

Translatable mRNA for GP140 (a Subunit of Type VI Collagen) Is Absent in SV40 Transformed Fibroblasts

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ABSTRACT Production of GP140, a major component of the extracellular matrix of cultured fibroblasts, is markedly decreased in SV40 transformed cells as compared with normal cells (Carter, W. G., 1982, *J. Biol. Chem.*, 257:13805–13815). To determine at what step the biosynthesis is inhibited, we compared the levels of functional mRNA for GP140 in normal and transformed fibroblasts. Translation of total RNA from WI38 cells in a reticulocyte lysate, followed by immunoprecipitation with affinity-purified antibodies to GP140, yielded a single polypeptide with an M_r of 125,000. This polypeptide was identified as GP140 based on its immunoreactivity, collagenase sensitivity, and co-migration on polyacrylamide gels with GP140 synthesized by cells in the presence of tunicamycin and 2,2'-bipyridyl. No cell-free synthesis of GP140 was observed with total RNA from SV40 transformed WI38 cells, indicating that these cells contain very low levels of GP140-specific mRNA. The biosynthesis of GP140 might therefore be blocked at the transcriptional level.

GP140 is a major glycoprotein of the extracellular matrix of cultured fibroblasts (1–4). The protein has been shown to form covalent and/or noncovalent interactions with several other matrix components including GP250, fibronectin, and collagen type I. When isolated under nonreducing conditions, GP140 can promote stable attachment and spread fibroblasts *in vitro* (2). This activity, however, is lost upon reduction, suggesting that the correct conformation of the protein provided by intact disulfide bonds is important for the interaction of GP140 with the cell surface. A soluble form of GP140, which differs from the matrix form in its lower degree of intermolecular disulfide bonding, is found in the conditioned media of cultured fibroblasts (3, 4).

GP140 contains equal amounts of hydroxyproline and hydroxylysine, indicating that it is a collagen-like protein (2). Recently, three different laboratories have demonstrated by immunological, biochemical, and electron microscopical techniques that GP140 is related to type VI collagen (5–7), a dumbbell-shaped molecule with a short, collagenous triple helix separating two large globular domains (8).

Previous work from this laboratory has established that GP140 is absent from the extracellular matrix of SV40 transformed fibroblasts and various human tumor cells, and that very little is found in the conditioned media of such cells (3, 4). The loss of GP140 appeared to be due to a decreased synthesis rather than an increased degradation, since matrix derived from normal cells was not degraded at an unusual

rate in the presence of transformed cells, and since virtually no cytoplasmic precursor form of GP140 was detected in transformed cells. The goal of the present study was therefore to determine at what step the production of GP140 may be inhibited. We show by *in vitro* translation that the level of functional mRNA for GP140 is decreased more than 20-fold in SV40 transformed WI38 cells as compared with normal cells. The biosynthesis of GP140 might thus be blocked at the transcriptional level.

MATERIALS AND METHODS

Cell Culture: Normal fibroblasts from embryonic human lung (WI38 cells) and SV40 transformants of these cells (WI38 VA13 cells) were obtained from the American Type Culture Collection (Rockville, MD). A204 cells from a human rhabdomyosarcoma and 251 MG cells from a human astrocytoma were provided by Dr. K. Alitalo (University of Helsinki). All cells were propagated at 37°C under an atmosphere of 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. For metabolic labeling, the cells were rinsed twice with methionine-free medium containing 0.5 mg/ml bovine serum albumin instead of fetal calf serum, and incubated for 5–14 h in this medium with 100 µCi/ml, 1,090 Ci/mmol [³⁵S]methionine (New England Nuclear, Boston, MA). For metabolic labeling in the presence of 2 µg/ml tunicamycin (Calbiochem-Behring Corp., La Jolla, CA) and 0.2 mM 2,2'-bipyridyl (Sigma Chemical Co., St. Louis, MO), cells were preincubated with these inhibitors for 2–5 h before addition of [³⁵S]methionine.

Cell-free Translation: Total cellular RNA was extracted by the guanidine HCl method (9) or by the SDS/proteinase K method (10). A reticulocyte lysate was prepared from anemic rabbits and treated with micrococcal nuclease

essentially as described by Pelham and Jackson (11). For in vitro translation, 19.6 μ l reticulocyte lysate was incubated for 2 h at 30°C in a final volume of 50 μ l containing 100 μ Ci [³⁵S]methionine (1,090 Ci/mmol) (New England Nuclear), 50–800 μ g/ml of total cellular RNA, 7.5 mM creatine phosphate, 1.25 mM spermidine, 1.87 mM dithiothreitol, 88 mM potassium acetate, 0.3 mM magnesium acetate, 23.4 μ M of all amino acids except methionine, and 18.7 mM HEPES (pH 7.6). Before addition to the translation reaction, the RNA was heated to 80°C for 6 s. In some experiments, 100 U/ml of the ribonuclease inhibitor RNasin (Promega Biotec, Madison, WI) was added.

