not react with anti-GP140 antibodies by immunoblot assay (Fig. 7) suggesting that it was antigenically unrelated to GP140 despite the co-precipitation. Reduction of the SDS extract before immune precipitation eliminated the higher molecular weight component.

Disulfide Bonding of GP140 to Exogenous Matrix

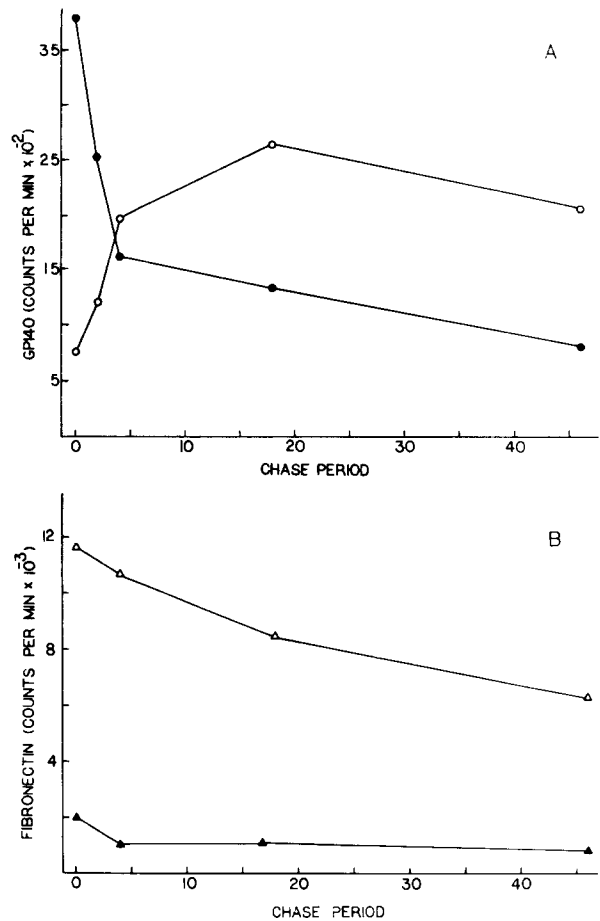The possibility that newly deposited GP140 may disulfidebond to matrix in adjacent cells or exogenous matrix, as a

Co-distribution and Sequence of Deposition of GP140 and Fibronectin in the Matrix

In newly confluent cells, in which GP140 was primarily Empigen BB-soluble (immunoblot control not shown), antibodies to GP140 were localized primarily in the cytoplasm (Fig. 5). In the same field of view, fibronectin was distributed predominantly as a fibrillar, extracellular matrix component with negligible levels in the cytoplasm agreeing with the results presented in Fig. 2. In addition, some of the antibodies to GP140 were also co-localized with the fibrillar fibronectin, but in spots or clumps along the continuous fibronectin fibers. The co-alignment of GP140 and fibronectin was clearly evident in the Empigen BB-insoluble matrix of identical cells that were cultured at confluence for an additional 3 d before immunofluorescence examination. In these older cells, the antibodies to GP140 were localized in bright spots along the continuous fibronectin fibers.

Disulfide Bonding of Multiple Forms of GP140

In sharp contrast to the disulfide-bonded, multimeric structures of GP140 found in matrix, the released forms of GP140 from normal and transformed cells exhibited a lower degree of polymerization (Fig. 6). Additional, small differences between the released GP140 from normal and transformed cells were also observed (Fig. 6) in molecular mass and degree of polymerization. Normal released GP140 was a mixture of monomeric, dimeric, and polymeric GP140, whereas released GP140 from transformed cells was primarily monomeric. At best, <50% of the cytoplasmic GP140 was ever found to be monomeric (see Fig. 6), and many samples analyzed were entirely polymeric. This result, although surprising, indicated that polymerization of GP140 may occur to a significant degree within the cytoplasm of the cells. The degree of polymerization of the cytoplasmic GP140 must, however, be con-

