Synthesis and Localization of Two Sulphated Glycoproteins Associated with Basement Membranes and the Extracellular Matrix

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ABSTRACT Two sulphated glycoproteins (sgps) of apparent molecular weight (M_r) 180,000 and 150,000, are synthesized by murine PYS and PF HR9 parietal endoderm and Swiss 3T3 cells. The M_r 150,000 sgp has a similar chemical structure to the sulphated glycoprotein, C, synthesized and laid down in Reichert's membrane by mouse embryo parietal endoderm cells (Hogan, B. L. M., A. Taylor, and A. R. Cooper, 1982, *Dev. Biol.*, 90:210–214). Both the M_r 180,000 and 150,000 sgps are deposited in the detergent-insoluble matrix of cultured cells, but they do not apparently undergo any disulphide-dependent intermolecular interactions and are not precursors or products of each other. They contain asparagine-linked oligosaccharides, but these are not the exclusive sites of sulphate labeling. Antiserum raised against the M_r 150,000 sgp C of Reichert's membranes has been used in an immunohistochemical analysis of rat skin. In early foetal and adult skin the antigen is present only in basement membranes, but transiently before and after birth it is also found throughout the upper part of the dermis. This suggests that 150,000 sgp C is at times synthesized by nonepithelial cells and contributes to the extracellular matrix of mesenchymal tissues.

Basement membranes are thought to play an important role in both establishing and maintaining tissue organization. The major components so far identified are Type IV procollagen, laminin (GP1 and GP2), and heparan sulphate proteoglycan of $M_r > 5 \times 10^5$ (1–6). More recently, sulphated glycoproteins (sgps) of M_r ~180,000 and 150,000 have been added to the list. Thus, Carlin et al. (7) have isolated a M_r 158,000 glycoprotein (gp), entactin, from the membrane sacs made by the murine parietal endoderm cell line, M1536-B3. Entactin was reported to be the major matrix gp labeled after incubating these cells with [^{35}S]sulphate. In vivo, entactin has been localized in a variety of basement membranes including kidney tubules, where the highest concentration is found immediately beneath the epithelial cell layer (7, 8).

While studying the biosynthesis of heparan sulphate proteoglycan by normal parietal endoderm (PE) cells from mouse embryos (6), we observed two heparitinase and chondroitin ABC lyase-resistant, sulphated glycoproteins (sgps) of M_r 175,000 and 145,000 in the basement membrane (Reichert's membrane) laid down by these cells. The lower molecular weight sgp appeared to be the same as the M_r 150,000 gp, C, originally described by us as immunoprecipitated from the culture medium of PE cells with antilaminin serum (4, 9). In the present paper, we report on the sgps of the murine parietal endoderm cell line, PYS. These cells incorporate some [^{35}S]-sulphate into the A- and B-chains of laminin and into the 150,000-M_r gp C. However, we find that the major sgp incorporated into the extracellular matrix has a M_r of 180,000. Extending these studies to other cultured murine cell lines, we found that Swiss 3T3 and STO cells also make M_r 180,000 and 150,000 sgps.

Antiserum raised against purified denatured M_r 150,000 sgp C has been used to localize the antigen in rat skin at different times of development. In adult and early fetal tissue, the antigen is restricted to basement membranes. However, during late embryonic stages and until 10 d after birth, 150,000-M_r sgp C is also present throughout the upper part of the dermis. This suggests that the 150,000-M_r sgp is at times synthesized by nonepithelial cells, and contributes to the extracellular matrix of mesenchymal tissues.

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MATERIALS AND METHODS

Cell Culture and Radioactive Labeling

Teratocarcinoma-derived PYS (10) and PFHR-9 (11) parietal endoderm cells were obtained from Drs J. Lehman (University of Colorado Medical Center, Denver, CO) and R. Oshima (La Jolla Cancer Research Foundation, La Jolla, CA), respectively. Swiss 3T3 cells (12) were from stocks held by the Cell Production Unit, Imperial Cancer Research Fund (Lincoln's Inn Field, London, England). STO is a thioguanine and ouabain-resistant line derived from 15–17-d-old SIM mouse embryo cells (13). It was originally obtained from Dr. A. Bernstein (Ontario Cancer Institute, Toronto, Canada). All cells were cultured in Dulbecco's modified Eagle's medium (DME) in 10% fetal bovine serum (FBS).

For labeling with [^{35}S]methionine (Met), cultures of ~3 × 10^5 cells in 35-mm tissue culture dishes were washed twice with DME without Met (DME-Met) and then incubated with 0.5 or 1.0 ml of DME containing 1 μg/ml cold Met, 10% dialyzed FBS, and 100–200 μCi/ml [^{35}S]Met (Amersham International; sp act 1,000 Ci/mmol).

For labeling with [^{35}S]SO_4, cultures of similar density in 35-mm dishes were washed twice with DME in which the $MgSO_4$ had been replaced with $MgCl_2$ (DME-SO_4) containing 10% dialyzed FBS and 200–500 μCi/ml [^{35}S]SO_4 (Amersham International, Amersham, Bucks, England; sp act 25–40 Ci/mg).

Tunicamycin (Sigma Chemical Co., St. Louis, MO) was prepared as a stock solution in dimethyl sulphoxide, and added to cells in DME containing only 1% FBS, at final concentrations of 5 or 2 μg/ml. These concentrations inhibited protein synthesis by 35% and 14% respectively. Control experiments showed that the minimum dose for complete inhibition of *N*-glycosylation of laminin in PYS cells and sgpC in 3T3 cells was 1 μg/ml; higher doses gave no further change in electrophoretic mobility.

Cell Fractionation

At the end of the labeling period the culture medium was removed, centrifuged to remove cells and debris and phenyl methyl sulphonylfluoride (PMSF), and *N*-ethylmaleimide (NEM) added to a final concentration of 2 and 5 mm, respectively. The cell layer was washed twice with 2 ml of Dulbecco's phosphate-buffered saline (PBSA), the dish placed on ice, and 500 μl of lysis buffer (0.15 M NaCl, 0.005 M EDTA, 0.05 M Tris, pH 8.0, 1% (vol/vol) Nonidet P-40 (NP-40), containing 0.002 M PMSF and 0.005 M NEM) added gently. After 10 min the cytoplasmic fraction was removed and centrifuged at 10,000 rpm for 10 min at 4°C. This detergent soluble fraction contained 92% of the total, cell-associated, acid-precipitable radioactivity after incubating cells for 2.25 h with [^{35}S]Met. The detergent-insoluble material remaining attached to the culture dish was washed with 2 ml of 0.01 M Tris-HCl, pH 6.8, at 4°C, and solubilized in 200 μl of electrophoresis sample buffer (see below) containing 2-mercaptoethanol at 100°C.