Immunoprecipitation: Antisera to a preparation of GP140 from the matrix of WI38 cells were raised in rabbits (3) and affinity-purified on Sepharose-immobilized GP140 that had been isolated from human placenta (5). A rabbit antiserum to vinculin was a generous gift from Dr. L. R. Rohrschneider (Fred Hutchinson Cancer Research Center, Seattle, WA). Proteins were precipitated from the translation mixtures (40- μ l aliquots) or from the conditioned media of WI38 cells (250- μ l aliquots) as described (12). The amount of antibody required for quantitative precipitation was determined in a previous experiment. When immunoprecipitation was successively repeated with the same aliquot of translation mixture, only negligible quantities of GP140 were recovered in the second precipitate. For control experiments, nonspecific rabbit immunoglobulins (Sigma Chemical Co.) were used. Digestion of the immunoprecipitates with bacterial collagenase (315 U/mg, chromatographically purified) (Worthington, Freehold, NJ) was done in the presence of 2 mg/ml bovine serum albumin as described (12).

Gel Electrophoresis: Protein was analyzed on 7% SDS polyacrylamide gels essentially as described by Laemmli (13). Gels were fixed in 50% methanol, 10% acetic acid, allowed to swell to their original size in 10% methanol, 10% acetic acid, and exposed to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY) after we dried them on filter paper. For quantitative comparison, areas corresponding to radioactive bands were excised from the dried gels, dissolved in 0.2 ml of 70% perchloric acid, 0.4 ml of 30% hydrogen peroxide (2–3 h at 75°C), and counted in 5 ml of Formula 963 scintillation fluid (New England Nuclear).

RESULTS

Synthesis of GP140 by Cells in Culture

Affinity-purified antibodies to GP140 precipitated two major polypeptides from the conditioned media of WI38 cells with apparent molecular weights of 140,000 and 240,000 on polyacrylamide gels (Fig. 1, lanes 1, 4, and 7). After heat denaturation, the lower molecular weight polypeptides was fully susceptible to digestion with bacterial collagenase, yielding fragments with molecular weights of 50,000–60,000; in its native conformation, however, the polypeptide partially resisted digestion with collagenase (Fig. 1, lanes 1–3). Immunoreactivity, molecular weight, and collagenase sensitivity clearly establish the M_r 140,000 polypeptide as GP140 (1–4). The higher molecular weight polypeptide was only slightly affected by bacterial collagenase, even after heat denaturation. Thus, this polypeptide shares characteristics with GP250, another component of the extracellular matrix of cultured fibroblasts (1–4), as well as with the pro α 1 chain of type VI procollagen (8). Experiments designed to establish its identity are in progress. In addition to the two polypeptides, a minor component with an M_r 190,000 was recovered in the immunoprecipitates in variable amounts. Whether this material is derived from the M_r 240,000 polypeptide or whether it represents a different entity is not yet known.

Two explanations could account for the co-precipitation of the M_r 240,000 polypeptide with GP140: either the M_r 240,000 polypeptide cross-reacts with the affinity-purified antibodies or it is specifically or nonspecifically bound to GP140. To address this question, we performed immunoprecipitation after dissociation of the proteins in the conditioned media with SDS and dithiothreitol (Fig. 1, lanes 4–6). Affinity-purified antibodies precipitated only GP140 from such pretreated media. When dissociation was done in the absence of reducing agents, however, both GP140 and the M_r 240,000