FIGURE 4 Quantitation of pulse-chase labeled GP140 and fibronectin. WI-38 cells were pulse-labeled with [^{35}S]methionine for 2 h then chased for the indicated times. Empigen BB-soluble extracts and insoluble matrix were prepared at each time point in the chase period and subsequently immune precipitated with specific antibodies to either GP140 or fibronectin. The precipitates were fractionated on polyacrylamide gels and the radioactive GP140 and fibronectin bands were quantitated. (A) O, matrix GP140 and ●, Empigen BB-soluble GP140; (B) △, matrix fibronectin and ▲, cytoplasmic fibronectin.
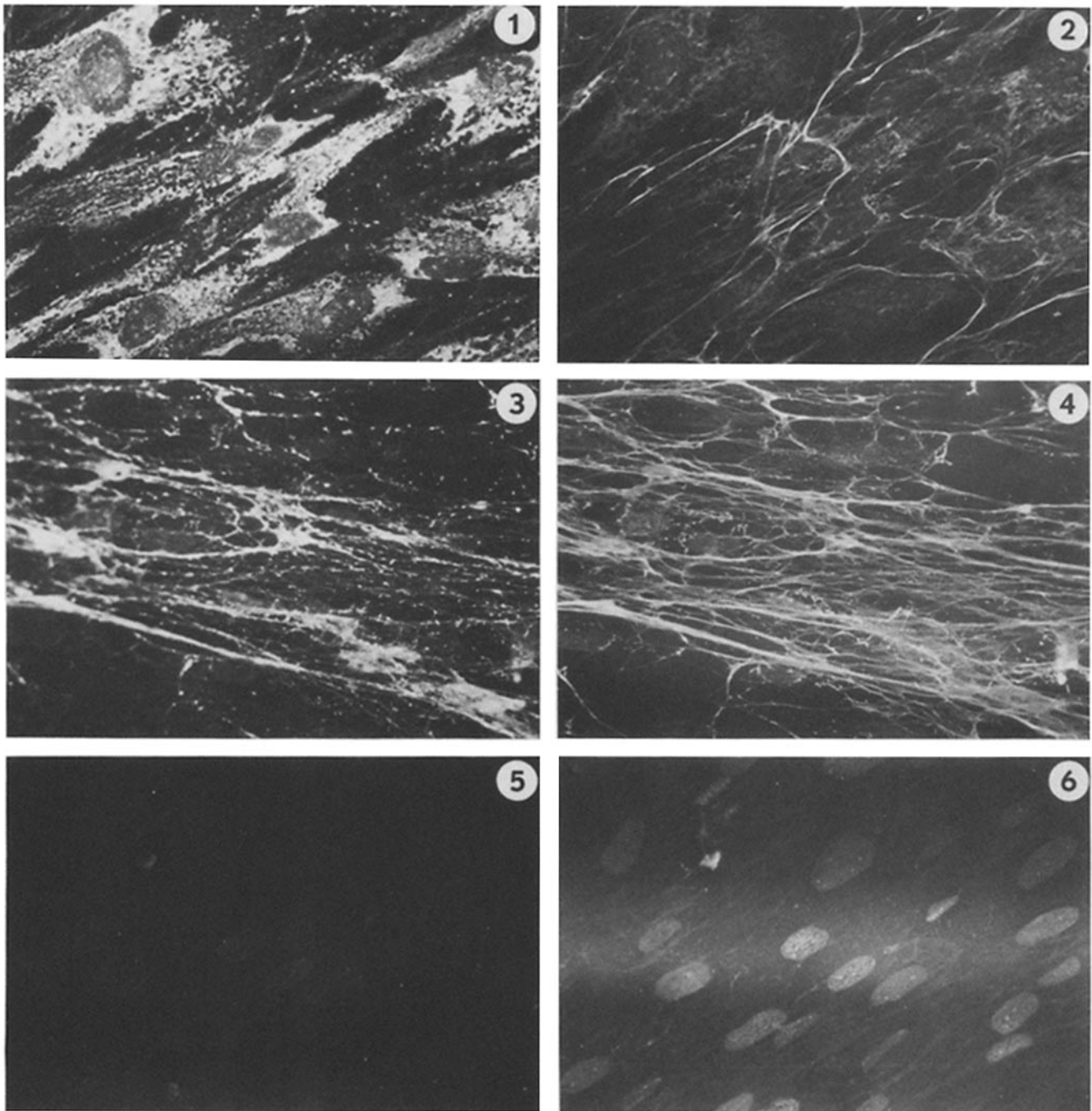
FIGURE 5 Localization of GP140 and fibronectin in cultured WI-38 cells utilizing immunofluorescence microscopy. Newly confluent cultures of WI-38 cells (confluent for 1 d), in which most of the cellular GP140 was Empigen BB-soluble, were examined after methanol fixation. Old confluent cultures of WI-38 cells (confluent for 4 d), in which most of the cellular GP140 was Empigen BB-insoluble, were examined after extraction with Empigen BB and methanol fixation. Affinity-purified (rabbit) anti-human GP140 antibodies were detected with rhodamine-conjugated (goat) anti-rabbit igG antibodies. Mouse monoclonal antibodies to human fibronectin were detected with fluorescein-conjugated (goat) anti-mouse IgG antibodies. In control experiments, nonimmune antibodies stained with both of the fluorescent second antibodies produced negligible background. Nonimmune antibody controls were photographed and printed with the same exposure settings as the immune antibody preparations. (1 and 2) Same field; whole, new confluent cells; 1, anti-GP140; 2, antifibronectin; (3 and 4) Same field; matrix, old confluent cells; 3, anti-GP140; 4, antifibronectin; (5 and 6) Same field; matrix, old confluent cells; 5, control for rhodamine; 6, control for FITC. × 400.