Polyethylene Glycol and Immunoprecipitation

Polyethylene glycol M_r 6,000 (Koch Light) was added to the culture medium to give a final concentration of 10% (wt/vol), together with 50 μg/ml gelatin (Sigma Chemical Co.; swine skin Type 1) as carrier. After 4 h at 4°C, the precipitate was collected by centrifugation, washed twice with 70% aqueous ethanol, and dissolved in electrophoresis sample buffer (see below).

Immunoprecipitation was carried out essentially as described (9), using 5–10 μl of antiserum and 30–50 μl of a 50% suspension of protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) in 1 ml of 0.4 M NaCl, 0.005 M EDTA, 0.05 M Tris, pH 8.0, 1% (vol/vol) NP-40.

Collagenase Digestion

High molecular weight molecules precipitated with 10% polyethylene glycol from the medium of 3 × 10^5 cells labeled for 19 h with [^{35}S]Met or -SO_4, were resuspended in 50 μl buffer (0.5 M NaCl, 0.5 mM $CaCl_2$, 0.05 M Tris, pH 7.5, with 5 mM NEM and 2 mM PMSF) with or without collagenase (Advance Biofactures Corp., Lynbrook, NY; Form III) at a concentration of 50 U/ml. The samples were incubated at 37°C for 2 h before analysis by SDS PAGE. All of the Type IV procollagen present was digested in this time.

SDS PAGE

This was carried out as described (9) by the method of Laemmli (14) using (unless otherwise stated) 5–10% gradient slab gels. Electrophoresis sample buffer contained 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.0625 M Tris, pH 6.8, and, unless otherwise stated, 5% (vol/vol) 2-mercaptoethanol. All samples were heated to 100°C for 3 min. Fluorography was carried out as described (9).

Immunoblotting

The method used was essentially that described by Towbin et al. (15). Approximately 20 μg of total protein from Reichert's membranes solubilized in 2% SDS, 0.0625 M Tris, pH 6.8, 5% 2-mercaptoethanol at 100°C (4), or 2 μg fibronectin isolated from human plasma by chromatography on gelatin-Sepharose (16) were analyzed on a 6% polyacrylamide slab gel under reducing conditions. Proteins were transferred to nitrocellulose using a constant current of 100 ma for 13–16 h and recovery assessed by staining part of the nitrocellulose sheet with Amido Black and the polyacrylamide gel with Coomassie Blue. The remaining nitrocellulose sheet was soaked in 3% haemoglobin in PBSA for 2 h, and strips then incubated with serial dilutions of antiserum (1:100–1:400) in 5 ml 3% Hb in PBSA for 2 h at room temperature. After extensive washing, the strips were incubated with 0.2 μCi ^{125}I-protein A (Amersham International; sp act 30 mCi/mg) in 5 ml 3% Hb in PBSA at room temperature for 2 h. Autoradiography was at −70°C using Fuji x-ray film and intensifying screens.

Antisera

Rabbit anti-hamster LETS protein (fibronectin) serum used for immunoprecipitation and immunoblotting experiments was kindly provided by Dr. R. O. Hynes (Center for Cancer Research, Department of Biology, Massachusetts Institute of Technology). Rabbit anti-mouse M_r 150,000 gp C (anti-C) serum was obtained by immunizing rabbits with denatured C-chains isolated from Reichert's membranes and purified by SDS PAGE (9). The antiserum shows no cross-

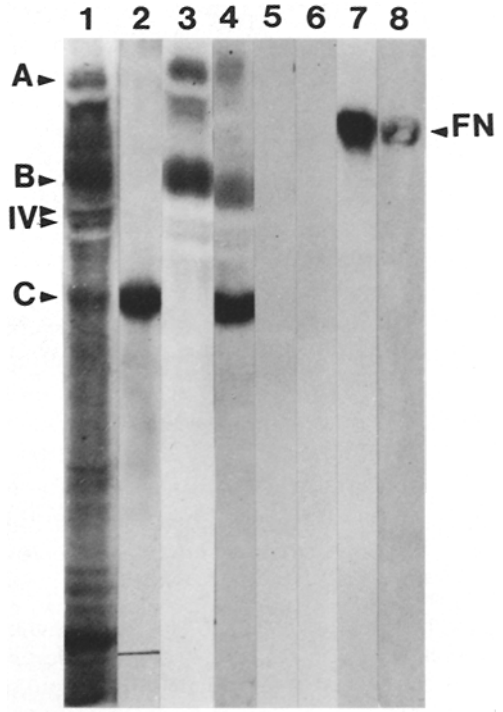

FIGURE 1 Specificity of antisera by immunoblotting. Human plasma fibronectin (2 μg/slot) and total protein from one batch of Reichert's membranes (~20 μg/slot) were analyzed on 6% SDS polyacrylamide gels under reducing conditions followed by transfer to nitrocellulose. Strips were incubated with antisera and ^{125}I-protein A as described. Lane *1*: Typical nitrocellulose strip showing Reichert's membrane protein stained with Amido Black. *A*, *B*, and *C* are the 450,000 and 230,000–240,000 chains of laminin, and the 150,000 gp C, respectively. *IV* marks the α_1- and α_2-chains of Type IV procollagen. Lane *2*: Autoradiogram of strip stained with anti-C′ serum at a dilution of 1:200. Lane *3*: Similar strip stained with anti-EHS sarcoma laminin antibody at a dilution of 1:400 (2.5 μg/ml). Lane *4*: Similar strip from a different transfer stained with anti-LC serum at a dilution of 1:200. In Lanes *5–8* purified fibronectin was transferred. Lane *5*: Autoradiogram of strip stained with anti-C serum at a dilution of 1:200. Film exposed for 3 d. Lane *6*: As for Lane *5* using anti-LC serum at a dilution of 1:200. Lane *7*: As for Lane *5* using antifibronectin serum diluted 1:400. Film exposed for 16 h. Lane *8*: Amido Black staining of nitrocellulose.