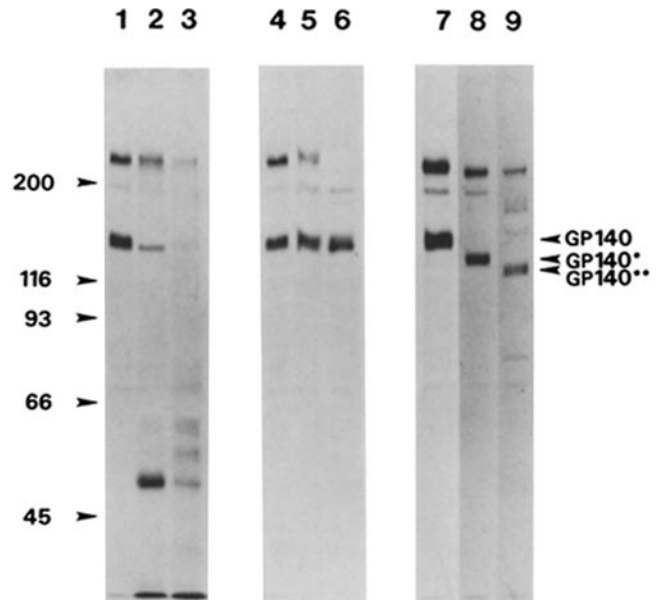


FIGURE 1 Characterization of GP140 synthesized by cells in culture. WI38 cells were metabolically labeled with [³⁵S]methionine and GP140 was immunoprecipitated from the conditioned media. Immunoprecipitates were resolved on 7% polyacrylamide gels in the presence of mercaptoethanol and visualized by autoradiography. (Lanes 1, 4, and 7) Immunoprecipitate obtained with anti-GP140 antibodies; (lane 2) collagenase digest of the immunoprecipitate; (lane 3) as for lane 2, but digestion performed after heating of the immunoprecipitate for 3 min to 100°C; (lane 5) immunoprecipitate obtained from media that had been boiled in the presence of 0.1% SDS (3 min at 100°C); (lane 6) as for lane 5, but media boiled in the presence of 0.1% SDS and 10 mM dithiothreitol; (lane 8) immunoprecipitate obtained from media of cells grown in the presence of tunicamycin; (lane 9) as for lane 8, but cells grown in the presence of tunicamycin and 2,2'-bipyridyl. GP140 and its unglycosylated (GP140*) and unglycosylated and unhydroxylated (GP140**) forms are identified in the right margin. Arrowheads in the left margin indicate the positions of migration of myosin (M_r , 200,000), β -galactosidase (M_r , 116,000), phosphorylase b (M_r , 93,000), serum albumin (M_r , 66,000), and ovalbumin (M_r , 45,000).

polypeptide were recovered in the immunoprecipitate. Thus, the higher molecular weight polypeptide does not cross-react with our antibodies.

GP140 has been reported to contain carbohydrates as well as hydroxyproline and hydroxylysine (2). To determine the effect of these posttranslational modifications on the electrophoretic mobility of GP140, we used conditioned media of cells that had been cultured in the presence of specific inhibitors for immunoprecipitation (Fig. 1, lanes 7–9). Tunicamycin, a potent inhibitor of *N*-glycosylation, increased the mobility of both GP140 and the M_r 240,000 polypeptide. 2,2'-Bipyridyl, which inhibits hydroxylation of prolyl and lysyl residues, had an additional effect on GP140 but not on the M_r 240,000 polypeptides. The mobility of GP140 synthesized in the presence of both inhibitors corresponded to a molecular weight of 125,000. The polypeptide still appeared as a fuzzy band on polyacrylamide gels, suggesting that it is modified by additional mechanisms other than glycosylation and hydroxylation. Preliminary data indicate, however, that GP140 is neither sulfated nor phosphorylated.

Cell-free Synthesis of GP140

For in vitro protein synthesis, we prepared total RNA from WI38 cells and translated it in a rabbit reticulocyte lysate.

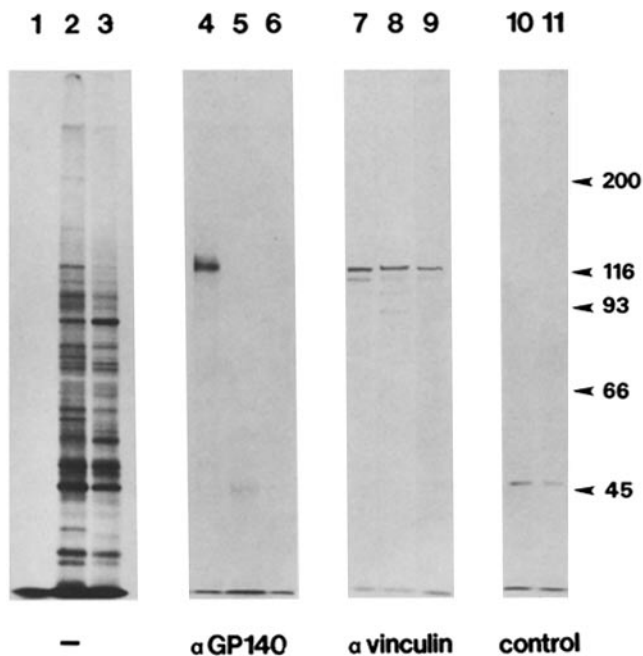


FIGURE 2 Cell-free translation of GP140 and of vinculin. Total cellular RNA from WI38 cells (lanes 2, 4, 5, 7, 8, and 10) or SV40 transformed WI38 cells (lanes 3, 6, 9, and 11) was translated in a rabbit reticulocyte lysate. Newly synthesized polypeptides were analyzed either directly (lanes 1-3) or after immunoprecipitation with anti-GP140 antibodies (lanes 4-6), antivinculin antiserum (lanes 7-9), or control immunoglobulins (lanes 10 and 11) on 7% polyacrylamide gels. Lane 1 depicts a control where no exogenous RNA was added to the reticulocyte lysate. The immunoprecipitates in lanes 5 and 8 were digested with bacterial collagenase.