means of stabilizing intercellular interactions was examined in Fig. 8. The protocol examined the ability of labeled cells to incorporate radioactive matrix components into an exogenous matrix that had been derivatized with biotin.

Biotinylated or nonbiotinylated matrix, when incubated with conditioned media but without cells, bound only two minor labeled proteins and these co-migrated with pro-α1, and pro-α2 procollagen. In contrast, when either biotinylated or nonbiotinylated matrix was incubated in the presence of cells and conditioned media, a number of labeled proteins including a major GP140 band were deposited in the matrix. Biotinylation of the matrix did not appreciably alter the

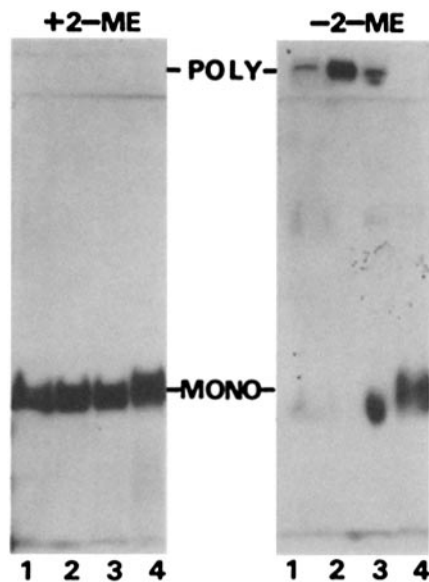—POLY—

—MONO—

1 2 3 4   1 2 3 4

Figure 6 (A) Disulfide bonding of cytoplasmic GP140, matrix GP140, and released GP140 from normal and transformed cells. Samples of various forms of GP140 were analyzed by polyacrylamide gel (5%) electrophoresis in the presence of SDS and 2-mercaptoethanol for reducing conditions (+2-ME) and SDS and N-ethylmaleimide (2 mM) for nonreducing conditions (−2-ME) followed by immunoblotting with antiserum to GP140. 1 Cytoplasmic GP140 of WI-38 cells; 2 Empigen BB-insoluble matrix from WI-38 cells; 3 conditioned medium from WI-38 cells; 4 conditioned medium from WI-38 VA13 cells.

of entrapment or artifactual cross-linking with biotinylated matrix. (b) Avidin precipitation of either the Empigen BB extracts or urea–NaCl extracts (see Materials and Methods) did not precipitate any specific radioactive components. The cross-linked components were recovered in the SDS extract of the matrix only.