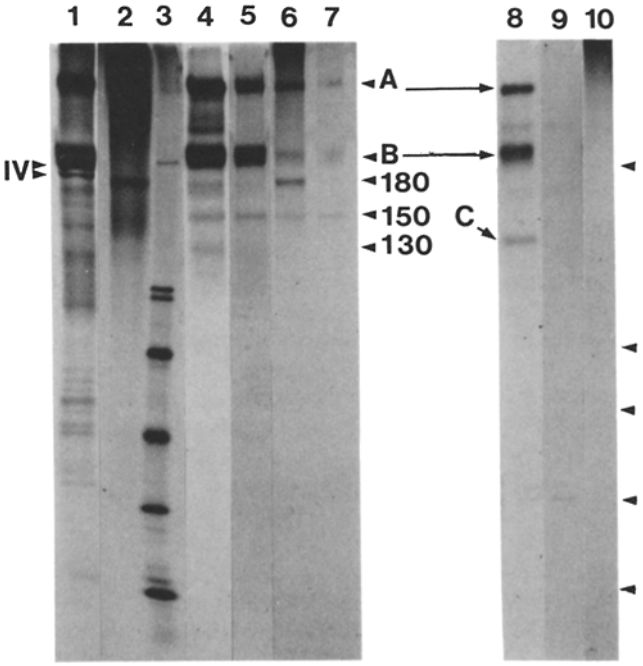

FIGURE 2 Sulphated macromolecules secreted by PYS cells. 3×10^5 PYS cells were labeled for 16 h in 0.5 ml of medium containing either 100 μCi [^{35}S]Met or 400 μCi [^{35}S]SO_4. At the end of the incubation, 60-μl aliquots of the medium were used for the isolation of high molecular weight components with 10% polyethylene glycol or for immunoprecipitation. Lanes *1-7* are from one experiment, lanes *8-10* from another. Lane *1*: [^{35}S]Met-labeled proteins precipitated with 10% polyethylene glycol. The α_1 and α_2 Type IV procollagen polypeptides (IV) migrate close to the B-chains of laminin. They could be clearly resolved on shorter exposure of the gel to film. The exposure in this figure was 16 h. Lane *2*: [^{35}S]SO_4-labeled material precipitated with 10% polyethylene glycol. The heterogeneous material migrating near the top of the gel is heparan sulphate proteolgycan (6). Film exposed for 10 d. Lane *3*: [^{14}C]-labeled protein M_r standard proteins (Amersham International). These are heavy chains of myosin (212,000), phosphorylase *b* (100,000 and 92,500), bovine serum albumin (69,000), ovalbumin (46,000) carbonic anhydrase (30,000) and lysozyme (14,300). Lane *4*: [^{35}S]Met-labeled polypeptides immunoprecipitated with anti-LC serum. Film exposed for 16 h. Lane *5*: [^{35}S]Met-labeled polypeptides immunoprecipitated with anti-C serum. Film exposed for 2 d. Lanes *6* and *7*: As for lanes *4* and *5*, but [^{35}S]SO_4 label. Lane *8*: [^{35}S]Met-labeled polypeptides immunoprecipitated with anti-C′ serum. Lane *9*: As for lane *8* using preimmune serum. Lane *10*: As for lane *9* using [^{35}S]SO_4-labeled medium.

reaction with fibronectin either in 1% immunodiffusion gels or by immunoblotting (Fig. 1). It does not react with Type IV procollagen either by immunoblotting (Fig. 1) or by immunoprecipitation (see Fig. 2). Unabsorbed serum does have weak cross-reaction with reduced and alkylated laminin, as described (9). However, after absorption with native Engelbreth-Holm-Swarm (EHS) sarcoma laminin (kindly supplied by Dr. R. Timpl, Max. Planck Institut Fur Biochemie, Martinsried, Federal Republic of Germany) covalently coupled to Sepharose (200 μg/100 μl of antiserum), the antiserum reacts only with the 150,000-M_r gp C component of total Reichert's membrane protein, as shown by immunoblotting (Fig. 1). This absorbed antiserum is designated anti-C′.

Rabbit anti-mouse laminin and 150,000-M_r gp C (anti-LC) serum was obtained by immunizing rabbits with native laminin and M_r 150,000 gp C isolated from Reichert's membranes (9). The antiserum shows no cross-reaction with fibronectin either in 1% immunodiffusion gels or by immunoblotting (Fig. 1). The antiserum does not react with Type IV procollagen, either in 1% immunodiffusion gels, or by immunoprecipitation (Fig. 2) or immunoblotting (Fig. 1). Immunoblotting experiments show that it reacts with both the A- and B-chains of laminin and the M_r 150,000 C polypeptide (Fig. 1). Affinity purified rabbit anti-EHS sarcoma laminin was kindly provided by Dr. R. Timpl. This does not react with M_r 150,000 C (Fig. 1).

Peptide Mapping

This was by a modification of the method of Cleveland et al. (17) as described previously (9).

In Vitro Translation of STO Cell mRNA

Total cellular RNA from STO cultures was prepared as previously described (18). For in vitro protein synthesis, 20 μl of nuclease treated rabbit reticulocyte lysate (a gift of Dr. J. Jenkins, Imperial Cancer Research Fund) was incubated at 30°C for 3 h in a final volume of 30 μl containing 10–30 μCi [^{35}S]Met, 0.2 mg/ml total cellular RNA, 50 μM of all amino acids except Met, 37.5 μg/ml calf liver tRNA, 37.5 μg/ml creatine kinase, 10 mM creatine phosphate, 5 mM 2-aminopurine, 0.1 mM spermine, 100 mM KCl, and 0.5 mM $MgCl_2$. Dog pancreas microsomal membranes were kindly provided by Dr. M. Owen (Imperial Cancer Research Fund) (19) and were added at a concentration of 9.6 A_{260} U/25 μl of total reaction mix.

Immunohistochemistry

Frozen sections of rat skin were prepared using previously described methods (20), but with paraformaldehyde fixation of the tissue after sectioning rather than before freezing. The anti-C serum was used at a 1:30 dilution. For antigen absorption experiments 1 μg 150,000 sgp C purified from Reichert's membranes by gel electrophoresis (9) was added to 30 μl of diluted anti-C serum and left overnight at 4°C before use. The preparation and characterization of the anti-bovine plasma fibronectin serum has been fully detailed elsewhere (21). Fluorescein isothiocyanate anti-rabbit IgG serum was obtained from Miles Laboratories (Slough, Bucks, England) and used at a 1:50 dilution. Photographs were taken on a Leitz Ortholux II microscope fitted with epi-illumination on Ilford HP5 film. The exposure and development times were identical for each micrograph.

RESULTS

Sulphated Glycoproteins in the Culture Medium of PYS Cells

Confluent cultures of PYS cells were labeled with either [^{35}S]Met or [^{35}S]SO_4. Equal aliquots of the culture medium were then treated with either polyethylene glycol, to precipitate high molecular weight molecules, or immunoprecipitated with antisera raised against proteins isolated from Reichert's membranes. Anti-LC serum reacts with the A- and B-chains of laminin and the 150,000-M_r gp C, while anti-C serum reacts only with the 150,000 gp C (Fig. 1). As shown in Fig. 2, the major [^{35}S]Met labeled high molecular weight proteins secreted by PYS cells are laminin (M_r, 450,000 A-chains and M_r, 230,000–240,000 B-chains) and α_1 and α_2 Type IV procollagen (M_r, 195,000 and 190,000) (9, 22). Labeling with [^{35}S]SO_4 reveals the synthesis of very high molecular weight heparan-sulphate proteoglycan (6, 22), some of which migrates into the top of the gel, and a prominent M_r 180,000 sulphated species (Fig. 2, lane *2*). This 180,000-M_r band is not sensitive to collagenase digestion (see Materials and Methods). Anti-LC serum immunoprecipitates several of the [^{35}S]Met-labeled polypeptides, including laminin A- and B-chains, a closely spaced doublet of M_r ~180,000, the 150,000-M_r, gp C, and a minor 130,000-M_r species. Similar results were obtained with anti-EHS sarcoma laminin serum (data not shown). Immunoprecipitation of medium from cells incubated with [^{35}S]SO_4 revealed labeling of laminin A-chains (obscured in lane *2* [Fig. 2] by the heparan-sulphate proteoglycan) and the slower migrating component of the 180,000-M_r doublet. Laminin B-chains and the 150,000-M_r polypeptide are labeled less strongly. A very similar complex of [^{35}S]SO_4 and ^{35}[S]Met-labeled proteins was immunoprecipitated with anti-C serum, except that this antiserum immunoprecipitates much less of the 180,000 sulphated protein. Anti-C′ serum absorbed with EHS sarcoma laminin also immunoprecipitates the A- and B-chains of laminin from the culture medium of PYS cells (Fig. 2, lane *8*).