The RNA directed the synthesis of a great variety of polypeptides with molecular weights of up to 200,000 as assessed by PAGE (Fig. 2, lanes 1-3). Affinity-purified antibodies to GP140 specifically precipitated an M_r 125,000 polypeptide from the translation mixture, whereas nonspecific immunoglobulins precipitated only minor low molecular weight material (Fig. 2, lanes 4 and 10). The precipitated polypeptide was sensitive to digestion with bacterial collagenase, yielding a fragment with a molecular weight of $\sim 50,000$ (Fig. 2, lane 5). Based on immunological reactivity, collagenase sensitivity, and co-migration on polyacrylamide gels with GP140 synthesized by cells in the presence of tunicamycin and 2,2'-bipyridyl, we conclude that the M_r 125,000 polypeptide represents a full-length translation product of GP140-specific mRNA. In vitro synthesis of GP140 as determined by incorporation of radioactivity into immunoprecipitable material was directly proportional to the amount of exogenous RNA added to the reticulocyte lysate between 50 and 400 $\mu\text{g}/\text{ml}$. Above 800 $\mu\text{g}/\text{ml}$, no further increase was observed. As had been noted above for GP140 synthesized by cells in culture, GP140 translated in vitro always revealed a fuzzy band on polyacrylamide gels. This may be due to an uncharacterized post-translational modification occurring in the reticulocyte lysate, rather than to unspecific degradation or premature termination during translation, since other polypeptides of the translation mixture such as vinculin gave sharp bands on polyacrylamide gels (see below). Addition of the ribonuclease inhibitor RNasin had no effect. Almost identical results were observed with three different preparations of RNA that had been obtained by the SDS/proteinase K or by the guanidine HCl method.

Absence of Functional mRNA for GP140 in Transformed Cells

Previous studies have indicated that the production of GP140 is markedly decreased in SV40 transformed WI38 cells (WI38 VA13 cells) (3). We investigated therefore whether GP140-specific mRNA can be isolated from such cells. When translated in the reticulocyte lysate, total RNA from WI38 VA13 cells directed the synthesis of a variety of polypeptides similar to total RNA from normal cells (Fig. 2, lane 3). Although the overall incorporation of radioactivity into newly synthesized proteins was equal when the two RNA preparations were used, some specific differences were noted. For quantitative comparison, we excised areas corresponding to discrete radioactive bands from the polyacrylamide gel and counted them in a liquid scintillation counter. Based on this method, functional mRNA for an M_r 95,000 protein was increased twofold in WI38 VA13 cells, and functional mRNA for an M_r 45,000 protein (probably actin) was decreased 1.8-fold.

Affinity-purified antibodies to GP140 did not precipitate any detectable material from the translation mixture containing RNA from WI38 VA13 cells (Fig. 2, lane 6). Since mRNA levels as low as one-twentieth the level found in normal cells can easily be detected by our method, the reduction of GP140-specific mRNA in WI38 VA13 cells must be more than 20-fold. Altering the RNA concentration or adding the ribonuclease inhibitor RNase had no effect. No in vitro translation of GP140 was observed, irrespective of whether the RNA had been prepared by the SDS/proteinase K or by the guanidine HCl method.

The RNA preparation from transformed cells can direct cell-free synthesis of proteins other than GP140. As an example we chose vinculin, a protein with a reported molecular weight of 130,000 (14) (Fig. 2, lanes 7-9). Crude antiserum to vinculin precipitated a major polypeptide with a molecular weight of 125,000 and several minor components of higher and lower molecular weights from a translation mixture containing RNA from normal WI38 cells. The M_r 125,000 polypeptide probably corresponds to a full-length translation product of vinculin-specific mRNA, whereas the minor components may represent unrelated polypeptides that cross-react with the crude antiserum. As expected, vinculin was not susceptible to digestion with bacterial collagenase. When total RNA from SV40 transformed WI38 cells was translated in vitro, and then immunoprecipitated, the same polypeptide of M_r 125,000 was recovered. Quantitation revealed that the mRNA activity for vinculin was reduced approximately threefold in the transformed cells.