The addition of 2-mercaptoethanol to the avidin-precipitation reaction completely abolished the precipitation of the [³H]GP140 but only partially inhibited the precipitation of the biotinylated matrix components. The incorporation of [³H]GP140 to the biotinylated matrix involved intermolecular disulfide bonding as the covalent interaction. Furthermore, the association required the presence of cells in that conditioned media had no detectable affect on deposition of either GP140 and/or fibronectin. Thus, labeled GP140 that deposited into the exogenous extracellular matrix was derived from the labeled cells and not from the released form GP140 present in conditioned media. In contrast, the [³H]pro-α1 and [³H]pro-α2 that was deposited in the matrix originated in the conditioned media, and their deposition did not require cell contact with the matrix and did not involve intermolecular disulfide bonding.

WI-38 VA13 cells did not synthesize matrix GP140 but did synthesize released form GP140. As expected, when WI-38 VA13 cells were metabolically labeled with [³H]glycine then grown on biotinyl matrix from WI-38 cell as described above, the transformed cells failed to incorporate any radioactive GP140 into the biotinyl matrix.
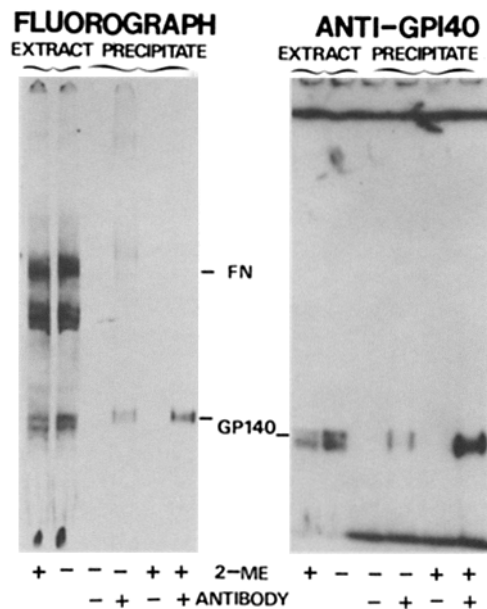
attachment of cells to the matrix or the pattern of major labeled proteins deposited in the matrix. In control experiments (results not shown), unlabeled cells were incubated on matrix with conditioned media from labeled cells. Under these conditions only labeled pro-α1 and pro-α2 from the conditioned medium were deposited in the matrix. These results combined with the above results suggested that pro-α1 and pro-α2 in conditioned medium bound to exogenous extracellular matrix with or without the presence of viable cells. Conversely, other matrix components such as GP140 and fibronectin were deposited in the matrix, to a detectable degree, only in the presence of cells, with or without condition medium.

In control experiments only biotinylated matrix coupled radioactively labeled avidin. Various matrix components including GP140 were derivatized with biotin during the labeling procedure (results not shown). Similarly, incubation of the extracts with avidin caused precipitation of matrix components only when the matrix was biotinylated. Furthermore, precipitation of ³H-labeled matrix only occurred when the biotinylated matrix had been incubated in the presence of cells and not just conditioned media. Thus, even after solubilization in SDS, radioactively labeled GP140 and possibly some fibronectin were specifically precipitated by avidin. These results indicated that [³H]GP140 had covalently bound to the biotinylated matrix in a manner that was resistant to disruption with hot SDS. Additional control experiments were also performed as follows (results not shown): (a) mixing of SDS extracts prepared from radioactively labeled matrix and SDS extracts of biotinylated matrix, followed by reaction with avidin, precipitated only biotinylated matrix and no radioactive label. Thus radioactive label is not precipitated as a result

FLUOROGRAPH   **ANTI–GP140**
EXTRACT PRECIPITATE   EXTRACT PRECIPITATE

— FN

— GP140_

+ − − + +   2—ME   + − − + +
− + − + ANTIBODY   − + − +

Figure 7 Immune precipitation of matrix GP140 from WI-38 cells. WI-38 cells were metabolic labeled with [³⁵S]methionine and the Empigen BB-insoluble matrix was prepared. The matrix was solubilized with SDS under nonreducing conditions, divided into equal aliquots, and incubated in the presence or absence of 2-mercaptoethanol (indicated as + or −2-ME) as described in Materials and Methods. The matrix extracts (indicated as EXTRACT, ±2-ME) were immune precipitated with or without antibodies to GP140 (labeled + or −ANTIBODY) and the resulting precipitates (PRECIPITATES) were fractionated by polyacrylamide gel electrophoresis (5% gel) and detected by fluorography or immunoblotting with antibodies to GP140.