Sulphated Glycoproteins in the Cell Layer of PYS and PFHR9 Cultures

We next investigated whether the sulphated M_r 180,000 and 150,000 species described above are also present in the extracellular matrix laid down by PYS and PFHR9 cultures. Cells were labeled with [^{35}S]Met or [^{35}S]SO_4, and the cytoplasm extracted with buffer containing the nonionic detergent NP-40. The detergent-insoluble residue attached to the culture dish was then analyzed by SDS PAGE. As shown in Fig. 3, lane *1*, this residue contains many [^{35}S]Met-labeled proteins, including myosin and actin. However, only three sulphated components are present; very high molecular weight heparan sulphate proteoglycan, a prominent 180,000-M_r species, and a minor 150,000-M_r band (Fig. 3, lane *2*). The 150,000-M_r component comigrates with the [^{35}S]Met-labeled polypeptide C in the culture medium immunoprecipitated with anti-C serum (Fig. 3, lane *3*). Similar results were obtained with the cell line PFHR9 (Fig. 3, lanes *4–6*), and with PYS cultures that were either subconfluent or dense (not shown).

The inhibitor tunicamycin was used to investigate whether the M_r 180,000 sulphated protein deposited in the matrix of PYS cells contains asparagine-linked carbohydrate residues, as shown earlier for the basement membrane sgps synthesized by normal parietal endoderm cells (6, 9). Tunicamycin reduces the M_r of the 180,000 sulphated protein to ~176,000, but does not abolish the sulphate labeling (Fig. 4*a*). To see if the M_r 180,000 and 150,000 sgps in the matrix are part of a higher molecular weight disulphide bonded complex or network, the [^{35}S]SO_4-labeled detergent insoluble residue was analyzed under reducing and nonreducing conditions. This produced only a small change in the migration of the M_r 180,000 and 150,000 sgps (Fig. 4*b*). The possibility was also considered that the M_r 180,000 and 150,000 sgps are precursors or products of each other. Some evidence against such a relationship comes from the results of the pulse-chase experiment described in Fig. 4*c*, which shows that over 24 h there was no apparent conversion of the 180,000 sulphated protein in the matrix into material with a M_r of 150,000. The results of *Staphylococcus aureus* V8 proteinase digestion of the two sgps is shown in Fig. 5. The larger, [^{35}S]Met labeled cleavage products differ in M_r by ~30,000. Some comigrating smaller peptides are also generated (open arrows).

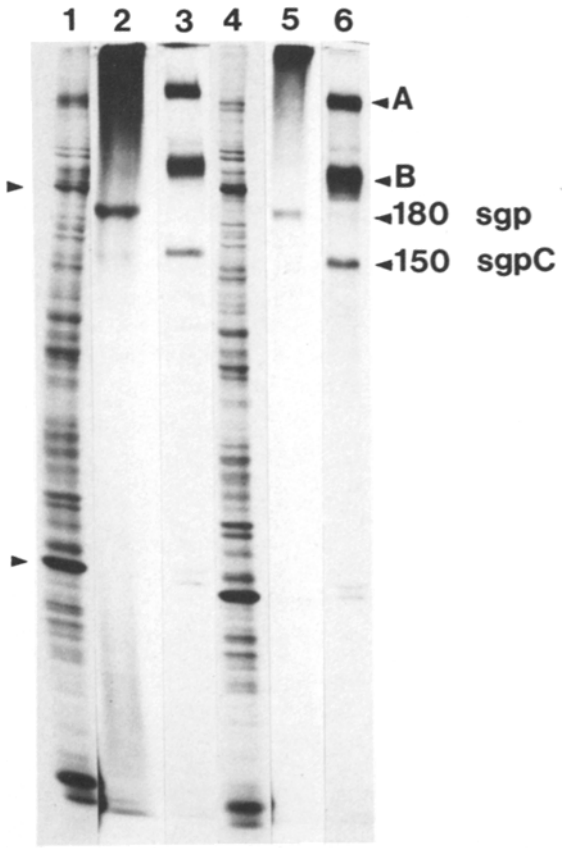

FIGURE 3 Sulphated macromolecules in the detergent insoluble residue of PYS and PF HR9 cells. 3×10^5 cells were labeled for 20 h in 1.0 ml of medium with either 100 μCi [^{35}S]Met or 200 μCi [^{35}S]-SO_4. After collecting the medium, the cells were washed and the cytoplasm extracted in 500 μl of buffer containing 1% NP-40. The detergent-insoluble residue remaining on the dish was washed, and solubilized in electrophoresis sample buffer at 100°C. Lane *1*: [^{35}S]Met-labeled polypeptides in the detergent-insoluble residue. The heavy chain of myosin and actin are marked with arrows. Lane *2*: [^{35}S]SO_4-labeled components of the detergent-insoluble residue. Lane *3*: [^{35}S]Met-labeled polypeptides immunoprecipitated from 400 μl culture medium with anti-C serum. Lanes *4–6*: As above, but using PF HR9 cultures.

Extracellular Sulphated Glycoproteins Synthesized by Swiss 3T3 and STO Cells

In a survey of different cell lines, we found that Swiss 3T3 cells synthesize and secrete 150,000 sgpC (Fig. 6). Immunoprecipitation of the culture medium with anti-LC serum reveals that these cells also synthesize some M_r 180,000 sgp, but less, relative to the 150,000 species, than PYS cells (not shown). Antifibronectin serum does not react with the M_r 150,000 and 180,000 sgps secreted by 3T3 cells (Fig. 6, lane *2*).

