

Mesosecrin: a Secreted Glycoprotein Produced in Abundance by Human Mesothelial, Endothelial, and Kidney Epithelial Cells in Culture

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Abstract. Human mesothelial cells, endothelial cells, and type II kidney epithelial cells growing in culture devote ~3% of their total protein synthesis to the production of an *M_r* ~46-kD, pI 7.1, secreted glycoprotein (designated Sp46). Fibroblasts make about 1/10th as much Sp46 as these cell types, and their synthesis is dependent upon hydrocortisone. Keratinocytes, urothelial cells, conjunctival epithelial cells, and mammary epithelial cells do not make detectable amounts of Sp46. Mesothelial cells secrete Sp46 onto the substratum, and from there it is subsequently released into the medium. Immunofluorescence analysis using specific antisera discloses that Sp46 is deposited beneath cells as a fine coating on the substratum. In sparse cultures, Sp46 is detected in trails behind

motile cells. In contrast, secreted fibronectin coalesces into fibers, most of which remain in contact with and on top of the cells; thus Sp46 does not preferentially bind to fibronectin. About 6 kD of the mass of human Sp46 is N-linked oligosaccharide, which is terminally sialated before secretion. Sp46 has a low glycine content, indicating that it is not a collagenlike protein. Its NH₂-terminal sequence over the first 40 amino acids does not resemble any protein for which sequence information is available. Sp46 appears to be a novel extracellular glycoprotein, high-level constitutive expression of which is restricted to mesoderm-derived epithelial and endothelial cells. We therefore propose for it the name "mesosecrin."

MANY differentiation-related proteins made by somatic cells are secreted, rather than remaining as permanent cellular constituents. A secreted protein may perform its function for the cell that synthesizes it, or for other cells in the immediate vicinity (e.g., extracellular matrix proteins such as collagens, fibronectin, and laminin; or clotting factors and wound repair growth factors such as those released by platelets). Alternatively, a secreted protein may enter the general circulation and act at a great distance from the cell that produces it (e.g., polypeptide hormones, immunoglobulins, and serum albumin). In examining the intact animal, it is often difficult to identify the cell responsible for secreting a particular protein, whether it is in the general circulation or in the extracellular matrix of a tissue. It is also possible for a prominent secreted protein made by a particular cell type to remain undiscovered. This might occur if the protein is produced only under special circumstances, is produced by a small number of cells, is quickly altered or cleared, or if it is simply diluted by the total blood volume of the animal.

The study of pure cell populations in culture can circumvent these problems. We have been studying several normal human epithelial cell types in serial culture in order to identify differentiation and growth control phenotypes that are

epithelial-specific or are characteristic of only certain epithelial cell types. One of these cell types is the mesothelial cell, which forms the simple squamous epithelium that lines the inner body cavities (pleura, pericardium, and peritoneum) and covers the organs contained therein. Mesothelial cells occasionally detach from their epithelium and are therefore present in fluid withdrawn from the body cavities. Many of these cells are proliferative and can be serially cultivated and expanded into large populations in culture if provided with a complex nutrient medium containing epidermal growth factor (EGF),¹ hydrocortisone (HC), and FCS (Wu et al., 1982; Connell and Rheinwald, 1983; LaRocca and Rheinwald, 1985). The mesothelial cell is rather unusual among the epithelia in that it is derived during development from the embryonic mesoderm, rather than the ectoderm or endoderm. The kidney cortex epithelial cells, which form the nephrons, or renal tubule filtration system, arise from a different region of the mesoderm. Two distinctive epithelial cell types proliferate in culture from explanted human kidney cortex (Rheinwald et al., 1984; Rheinwald and O'Connell,

1. *Abbreviations used in this paper:* CM, conditioned medium; EGF, epidermal growth factor; HC, hydrocortisone; M199, medium 199; THS, Triton/high salt buffer; TTBS, Tween/Tris-buffered saline; 2D NEPHGE, two-dimensional nonequilibrium pH gradient electrophoresis.

1985). Type II kidney epithelial cells closely resemble, but are not identical to, mesothelial cells with respect to characteristics of growth regulation and intermediate filament protein expression in culture.