DISCUSSION

Interrelation of Multiple Forms of GP140

WI-38 cells in culture produce three forms of GP140; cytoplasmic, matrix, and released. In addition, GP140 found in various fetal and adult human tissues appears to be similar in molecular weight, intermolecular disulfide bonding, and antigenic reactivity to the matrix form of GP140 from WI-38 fibroblasts. Extraction of cultured WI-38 cells with Empigen BB solubilized the cytoplasmic GP140 and left the matrix GP140 in the insoluble residue. Evidence for the localization of the Empigen BB-soluble form of GP140 in the cytoplasm included: (a) the resistance of the Empigen BB soluble GP140 to pronase digestion of intact cells under conditions that degraded the matrix form of GP140; and (b) prominent immunofluorescence staining of the cytoplasm of newly confluent cells with subsequent removal of the cytoplasmic staining by extraction with Empigen BB.

Although a minor quantity of GP140 may have been directly associated with the plasma membrane, no such localization was observed by immunofluorescence microscopy. Thus, GP140 is probably unrelated to the various calcium-dependent cell adhesion molecules of similar molecular weight described by others (23–28).

Characteristically, the cytoplasmic and matrix GP140 both accumulated in the cell layer with prolonged periods of culturing and existed primarily as polymeric structures. In contrast, the released form of GP140 possessed very little intermolecular disulfide bonding. These observations suggested that the accumulated cytoplasmic GP140 was the direct precursor of the matrix GP140. This suggestion was confirmed by: (a) pulse-chase experiments that indicated that the accumulation of GP140 in the matrix was paralleled by corresponding decreases in cytoplasmic GP140; (b) the intermolecular disulfide bonding of GP140 into exogenous matrix required cell contact with the exogenous matrix and was independent of the released form of GP140 present in the conditioned media.

Released form GP140 may be derived from a relatively small pool of unpolymerized, cytoplasmic GP140 as seen in the WI-38 VA13 cells that produced only released form GP140. It is unlikely that the released form of GP140 derived from dissociation of the matrix GP140. The half-life of GP140 in the matrix ($t_{1/2}$ = 73 h; 9) is too long to account for the relatively large quantities of GP140 present as a nondisulfide-bonded form in the condition media (2 ng/µg of cell protein/ 24 h). It is unclear at this time if released GP140 represents a biologically significant form of GP140 or if it is simply a cell culture artifact, generated by insufficient intermolecular disulfide bonding. The lack of intermolecular disulfide bonding was most pronounced in the released form of GP140 from transformed cells.

Interaction of GP140 and Fibronectin

The following observations have suggested that GP140 and fibronectin may specifically interact with each other in the extracellular matrix of cultured human fibroblasts either directly or through some intermediary component(s): (a) both GP140 and fibronectin were recovered in the Empigen BB-insoluble matrix of confluent human fibroblasts along with GP250 (8–10). (b) Isolated, nonreduced GP140 induced stable cell attachment in cell adhesion assays. However, trypsiniza-

tion of the cell surface under conditions that released the external fibronectin also decreased the ability of the cells to attach to GP140-coated surfaces. (c) Pro-α_1 and pro-α_2 procollagens and, to a lesser degree, GP140 all bound fibronectin and the gelatin-binding domain of fibronectin in both ligand blot assays and affinity chromatography experiments (10). (d) Immunofluorescence microscopy revealed extensive co-distribution of fibronectin and GP140 in the matrix of confluent fibroblasts. However, demonstration of co-distribution of both proteins at molecular distances would require immuno-electron microscopy.