In the cytoplasmic fraction of 3T3 cells labeled for 2 h with [^{35}S]Met, gp C exists as two species, of M_r ~150,000 and 145,000 (Fig. 6, lane *6*). By analogy with experiments on endoderm cells (9), the larger molecules are those which have undergone terminal glycosylation in the Golgi apparatus, while the smaller ones are partially glycosylated molecules not yet fully processed. Both in the medium (Fig. 6, lane *3*) and cytoplasmic fraction (not shown), [^{35}S]SO_4 label is associated only with the larger 150,000 molecules. After treating 3T3 cells with tunicamycin, the glycoprotein C secreted into the culture medium has a M_r of ~145,000 and is still labeled with [^{35}S]-SO_4 (Fig. 6, lanes *4* and *7*). Immunoprecipitation of the cytoplasmic fraction of tunicamycin treated cells labeled with [^{35}S]Met for 2 h reveals two C polypeptides, both of which migrate faster than the corresponding chains in untreated cells (Fig. 6, track *8*). Control experiments gave identical results with between 1 and 5 μg/ml tunicamycin (see also Fig. 6, lane *9*), and showed that these doses yielded only single intracellular precursors of laminin A-, B_1- and B_2-chains in PYS cells (9).

To test the possibility that the rate of synthesis of 3T3 sgps varies with cell density, sparse, touching, and confluent cultures were labeled with [^{35}S]Met and aliquots of the culture medium containing equal amounts of acid precipitable radioactivity were immunoprecipitated with anti-C serum. As shown in Fig. 7, there does not appear to be a significant change in the specific accumulation of the 150,000 sgp in the culture medium with cell density. Similarly, the ratio of the M_r 180,000 and 150,000 sgps in the detergent insoluble residue attached to the culture dish was apparently unaffected by cell density.

As with PYS cells, the 150,000 sgp in the medium and cytoplasm of 3T3 cells is not disulphide bonded into higher

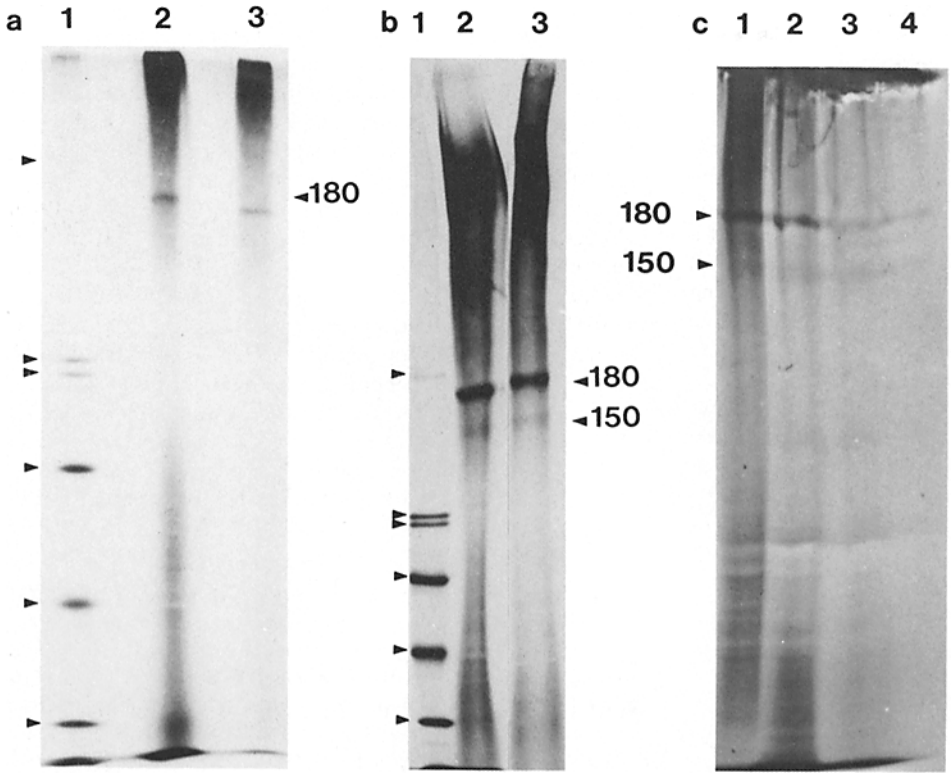

FIGURE 4 Sulphated glycoproteins in the detergent-insoluble matrix of PYS cells. (*4a*) Effect of tunicamycin. 3 × 10^5 PYS cells were preincubated for 3.5 h with or without 5 μg/ml tunicamycin. The cells were then labeled for 10 h with 400 μCi [^{35}S]SO_4 in 0.5 medium, also in the presence or absence of inhibitor. The detergent-insoluble residue was isolated and analysed under reducing conditions. Lane *1*: [^{14}C]-labeled M_r standard proteins (arrowed). Lane *2*: Detergent-insoluble residue of control cells. Lane *3*: Detergent-insoluble residue of cells incubated with tunicamycin. (*b*) Absence of disulphide bonding. Dense cultures of PYS cells (~1 × 10^6/35-mm dish) were labeled for 11 h in 1 ml of medium containing 200 μCi [^{35}S]SO_4. The detergent-insoluble residue was prepared as described, and solubilized in SDS electrophoresis sample buffer with or without 2-mercaptoethanol. Equal aliquots were analyzed on the same 5% gel. The similar intensity of the M_r 180,000 band in lanes *2* and *3* is evidence that the gp is as efficiently extracted from the matrix in the absence of 2-mercaptoethanol as in its presence. Lane *1*: [^{14}C]-labeled M_r standard proteins (arrowed). Lane *2*: [^{35}S]SO_4-labeled detergent-insoluble residue analyzed under reducing conditions. Lane *3*: [^{35}S]SO_4-labeled detergent-insoluble residue analyzed under nonreducing conditions. (*c*) Pulse-chase analysis. Cultures of 4 × 10^5 PYS cells were labeled for 6 h in 0.5 ml of sulphate free medium containing 250 μCi of [^{35}S]SO_4. The medium was then removed, the cells washed, and incubation continued with 2 ml of DME containing the normal amount of $MgSO_4$. 40-μl aliquots of the detergent-insoluble residue of each culture were analyzed under reducing conditions. Lane *1*: [^{35}S]SO_4-labeled components of the detergent-insoluble residue after 6 h of labeling. Lane *2*: As above, after 8.5-h chase. Lane *3*: After 18-h chase. Lane *4*: After 24-h chase.

molecular weight aggregates, although a small shift in electrophoretic migration is observed under nonreducing conditions (not shown). Further evidence for a similar chemical structure between the 150,000 sgp synthesized by 3T3 cells and parietal endoderm cells comes from a comparison, by one-dimensional SDS cell electrophoresis, of the peptides generated by *S. aureus* V8 protease digestion (Fig. 8).

Similar results were obtained using the murine STO cell line, and Swiss 3T6 cells (not shown).

In Vitro Translation of Messenger RNA for 150,000 Glycoprotein C

Total RNA, isolated from STO cells, was translated in the rabbit reticulocyte lysate with or without supplementation by dog pancreas microsomal membranes. Polypeptides were then immunoprecipitated from the total translation products using anti-C serum, and analyzed by SDS gel electrophoresis. As shown in Fig. 9, the in vitro synthesized C polypeptide has a M_r equal to 140,000 (Fig. 9, lane *3*). Translation in the presence of microsomal membranes yields a C polypeptide with a M_r of ~143,000 (Fig. 9, lane *2*).