During our studies of the intermediate filament proteins of these cell types (Wu et al., 1982; our unpublished observations) we noticed, in addition to vimentin and the four simple epithelial keratins, a prominent, metabolically labeled 46-kD protein in the Triton/high salt (THS)-insoluble fraction. We initially thought that this 46-kD protein was another keratin, but found in later pulse-chase experiments that it disappears from the cell extract soon after synthesis (Connell and Rheinwald, 1983). We report here that this protein is actually a glycoprotein which is initially secreted onto the substratum and subsequently accumulates in the medium. Next to β -actin, it is the most prominent protein synthesized by human mesothelial cells and several other mesoderm-derived cells in culture, and we therefore have named it "mesosecrin."

Materials and Methods

Normal Human Cell Strains and Culture Conditions

Peritoneal mesothelial cell strains LP-9 and LP-3 (Wu et al., 1982) and pleural mesothelial cell strain HPM-1 (cultured from pleural effusion fluid withdrawn from a 63-yr-old man) were cultured in a 1:1 (vol/vol) mixture of medium 199 (M199) and medium MCDB202 plus 15% FCS, 0.4 μ g/ml HC, and 10 ng/ml EGF, as described previously (Connell and Rheinwald, 1983; LaRocca and Rheinwald, 1985). In this paper, M199/MCDB202 plus 15% FCS and HC will be referred to as "standard medium." (LP-9 and LP-3 are now available as strain numbers AG7086 and AG7090, respectively, from the Aging Cell Repository of the Coriell Institute for Medical Research, Camden, NJ.)

Fetal kidney cortex type I epithelial cell strain HKi-12 clone D and kidney cortex type II epithelial strains HKi-12 clone 2.1 (fetal) and HKi-14 clone 8 (adult) were cultured in standard medium plus EGF, as described previously (Rheinwald and O'Connell, 1985). Dermal fibroblast strains A1-F (newborn), R2-F (adult), and P1-F (adult) (Didinsky and Rheinwald, 1981; O'Connell and Rheinwald, manuscript in preparation) were cultured either in a 1:1 (vol/vol) mixture of DME (Flow Laboratories, Inc., McLean, VA) and F12 (Gibco, Grand Island, NY) media plus 15% FCS, HC, and EGF or in standard medium plus EGF.

Epidermal keratinocyte strain N (newborn) (Rheinwald and Beckett, 1980) and A4-Ep (adult) (O'Connell and Rheinwald, manuscript in preparation), adult urinary bladder urothelial strains HB1-4 and HB1-8 (Wu et al., 1982; Rheinwald et al., 1984), fetal conjunctival epithelial cell strain AX (Wu et al., 1982), and adult mammary epithelial cell strains H116 (754AB) and H30 (464AA) (both initiated from collagenase-digested mammary gland cell clusters provided by Martha Stampfer, University of California, Berkeley) all were cultured in the presence of mitomycin-treated 3T3 mouse fibroblast feeder cells (Rheinwald and Green, 1975; Rheinwald, 1980) in DME/F12 medium plus 5% FCS, 0.4 μ g/ml HC, 10 ng/ml EGF, 10^{-9} M cholera toxin (Schwarz-Mann, Orangeburg, NY), 2×10^{-8} M adenine, and 5 μ g/ml insulin (Sigma Chemical Co., St. Louis, MO) as described previously (Allen-Hoffman and Rheinwald, 1984; Wu et al., 1982). In some experiments, mammary epithelial cells were cultured without 3T3 feeder cells in standard medium plus EGF.

Two umbilical vein endothelial cell populations in early passage (kindly provided by Dr. Jordan Pober of Brigham and Women's Hospital, Boston, MA) were cultured in M199 plus 20% FCS, 5 μ g/ml Endothelial Cell Growth Factor (Meloy Laboratories, Inc., Springfield, VA) and 10 μ g/ml heparin (Sigma Chemical Co.) (Thornton et al., 1983).