We next looked for GP140-specific mRNA in cells derived from two spontaneous human tumors of mesodermal origin. Based on in vitro translation, cells from an astrocytoma (251 MG cells) exhibited a 13-fold lower level of mRNA for GP140 than normal WI38 cells, and cells from a rhabdomyosarcoma (A 204 cells) exhibited a 20-fold lower level. In both cases, however, translation products of GP140-specific mRNA could readily be detected (not shown). These reductions in mRNA activity correlate with the published observations that 251 MG and A 204 cells do not produce any matrix form of GP140 and only minor quantities of the released form (4, 7).

DISCUSSION

Transformation of fibroblasts by viruses causes a number of cellular changes including alterations in cell growth and shape,

and changes in the synthesis rate of a discrete group of proteins (see reference 15 for review). Two proteins whose synthesis is markedly reduced after viral transformation are fibronectin and collagen type I. This change appears to be specific, for the synthesis of most other cellular proteins is unaltered.

Recently, one of us reported that production of GP140, another glycoprotein of the extracellular matrix, is decreased in SV40 transformed fibroblasts to an extent similar to or even higher than that of fibronectin and collagen type I (3). In this study, we demonstrated that this reduction can be fully accounted for by a decrease in the level of translatable mRNA for GP140. Many steps in RNA metabolism could play an active part in regulating the ultimate steady state level of functional mRNA in the cytoplasm of a cell. Possible mechanisms for a decrease in the mRNA activity of GP140 include decreased transcription, increased degradation, or failure to process the mRNA to an active translatable form. It seems unlikely that the RNA preparations from transformed cells are contaminated with a specific inhibitor of translation that could cause a decrease in mRNA translation activity since (a) translation of other proteins including vinculin could readily be demonstrated; (b) several preparations of RNA that had been obtained by two different methods gave the same results; (c) additional extraction of the RNA with phenol/chloroform or addition of the ribonuclease inhibitor RNasin had no effect; and (d) the RNA was always denatured before it was added to the translation reaction.

In the case of fibronectin, it has been shown by *in vitro* translation and by use of a cloned cDNA probe that the level of fibronectin-specific mRNA is decreased in virally transformed fibroblasts to ~10% of that in normal cells (9, 15, 16). Similar techniques have been used to demonstrate that the decrease of type I collagen synthesis in Rous sarcoma virus transformed fibroblasts is due to a 10-fold reduction in the level of collagen-specific mRNA (9, 10, 15, 17, 18). Since a thermosensitive mutant of the Rous sarcoma virus induces a drop of collagen mRNA levels only at the permissive but not at the nonpermissive temperature, it was suggested that the control of collagen synthesis occurs at the transcriptional level (17, 18). This conclusion is supported by the fact that an unrelated bacterial gene linked to the collagen promoter is regulated in a similar way as the collagen gene after viral transformation (19).

A reduction in the biosynthesis of GP140 may have profound effects on the adhesive properties of transformed fibroblasts, since GP140 is known to promote cell attachment and cell spreading (2). Furthermore, low levels of GP140 may disturb the integrity of the extracellular matrix, for GP140 is normally associated with other matrix components (1-4). Several publications have appeared supporting the idea that GP140 represents a subunit of type VI collagen (5-7). Type VI collagen on the other hand was shown to be composed of three different polypeptide chains (20). It would therefore be interesting to investigate whether all three subunits of type VI collagen are coordinately regulated in transformed fibroblasts, or whether any of the subunits is preserved during transformation. A covalent interaction of GP140 with an M_r 240,000 polypeptide is indicated by the co-precipitation of the two proteins from the conditioned media of WI38 cells (Fig. 1,

lanes 4-6). The possibility that the polypeptides covalently linked to GP140 is identical to GP250, another component of the extracellular matrix of cultured fibroblasts (1-4), or to the $\text{pro}\alpha 1$ chain of type VI procollagen (8) is currently under investigation. Immunoblotting experiments have previously revealed that GP250 is also absent from the matrix of transformed fibroblasts; yet transformed fibroblasts secrete a soluble form of GP250 into the culture medium (3). We attempted to correlate the level of functional mRNA of GP250 with that of GP140. Cell-free synthesis of GP250, however, was not successful using the rabbit reticulocyte lysate system as has been reported for fibronectin (21). Such problems will certainly be overcome by preparation of specific cDNA probes to this new class of extracellular matrix proteins.

This work was supported by grant BC-419 from the American Cancer Society and by a grant from the Swiss National Science Foundation.

Received for publication 18 July 1984, and in revised form 22 October 1984.

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