Immunohistochemical Localization of 150,000 sgp C in Rat Skin

Frozen sections of rat skin at various developmental stages were stained by indirect immunofluorescence using anti-C serum. In 15-18-d old embryos, the 150,000 sgp is located only in basement membranes (Fig. 10*a*). However, at later stages of prenatal development until ~10 d postpartum, there is staining of short fibrils throughout the papillary dermis (Fig. 10*c*). This pattern is in addition to, and quite distinct from, the staining of basement membranes, including those of capillaries. It is also distinct from the pattern obtained with antifibronectin serum (Fig. 10*b*). The reticular dermis does not show staining with anti-C serum at this stage. Anti-C serum preabsorbed with purified M_r 150,000 sgp C antigen gave negative results on skin of all ages (Fig. 10*d*), but staining was not removed by preadsorption with EHS sarcoma laminin (not shown). In adult rats, the pattern of staining is the same as that of 15–18-d old embryos (not shown).

DISCUSSION

Here we show that murine PYS and PFHR9 parietal endoderm, and Swiss 3T3, cells synthesize two extracellular matrix sgps of M_r 180,000 and 150,000. These appear to be the same as the two sgps made by mouse embryo parietal endoderm cells and deposited in vivo into the underlying Reichert's membrane (6). The 150,000 sgp C is probably the same as entactin, a basement membrane M_r 158,000 sgp recently described by Carlin et al. (7). However, the M_r 180,000 sgp has not previously been identified as a product of cells in culture. In addition, our novel observation that the A- and B-chains of PYS laminin are sulphated (Fig. 1, lane *6*) and the sulphation of fibronectin (Fig. 6 and reference 23), raise the possibility that sulphation of noncollagenous extracellular matrix gps is a

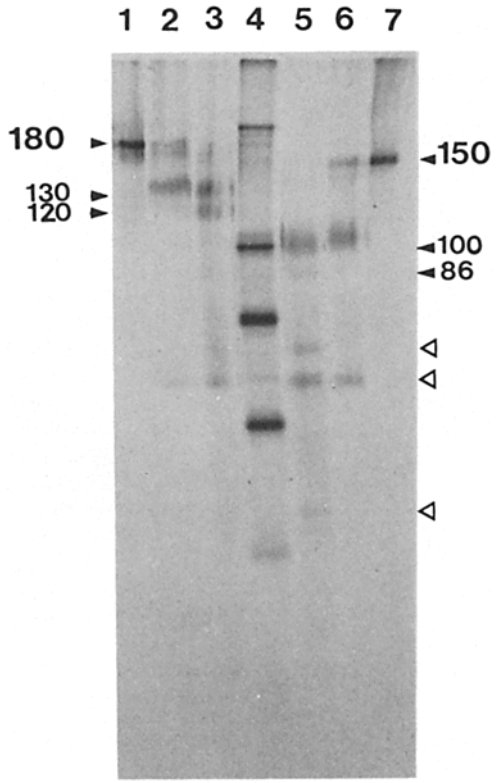

FIGURE 5 *S. aureus* V8 proteinase digestion of 150,000 and 180,000 sgps made by PYS cells. 3×10^5 PYS cells were incubated for 5 h in 0.5 ml of medium containing 250 μCi [^{35}S]Met. The medium was immunoprecipitated with antilaminin serum and the samples fractionated on a 5–7.5% gradient SDS polyacrylamide gel. Bands corresponding to the M_r 180,000 and 150,000 sgps were cut out and digested with *S. aureus* V8 protease at 37°C for 2 h. The digests were analyzed on a 10–15% gradient gel. Lanes *1–3*: 180,000 s gp digested with 0, 10, and 100 μg/ml V8 protease, respectively. Lane *4*: [^{14}C]-labeled M_r standard proteins (from New England Nuclear). Lanes *5–7*: 150,000 sgp digested with 100, 10, and 0 μg/ml V8 protease, respectively. The position of possible common peptides is marked with open arrows, and the approximate M_r's of the major cleavage products indicated.

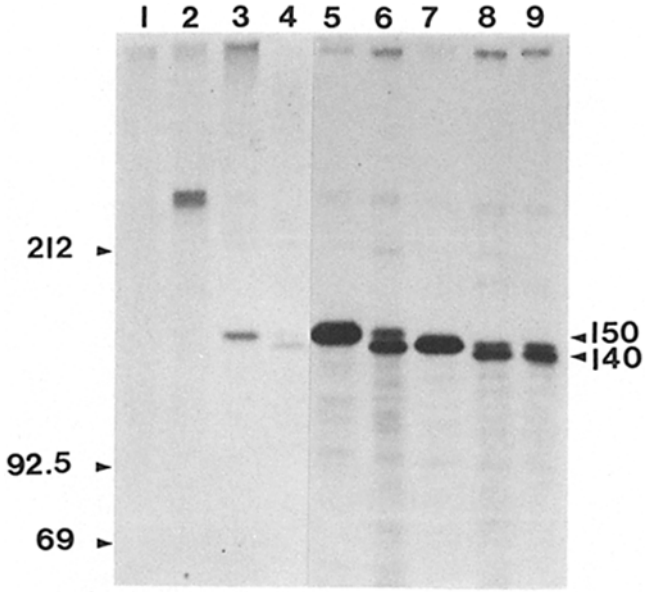

FIGURE 6 Synthesis of 150,000 sgp C by Swiss 3T3 cells. Cultures of 3×10^5 3T3 cells were preincubated for 2 h with or without 5 or 2 μg/ml tunicamycin and then labeled in the presence or absence of inhibitor for a further 16 h or 2 h with 500 μCi/ml [^{35}S]SO_4 or 200 μCi/ml [^{35}S]Met, respectively. Equal aliquots of the culture medium or cytoplasmic extract were immunoprecipitated and samples analyzed on a 5–7.5% gradient gel. Lane *1*: [^{35}S]SO_4-labeled control culture medium immunoprecipitated with preimmune rabbit serum. Lane *2*: As for lane *1*, using anti-fibronectin serum. Lane *3*: As for lane *1*, using anti-C′ serum. Lane *4*: As for lane *3*, using an equal aliquot of culture medium from cells incubated with 2 μg/ml tunicamycin. Lane *5*: [^{35}S]Met-labeled culture medium of control cells immunoprecipitated with anti-C serum. Lane *6*: [^{35}S]Met-labeled cytoplasmic fraction of control cells immunoprecipitated with anti-C serum. Lane *7*: As for lane *5*, using medium of cells treated with 5 μg/ml tunicamycin. Lane *8*: As for lane *6*, using the cytoplasmic fraction of cells treated with 5 μg/ml tunicamycin. Lane *9*: As for lane *8*, using cells treated with 2 μg/ml tunicamycin.