Metabolic Labeling and Electrophoretic Separation of Proteins

Cultures were labeled for 1 h (unless otherwise indicated) with 50 μ Ci/ml [35 S]methionine (specific activity 800 Ci/mmol; New England Nuclear, Boston, MA) in otherwise methionine-free MEM medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% dialyzed FCS and the same growth factors and hormones with which the cells had been cultured. Some

labeled cultures were lysed immediately. Others were "chased" by replacing the labeling medium with unlabeled growth medium containing 1% FCS and the appropriate growth factors, and returning the cultures to the incubator for various periods of time before collecting the medium and lysing the cells. The conditioned medium (CM) from these chased cultures was examined electrophoretically for secreted proteins.

Cultures were dissolved in O'Farrell lysis buffer, containing 8M urea (O'Farrell, 1975), or in Laemmli lysis buffer, containing SDS (Laemmli, 1970), or they were lysed in THS, containing 20 mM Tris-HCl (pH 7.3), 1% Triton X-100, 0.6 M KCl, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (a modification of the THS buffers described previously) (Franke et al., 1979; Wu et al., 1982). THS-lysed cells were sonicated at 4°C and the insoluble "cytoskeletal" fraction was separated from the soluble material by centrifugation at 5,000 g for 5 min. THS-soluble and -insoluble fractions were dissolved in Laemmli lysis buffer and the proteins were separated by one-dimensional SDS PAGE (1D SDS PAGE) in 10% acrylamide gels (Laemmli, 1970), or were dissolved in O'Farrell lysis buffer and separated by two-dimensional, nonequilibrium pH gradient electrophoresis (2D NEPHGE) (O'Farrell et al., 1977). Gels were stained with Coomassie Brilliant Blue R (Sigma Chemical Co.) in order to detect total protein. To detect metabolically labeled proteins, gels were soaked in the fluor EnHance (New England Nuclear), dried, and autoradiographed by placing against Kodak SB-5 X-ray film (Eastman Kodak Co., Rochester, NY).

Glycoprotein Analysis

Mannose-containing proteins were detected by labeling cells for 4 h with 0.5 mCi/ml [3 H]mannose (specific activity 50 Ci/mmol; New England Nuclear) in medium containing a reduced glucose concentration (50 μ g/ml). To block the addition of oligosaccharide to asparagine residues in potential glycoproteins, cells were preincubated with 0.25 μ g/ml tunicamycin (Sigma Chemical Co.) for 24 h and then were labeled for 1 h with [35 S]methionine in the presence of tunicamycin.

Asparagine-linked oligosaccharides (N-glycans) were cleaved from Sp46 with endoglycosidase F (Elder and Alexander, 1982). Electrophoretically purified Sp46 was incubated with 1 U of endoglycosidase F (New England Nuclear) in a buffer consisting of 0.1 M sodium phosphate (pH 6.1), 0.05 M EDTA, 0.5% NP40, 0.1% SDS, and 1% 2-mercaptoethanol for 2 h at 37°C. Terminal sialic acid residues were removed from N-glycans by incubation with 50 mU of *Vibrio cholerae* neuraminidase (Calbiochem-Behring Corp., San Diego, CA) in 50 mM sodium acetate buffer (pH 5.6) containing 1 mM CaCl₂ for 24 h at 37°C.

Purification of Sp46/Mesosecrin from CM

Cultures of LP-9 were grown to confluence in 100-mm diam dishes in standard medium without EGF. They then were rinsed twice with serum-free medium and incubated for 3 d with 8 ml per dish of M199/MCDB202 medium, 0.1% FCS, and 0.4 μ g/ml HC. The CM was then harvested and the cultures were refed for 1 d with standard medium without EGF. Then the medium was replaced with low serum medium for another 3-d incubation. Immediately after collection, Hepes was added to a concentration of 20 mM, the CM was passed through a 0.2- μ m sterilization filter (Nalge Co., Rochester, NY), and then was stored at 4°C for no more than 10 d. CM was concentrated 50-fold by pressure ultrafiltration over a PM10 filter (Amicon Corp., Danvers, MA) and dialyzed by diluting with 20 vol of 20 mM Tris-HCl (pH 7.2) buffer and reconcentrating. Concentrated CM was stored at -20°C.