The collagenlike nature of GP140 (9, 10) would obviously suggest a direct interaction of fibronectin and GP140 via the gelatin-binding domain of fibronectin (19). Results to be presented elsewhere (11) have detected both immunological and biochemical similarities of GP140 to the collagenous peptide termed short-chain collagen or type VI (29, 30), prepared by pepsin digestion of human placenta. The designation of short chain collagen (30) as a new type of collagen (VI) was based on characterization of pepsin-resistant, collagenase-sensitive peptides of ~60 kilodaltons in relative molecular mass. Our own results (11) indicated that the pepsin-resistant, collagenase-sensitive domain of GP140 is 64 kilodaltons. Thus, the noncollagenous domain of GP140 comprises over half of the native protein found in both extracellular matrix and tissue (11). GP140 may represent a hybrid structure with characteristics of both collagens and other extracellular matrix glycoproteins like fibronectin. These unique hybrid qualities of GP140 may play a major role in its assembly and interactions with various components of the extracellular matrix.

Assembly of GP140 in the Extracellular Matrix

An analysis of the sequence of appearance of GP140 and fibronectin in the cytoplasm and matrix of fibroblasts utilizing both pulse-chase experiments and immunofluorescence microscopy yielded similar results by both techniques. Fibronectin appeared in the matrix before GP140 with no prior accumulation of an extensive cytoplasmic pool of fibronectin. After accumulation of the cytoplasmic GP140, the matrix GP140 appeared and co-localized with the already established fibronectin matrix. These data strongly suggested that the major matrix form of GP140 did not function as a receptor for fibronectin. Rather, fibronectin may have functioned as a receptor for GP140 either directly or through some intermediary component. Fibronectin may have provided a template for the proper deposition of GP140 through noncovalent interactions, followed by the intermolecular disulfide bonding of matrix GP140. A number of reports (4, 5, 7, 31–33) have indicated that type I and III collagens and/or procollagens also co-distribute with fibronectin in the extracellular matrix.

Disulfide Bonding of GP140 to Exogenous Matrix

The results presented in Fig. 8 indicated that normal cells synthesized and covalently bound GP140 into an exogenous matrix. Most likely, GP140 disulfide bonded directly to exogenous GP140 and not other matrix components in that, as seen in Fig. 7, GP140 forms primarily homomultimers. This bonding was dependent on cell contact with the exogenous matrix. These experiments were unable to detect significant levels of released form GP140, that deposited in the exogenous matrix. Conceivably, small quantities of released GP140 may