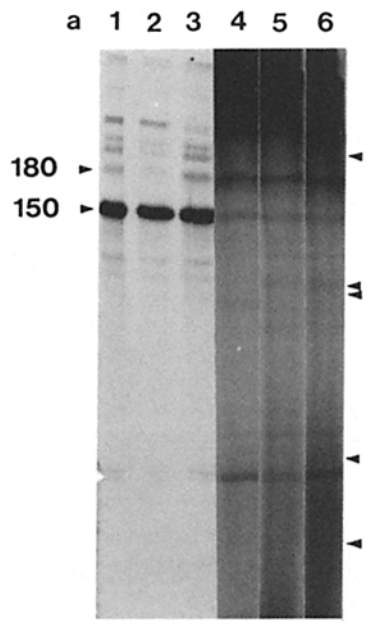

FIGURE 7. 150,000 sgp of 3T3 cells synthesized by sparse, touching and confluent cultures. Cultures of 3T3 cells containing 5×10^4, 1×10^5, and 5×10^5 cells were labeled for 15 h in 0.5 ml of medium with either 100 μCi [^{35}S]Met or 300 μCi [^{35}S]SO_4. Aliquots of the culture medium containing equal amounts of acid precipitable [^{35}S]Met radioactivity were immunoprecipitated with anti-C antiserum. Aliquots of the detergent-insoluble matrix containing equal amounts of acid precipitable [^{35}S]SO_4 radioactivity were also analysed on the same gel. Lanes *1–3*: [^{35}S]Met-labeled proteins immunoprecipitated from the medium of sparse, touching, and dense cultures, respectively. Lanes *4–6*: [^{35}S]SO_4-labeled components of the detergent-insoluble residue of the cultures used in lanes *1–3*. The positions of the [^{14}C]-labeled M_r standard proteins are indicated by arrows.

rather general phenomenon.

Two lines of evidence argue against a precursor-product relationship between the M_r 180,000 and 150,000 sgps. Firstly, anti-C serum recognizes only a single polypeptide of M_r 140,000 or 143,000 when RNA from STO cells (Fig. 9) or normal parietal endoderm cells (M. Kurkinen, unpublished results) is translated in vitro in the absence or presence of microsomal membranes, respectively. Secondly, there appears to be no interconversion of the two species in the matrix of PYS cells during a pulse-chase experiment (Fig. 4*c*). The one-dimensional peptide maps obtained by *S. aureus* V8 proteinase digestion (Fig. 5) are consistent with the larger sgp being a precursor of the smaller. However, taken with the other results, they can also mean that the two species are discrete proteins which share common sequences but differ by a 30,000-polypeptide extension.

Anti-C′ serum, which has been absorbed with EHS sarcoma laminin and does not react with A- and B-chains by immunoblotting (Fig. 1), nevertheless immunoprecipitates laminin from the culture medium of PYS cells. Similarly, antilaminin serum, which does not react with 150,000 C (Fig. 1) immunoprecipitates C from the culture medium of PE cells (4). These results

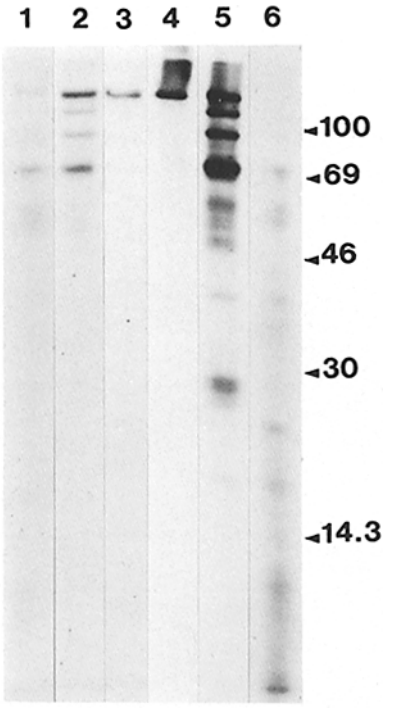

FIGURE 8. *S. aureus* V8 proteinase digestion of 150,000 glycoprotein C from 3T3 cells and normal parietal endoderm cells. 3×10^5 3T3 cells and 30 Reichert's membranes dissected from 13.5-d-old mouse embryos (4) were labeled for 16 h with 200 μCi [^{35}S]Met. The medium was then immunoprecipitated with anti-C serum, and the samples fractionated on a 5–10% gradient SDS polyacrylamide gel. Bands corresponding to the 150,000 gp C were cut out and digested with *S. aureus* V8 protease at 37°C for 2 h. The digests were analyzed on a 10–15% gradient gel. Lanes *1–3*: 150,000 sgp from 3T3 cells digested with 100, 10, and 0 μg/ml of V8 protease respectively. Lanes *4–6*: 150,000 sgp from normal parietal endoderm cells digested with 0, 10, and 100 μg/ml of V8 protease respectively.

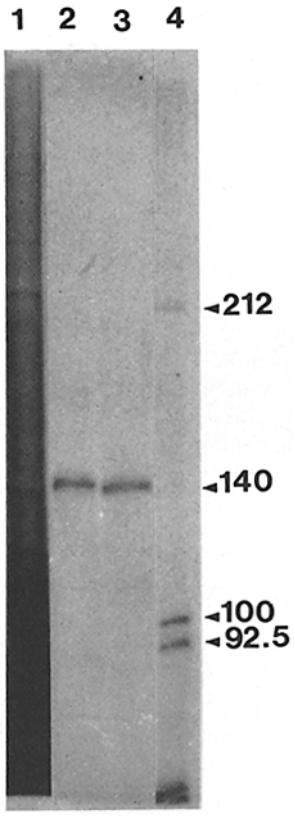

FIGURE 9 In vitro translation of gp C from total RNA of STO cells. Total cellular RNA isolated from STO cultures was translated in the nuclease treated rabbit reticulocyte lysate with and without dog pancreas microsomal membranes as described in Materials and Methods. Lane *1*: Total in vitro synthesized polypeptides. Lane *2*: Translation in the presence of membranes, immunoprecipitated with anti-C serum. Lane *3*: Translation in the absence of membranes, immunoprecipitated with anti-C serum. Lane *4*: [^{14}C]-labeled M_r standard proteins (Amersham International).