Aliquots of concentrated CM were separated by 1D SDS-PAGE, the gels were stained with Coomassie Blue, the 46-kD band was excised with a razor blade, and the Sp46 was electroeluted in an Isco model 1750 sample concentrator (Isco, Inc., Lincoln, NE). Purity of the Sp46 was determined by separating an aliquot of electroeluted material on another gel, and its concentration was estimated by comparing its Coomassie Blue staining intensity on gels with that of BSA standards of known concentration.

In later preparations, concentrated CM was mixed with concanavalin A (Con A)-Sepharose beads (Pharmacia, Inc., Piscataway, NJ), which had been equilibrated in 20 mM Tris-HCl buffer (pH 7.3), 0.5 M NaCl, 1 mM CaCl₂, and 1 mM MnCl₂. The beads were then rinsed twice in this buffer, and bound proteins were eluted by the addition of α -methylmannoside to 0.5 M. The eluted proteins were concentrated by ultrafiltration and Sp46 purified from this material by preparative 1D SDS PAGE as described by Hunkapillar et al. (1983) to minimize cleavage and chemical modification. Electrophoresis-grade gel and buffer reagents from BioRad Laboratories (Richmond, CA) and Sigma Chemical Co. were used, and electrophoresis was performed in the presence of 0.1 M sodium thioglycolate. Sp46 was lo-

cated on gels by brief staining with highly purified Coomassie Blue (Serva Fine Biochemicals, Inc., Garden City Park, NY) in acetic acid/isopropanol/water (1:3:6, vol/vol/vol), and the bands were excised. The Sp46 was electroeluted and then electrodialed against 0.01 M NH_4HCO_3 and 0.02% SDS. This material was used for amino acid analysis and NH_2 -terminal sequencing.

Preparation of Antisera

Two rabbits were immunized by three subcutaneous injections of 100 μg of gel-purified Sp46. Because the antisera cross-reacted with some 60–70 kD FCS proteins in immunoblot experiments, the antiserum from one rabbit (J1) was passed through a column of FCS protein covalently linked to cyanogen-Sepharose beads (Pharmacia Fine Chemicals). The unbound fraction exhibited greatly reduced cross-reaction with serum proteins in immunoblots but undiminished reactivity to Sp46. Two mice were immunized by four injections of 30–50 μg of Sp46.

Immunochemical Methods

Immunoprecipitation. [^{35}S]methionine-labeled THS-soluble cell protein, CM, and purified Sp46 were diluted to a final volume of 0.4 ml in 20 mM Tris-HCl (pH 7.35) buffer containing 0.14 M NaCl, 2 mM EDTA, and 0.5% NP-40. 5 μl of undiluted antiserum was then added and the solution was incubated for 30 min at room temperature. Then 50 μl of hydrated protein A-Sepharose beads (Pharmacia Fine Chemicals) was added and the mixture was incubated for 30 min with shaking. The beads and bound material were pelleted in a microcentrifuge and rinsed twice with neutral, isotonic PBS. The beads were then treated with 100 μl of either O'Farrell lysis buffer or twice-concentrated Laemmli sample buffer, the eluted material was separated electrophoretically, and the immunoprecipitated labeled proteins were detected by autoradiography.

Immunoblotting. Proteins were blotted onto nitrocellulose paper from 1D slab gels by electrotransfer using an E-C Apparatus Corp. (St. Petersburg, FL) electroblot set at 300 mA for 2 h at 4°C. Blots were stained with 0.1% Fast Green in methanol/acetic acid, destained in water, and photographed. Blots were preblocked by soaking them in a buffered solution of nonfat dry milk (Blotto) (Johnson et al., 1984) for at least 1 h, and were sometimes stored in Blotto overnight before use. Blots were incubated for 1 h at room temperature with antiserum diluted 1:1,000 in Tween/Tris-buffered saline (TTBS), which consisted of 0.05% Tween-20 in 50 mM Tris and 150 mM NaCl (pH 8.0). After rinsing for 15 min in TTBS, the blots were incubated for 1 h with biotinylated secondary antibody (Vector Laboratories, Inc., Burlingame, CA) diluted 1:200 in TTBS, rinsed in TTBS for 15 min, and then incubated in the avidin-biotin peroxidase complex (Vector Laboratories) for 30 min. The blots were then rinsed in 50 mM Tris-HCl (pH 7.6) for 15 min and immersed in a freshly prepared solution of 0.01% hydrogen peroxide plus 50 $\mu\text{g}/\text{ml}$ diaminobenzidine (Sigma Chemical Co.) in 50 mM Tris-HCl (pH 7.6) for 5–15 min. When the bands containing antigen were suitably dark, the reaction was stopped by rinsing in distilled water and air-drying.