PROTEIN STAIN

EXTRACTS PRECIPITATES

FLUOROGRAPH

EXTRACTS PRECIPITATES

— FN —
— GP190 —
— GP170 —
— GP140 —

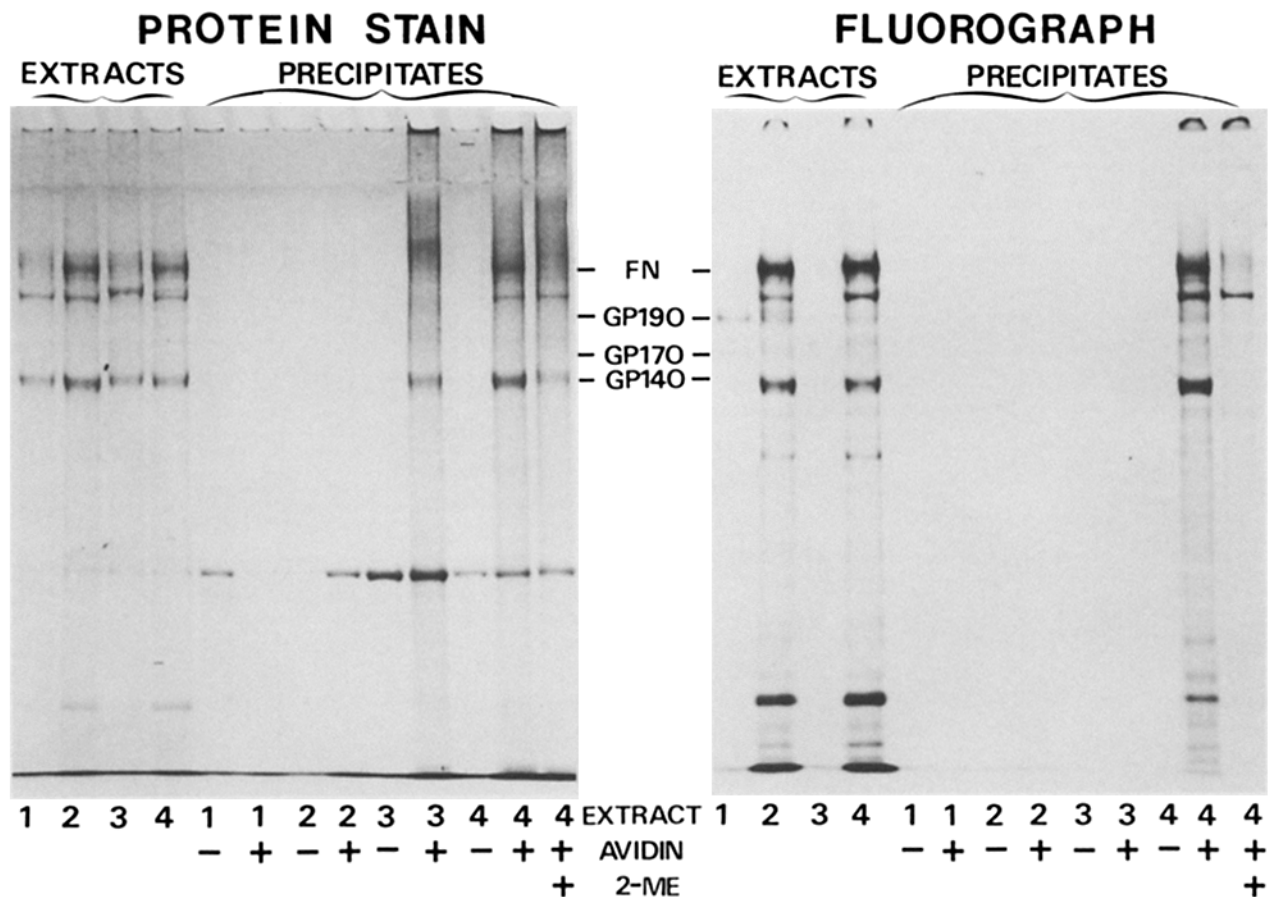| 1 2 3 4	1 1 2 2 3 3 4 4 4	EXTRACT	1 2 3 4	1 1 2 2 3 3 4 4 4
	− + − + − + − + +	AVIDIN		− + − + − + − + +
	+	2-ME		+

FIGURE 8 Disulfide bonding of GP140 to biotinylated exogenous matrix. Empigen BB-insoluble matrix of WI-38 cells was prepared on divided culture plates (See Materials and Methods). The two plates were reacted with and without N-hydroxysuccinimidobiotin. One side of each divider plate was then seeded with WI-38 cells that had been metabolically labeled with [³H] glycine. Culture media, but not cells, had free passage across the plate divider. The plates were cultured for 2 d then extracted with Empigen BB followed by 1 M urea in 1 M NaCl. The insoluble matrix from each side of the divider on both plates were then solubilized with SDS (0.5% at 100°C) yielding four extracts (*EXTRACTS*) indicated *1*, − biotin, − cells, + media; *2*, −biotin, + cells, + media; *3*, + biotin, − cells, + media; and *4*, + biotin, + cells, + media. Each extract was then incubated with or without avidin (indicated + or − *AVIDIN*) to precipitate biotinylated matrix and any covalently bound radioactive components. All precipitates were fractionated by polyacrylamide gel electrophoresis (7% gel) and detected by staining for protein with Coomassie Blue (*PROTEIN STAIN*) and fluorography (*FLUOROGRAPH*). Radioactive matrix components precipitated with avidin only from extract *4* (+ biotin, + cells, + media). The precipitation of the radioactive matrix (as seen in *FLUOROGRAPHY*) but not the biotinylated matrix (as seen in *PROTEIN STAIN*) could be inhibited with 2-mercaptoethanol (indicated as +2-ME).

incorporate into the exogenous matrix. However, relative to GP140 depositing directly from the cells, contributions from the conditioned media are minor. In contrast to normal cells, transformed cells lacked the ability to incorporate their own GP140 into the exogenous matrix. Cells may stabilize their association with extracellular matrix of adjacent cells by covalently bonding their own matrix components to the matrix of adjacent cells. This disulfide-dependent cross-linking required direct cell contact or at least cell proximity to the exogenous matrix.

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