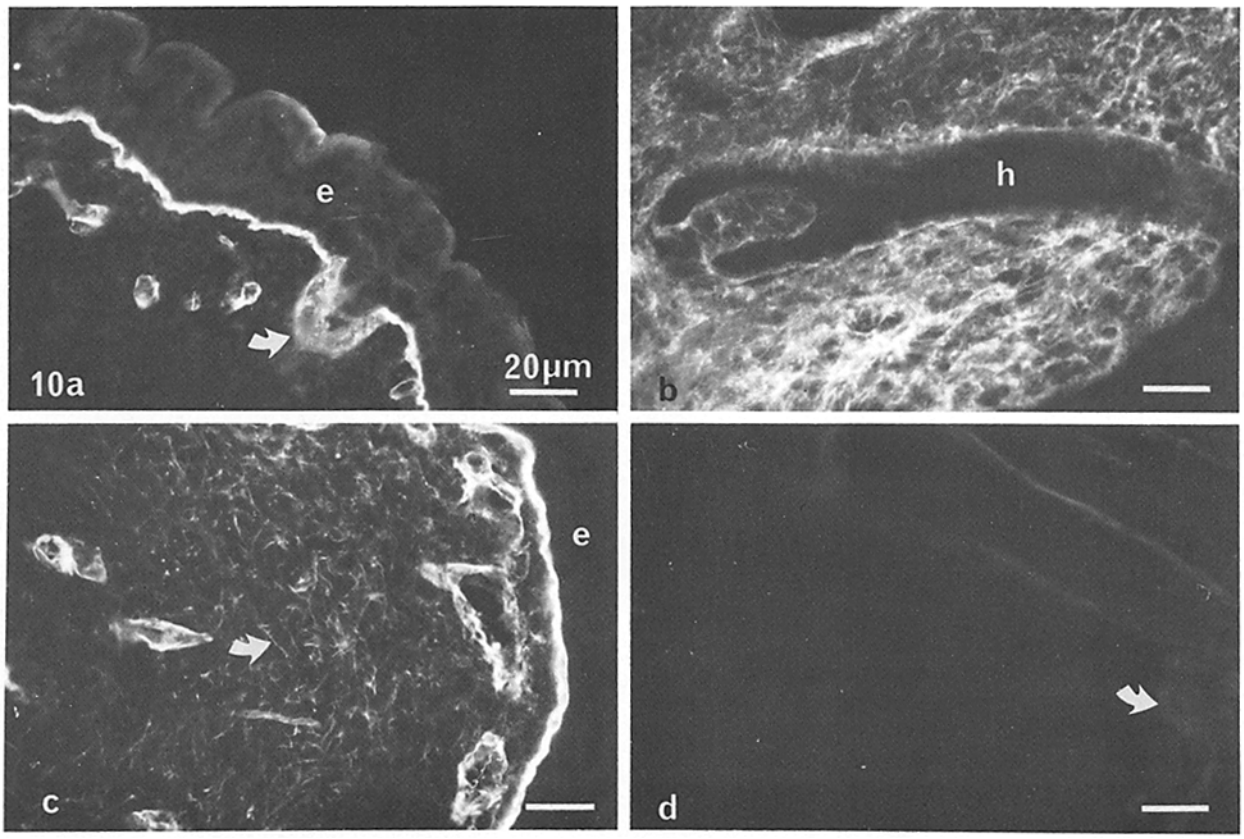

FIGURE 10 Antigen localization in rat skin by indirect immunofluorescence. (*a*) Distribution of the 150,000 sgp in 18-day rat embryo skin. The staining is restricted to basement membranes of the dermal/epidermal junction, including a developing hair follicle (arrow), and around capillaries and nerve and muscle fibers. *e*, epidermis. (*b*) Fibronectin distribution in neonate rat skin, characterized by widespread fibrillar staining of the dermis. The hair follicle (*h*) and epidermis are not stained. (*c*) 150,000 sgp distribution in neonate rat skin. In addition to basement membrane staining of the dermal/epidermal junction and capillaries (cf *a*) fine fibrils containing 150,000 sgp are visible (arrow). (*d*) Section of neonate rat skin treated with anti-C serum preabsorbed with the 150,000 sgp C. No positive stain is visible. The dermal/epidermal junction is pointed out by an arrow. Bars, 20 μm. × 460.

suggests that laminin and 150,000 sgp C form a noncovalent complex resistant to dissociation in 0.4 M NaCl (as used in the immunoprecipitation reaction). Carlin et al. (7) have also reported that laminin (GP1 and GP2) and entactin are immunoprecipitated as a complex from the medium of M1536-B3 parietal endoderm cells. It seems likely that in the culture medium these proteins would also form a complex with heparan-sulphate proteoglycan, since laminin binds to heparin at physiological salt concentrations (24).

Although 3T3 cells have been shown by radioimmune assay to synthesize laminin (3) this is the first report that these cells also produce 150,000 sgp C (entactin). Experiments described in this paper provide some information about the intracellular processing of this gp. Tunicamycin reduces the M_r of the secreted and intracellular polypeptides, but does not abolish labeling with [^{35}S]SO_4. This suggests that asparagine-linked oligosaccharides are present in the molecule, but are not the exclusive site of sulphation. Further evidence for the presence of N-linked oligosaccharides in C-chains comes from earlier experiments on the sensitivity of intracellular molecules to digestion by Endo-β-*N*-acetylglucosaminidase (Endo-H) (9). The observation that two intracellular polypeptides are present after labeling tunicamycin-treated cells with [^{35}S]Met for 2 h (Fig. 6, lanes *8* and *9*) suggests that the protein contains O-linked oligosaccharides, which may be sulphated. The unmodified C polypeptide synthesized in vitro in the rabbit reticulocyte lysate has an M_r of approximately 140,000 (Fig. 9). Preliminary experiments show that it migrates slightly slower than the smallest C-chains present in tunicamycin-treated cells (D. Barlow, unpublished observations). This suggests that the mRNA encodes a leader sequence which is removed during intracellular processing.

When anti-C serum was used to investigate the distribution of the 150,000 sgp C in rat skin, two quite distinct patterns were seen. In embryonic and adult animals, staining is confined to basement membranes, but during a transient period from ~3 d before birth to ~10 d after, there is also fibrillar staining of the papillary dermis (Fig. 10*c*). The significance of this transient dermal localization of 150,000 sgp C, and the qualitative change in fibronectin distribution also seen at this time (Couchman, J. R., A. R. Cooper, B. L. M. Hogan, A. C. Weaver, D. A. Rees, manuscript in preparation) is at present unclear. Preliminary experiments suggest that dermal fibroblasts synthesize both laminin and 150,000 sgp C (Couchman, J. R., unpublished results). The exact phenotype of Swiss 3T3 and STO cells is uncertain, but they were both originally derived from 15–17-d old mouse embryos (12, 13) and are generally considered to be of mesenchymal origin. Ekblom et al. (26) have localised laminin in embryonic kidney mesenchymal rudiments by indirect immunofluorescence, and Carlson et al. (27) have shown an increased level of laminin in sinusoids of regenerating mouse liver. Taken together, these results point to a role for 150,000 sgp C and laminin in mesenchymal tissues during processes such as cellular reorganization or repair.

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Note Added in Proof: We have now observed that antientactin serum, kindly provided by Dr. A. E. Chung (University of Pittsburg), immunoprecipitates the 150,000 sgp C made by Swiss 3T3 cells. It therefore appears that sgp C and entactin are identical molecules.

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