Indirect Immunofluorescence

Cultures grown on plastic dishes were fixed for 10 min to 1 mo in -20°C methanol, or were fixed for 10 min in 3.7% formaldehyde/PBS followed by a 2-min treatment with 0.5% Triton X-100 in PBS. Cultures were then rinsed in PBS, briefly air-dried, and incubated with antiserum (diluted 1:30 to 1:50 in PBS) for 30 min at room temperature. The cultures then were rinsed for 15 min in PBS, incubated with fluorescent secondary antibody for 30 min, rinsed for 30 min in PBS, and mounted under coverslips with glycerin jelly (Sigma Chemical Co.) containing 1 mg/ml *p*-phenylenediamine to reduce photobleaching (Johnson and Noguiera-Araujo, 1981). Cells were examined for immunofluorescence with a Zeiss epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with rhodamine and fluorescein barrier filters and a $\times 25$ immersion objective.

Microscopy and Photography

Cells processed for immunofluorescence were photographed on Kodak Tech Pan 2415 film exposed at ASA 160 for phase-contrast and fluorescein immunofluorescence, and at ASA 80 for rhodamine immunofluorescence. Stained gels, blots, and autoradiograms were photographed on Tech Pan film exposed at ASA 80. Coomassie Blue-stained gels were photographed through an orange filter, Fast Green-stained blots through a red filter, and peroxi-

dase/diaminobenzidine-reacted blots through a blue filter. Film was developed in Kodak D19 developer.

Results

Mesothelial Cells Synthesize a 46-kD Protein at a High Rate and Secrete It into the Medium

Cultures of the normal human mesothelial cell strain LP-9 were labeled for 1 h with [^{35}S]methionine and then were refed with unlabeled medium. The cells, the substratum-associated material, and the culture medium (CM) were examined at various times thereafter by SDS PAGE (Fig. 1), which disclosed a secreted protein of apparent molecular mass 46 kD (size estimated by its coelectrophoresis with the 46-kD keratin no. 17 of cultured human epidermal keratinocytes [Sun and Green, 1978; Fuchs and Green, 1978; see also Moll et al., 1982]) (data not shown). Scans of the autoradiograms indicated that this protein represented $\sim 3\%$ of total protein synthesis and was the major protein secreted by mesothelial cells. (In the Results section we will hereafter refer to this 46-kD secreted protein as Sp46.) Even after only 1 h of labeling, 35% of the Sp46 synthesized during that period had already been secreted by the cells onto the culture vessel surface (Fig. 1). During the first hour after the label was removed from the medium, slightly higher levels of the previously synthesized Sp46 accumulated on the dish. Then over the subsequent 6 h almost all of the dish-associated, labeled Sp46 material was released into the medium. Its half-life of association with the substratum was ~ 2 h. The Sp46 that accumulated in the medium was not significantly degraded for at least several days. Similar kinetics of secretion and movement of Sp46 from the substratum into the medium were measured for cells at preconfluence or post-confluence, in the presence or absence of EGF, or when cultured on standard tissue culture dishes (negatively charged surface) vs. positively charged dishes (Primaria, Falcon Plastics, Oxnard, CA).

Glycosylation, Processing, and Secretion of Sp46

Sp46 is a glycoprotein, as evidenced by its metabolic labeling with [^3H]mannose and by its binding to the mannose-specific lectin Con A (Fig. 2). Sp46 could be concentrated from CM and purified from the major serum contaminant, BSA, by elution from Con A-Sepharose beads with α -methylmannoside.

2D NEPHGE separation of labeled cell proteins revealed size and charge heterogeneity of Sp46 within the cell prior to secretion (Fig. 3 A). Extracts of cells that were labeled for 1 h contained two major forms, one of pI 7.4 and one which migrated as a slightly larger (~ 1 kD) protein of pI 7.1. (Isoelectric points were estimated by migration in NEPHGE of Sp46 relative to the 56-kD keratin no. 6 and the 58-kD keratin no. 5 during coelectrophoresis with the THS-insoluble fraction of cultured human bladder urothelial cells (Wu et al., 1982; Rheinwald et al., 1984; see also Moll et al., 1982)(data not shown). Both labeled species disappeared from the cells during an overnight chase and a labeled protein of size and charge identical to that of the larger, more acidic cellular form appeared in the medium. These experiments also revealed that mesothelial cells synthesize Sp46 at the same rate when growing rapidly in the presence of EGF

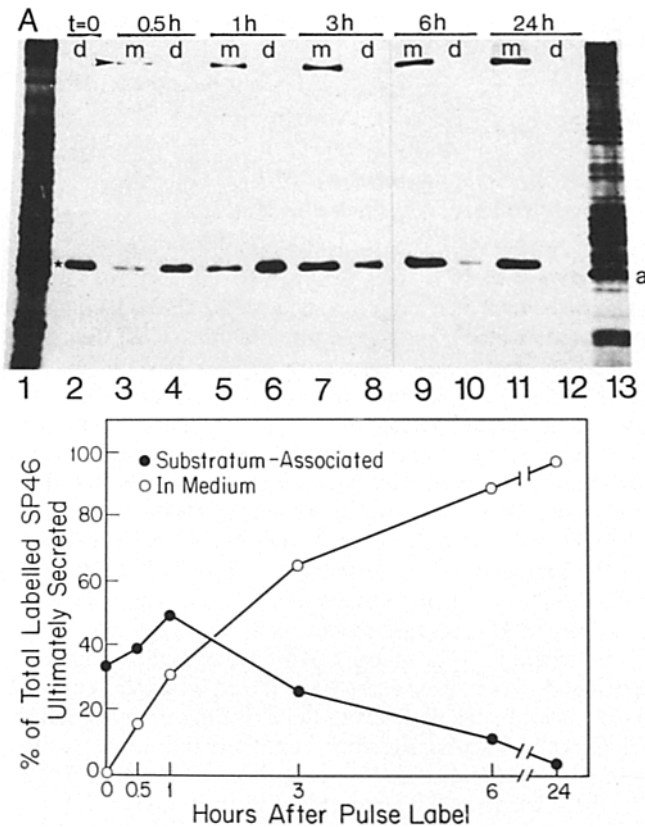


Figure 1. Kinetics of Sp46 secretion onto the substratum and accumulation in the medium. (A) A set of LP-9 cultures that had just reached confluence in the presence of EGF was labeled with [³⁵S]Met for 1 h. One culture was immediately rinsed, the cells were detached from the dish with EDTA, and the cells (lane 1) and dish surface (lane 2) were extracted with Laemmli SDS lysis buffer. The remaining cultures were refed with unlabeled medium and returned to the incubator. At various times thereafter (0.5 h, lanes 3 and 4; 1 h, lanes 5 and 6; 3 h, lanes 7 and 8; 6 h, lanes 9 and 10; and 24 h, lanes 11, 12, and 13), the medium was harvested from one of these cultures, the cells were removed with EDTA, and the dish surface was extracted with SDS buffer. 1/40th of the total volume of cell extract (lanes 1 and 13), of the CM (lanes 3, 5, 7, 9, and 11), and of the dish extract (lanes 2, 4, 6, 8, 10, and 12) were separated by SDS PAGE and autoradiographed. Asterisk indicates Sp46; a indicates actin; arrowhead indicates fibronectin. (B) Graphic depiction of the experiment shown in A, quantitated by scanning gels of appropriate dilutions of each sample.

as they do when quiescent at confluence in the absence of EGF (compare panels *a* and *c* of Fig. 3 A). In contrast, fibronectin was synthesized at much higher levels by rapidly growing than by quiescent cells.

To study the intracellular processing of Sp46 more closely, cells were pulse-labeled for various lengths of time and either extracted immediately or after a brief chase in unlabeled medium. 2D NEPHGE separation of proteins (Fig. 3 B) revealed that Sp46 is translated as the smaller, more basic form and then during the next 1–2 h is converted within the cell to the larger, more acidic form that is secreted into the medium. A minor intracellular form, intermediate in size and charge between the initially synthesized and the ultimately secreted forms, was detectable in some well-resolved electrophoretic separations of 1-h pulse-labeled cell proteins (Fig. 3 B, panel *b*). All three forms were immunoprecipitated (Fig. 3 B, panel *d*) by an anti-Sp46 antiserum (described below), thus confirming their identity as processing intermediates of Sp46.

A very minor form (indicated by the small arrowhead in Fig. 3 A, panel *e*, and Fig. 3 B, panel *b*), slightly larger and more acidic than the predominant secreted form, was also detectable (Fig. 3 A, panels *e* and *f*) in heavily labeled cell extracts and in some CM samples. This form was preferentially retained by the substratum, as revealed by electrophoretic and immunochemical analysis of labeled material remaining dish-associated after a 24-h chase (data not shown). This form was produced as a higher proportion of the total Sp46 synthesized by type II kidney epithelial cell extracts and CM, representing ~25% of the total Sp46 secreted by this cell type (see below).

Tunicamycin inhibits the coupling of *N*-acetylglucosamine (GlcNAc) to dolichol (see Duksin and Mahoney, 1982), which is the first step in the pathway leading to addition of high-mannose (Man) oligosaccharide units to certain asparagine residues in glycoproteins. In the presence of tunicamycin (Fig. 3 B, panel *e*), cells synthesized and secreted a 40-kD form of Sp46 (size estimated by coelectrophoresis in SDS PAGE with the 40-kD keratin no. 19 of cultured mesothelial cells (Wu and Rheinwald, 1981; Wu et al., 1982; Moll et al., 1982) (data not shown). Treatment with tunicamycin did not result in a significant reduction of either synthesis or secretion of unglycosylated Sp46 compared with that of the normal form by control cells. The apparent 6-kD difference in mobility in SDS PAGE indicates that Sp46 probably contains two *N*-glycan units and certainly no more than three, taking into consideration a molecular mass of ~2 kD per complex *N*-glycan unit (Kornfeld and Kornfeld, 1985) and the very slightly slower predicted mobility of a glycoprotein of this size and carbohydrate content in 10% SDS gels as compared with that of an unglycosylated protein having the same total mass (Segrest and Jackson, 1972).

The 40 kD form secreted by tunicamycin-treated cells had the same charge as the normal translation product of Sp46 (Fig. 3 B, panel *e*). As for most *N*-linked glycoproteins (Kornfeld and Kornfeld, 1985), the *N*-glycan units appear to be added to Sp46 as it is being translated, because no 40-kD form is normally detectable in cell extracts, even after a very brief pulse label (Fig. 3 B, panel *a*). Posttranslational processing to a larger and more acidic form is therefore dependent upon the presence of the added *N*-glycan. Treatment of [³⁵S]Met-labeled CM with neuraminidase resulted in the

conversion of secreted Sp46 to the size and charge of the newly translated, intracellular form (data not shown). Thus the change in charge and SDS PAGE mobility that occurs posttranslationally under normal conditions is a consequence of the addition of terminal sialic acid residues. This is typically the final step of a trimming and addition process in the Golgi apparatus, which converts the initial (GlcNAc)₂ (Man)₉ (Glc)₃ "high mannose" glycan to the ultimate (GlcNAc)₂ (Man)₃ (GlcNAc)₂ (Gal)₂ (Sial)_{1,2} "complex" glycan on many glycoproteins (reviewed in Kornfeld and Kornfeld, 1985; Hughes, 1983).

Preparation and Immunochemical Characterization of Antisera against Sp46

Mesothelial cells that had grown to confluence in standard medium without EGF were refed with medium containing 0.1% FCS. They appeared healthy during a 3-d incubation in this low serum medium, and electrophoretic analysis of the CM disclosed that the $\sim 2 \times 10^6$ cells on each 100-mm diam culture dish usually secreted about 10 μ g of soluble Sp46 over the course of 3 d.

The only other prominent protein secreted by mesothelial cells into the CM was fibronectin, and it was synthesized at a relatively low rate when the cells were confluent in the absence of EGF. Most of the total protein in the CM was from the FCS (see Figs. 2 and 4 B). No 44–48-kD proteins were detectable by Coomassie Blue or Fast Green staining in serum-containing medium that had been incubated for 3 d without cells, however (compare lanes 3 and 4 of Fig. 4 B), indicating that Sp46 could be purified from CM by preparative ID SDS PAGE (see Materials and Methods).

A rabbit antiserum (J1) raised against the gel-purified protein immunoprecipitated Sp46 from mesothelial cell extracts and from CM (Fig. 4 A). A mouse anti-Sp46 antiserum (M3) showed the same specificity for Sp46 in immunoprecipitation when a rabbit anti-mouse IgG antibody was used as a bridge between the mouse anti-Sp46 and the protein A (data not shown). The rabbit antiserum also recognized the unglycosylated form of Sp46, which is synthesized and secreted by cells in the presence of tunicamycin (Fig. 4 A, lane 8). Thus the antiserum recognized the polypeptide of Sp46, and not only its oligosaccharide. Immunoblotting with the rabbit (Fig. 4 B) and mouse (data not shown) antisera disclosed no Sp46 in FCS and no cross-reaction with proteins of size other than 46 kD in cell extracts. Reaction of the J1 antiserum with endoglycosidase F-treated Sp46 also indicated specificity for the polypeptide portion of the glycoprotein.

Immunofluorescence Analysis of Substratum-associated Sp46. Immunofluorescence staining revealed that the substratum-associated Sp46 was localized in the vicinity of cells as a finely divided or speckled coating (Fig. 5). As expected from the electrophoretic analysis described above, release of cells with EDTA (Fig. 5 f) or lysis and solubilization of the cultures with THS buffer (not shown) left the dish-associated material undisturbed. Our time-lapse video microscopic studies had disclosed that mesothelial cells are mobile, and that migrating cells usually extend a very broad ruffled membrane at their leading edge. Although the quantity of Sp46 around individual cells varied (as, for example, the two cells shown in Fig. 5 c), in sparse cultures Sp46 frequently was concentrated in a trail behind the cells (Fig. 5, a and b). Con-

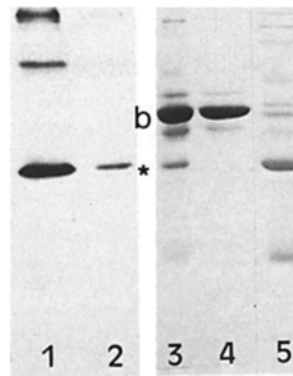


Figure 2. Characterization of Sp46 as a glycoprotein containing mannose. [³H]mannose labeling: two LP-9 cultures (confluent, + EGF) were labeled with either [³H]mannose or [³⁵S]methionine. The labeled cultures were refed with unlabeled medium and 24 h later the medium was harvested and analyzed by SDS PAGE and autoradiography. Lane 1, [³H]mannose-labeled CM; lane 2, [³⁵S]met-labeled CM. Con A-Sepharose bind-

ing of Sp46: concentrated mesothelial cell CM (a sample of which is shown in lane 3) was mixed with Con A-Sepharose beads. Proteins remaining unbound and in solution are shown in lane 4. Proteins bound to the Con A-Sepharose beads and eluted by 0.5 M α -methylmannoside are shown in lane 5. Samples were separated by SDS PAGE and stained with Coomassie Blue. Asterisk indicates Sp46; b indicates BSA.

sidering the rate of Sp46 dissociation from the substratum (Fig. 1), such trails probably represent the migratory paths of cells during the 3–6 h preceding fixation.

The cytoplasm of the cells also was stained, but the very flat periphery of mobile cells and the underlying substratum in these areas were unstained (Fig. 5, d and e). Mesothelial cells spread out and become more flattened as they approach confluency in the absence of EGF (Connell and Rheinwald, 1983). In such cultures, regions of the substratum unoccupied by cells at the time of fixation stained rather uniformly for Sp46, whereas the areas occupied by cells stained more heterogeneously (Fig. 5, e and g). The peripheral margins of some of the cells were unstained as was also a dartlike pattern at the cell-substratum interface of large, less motile cells.

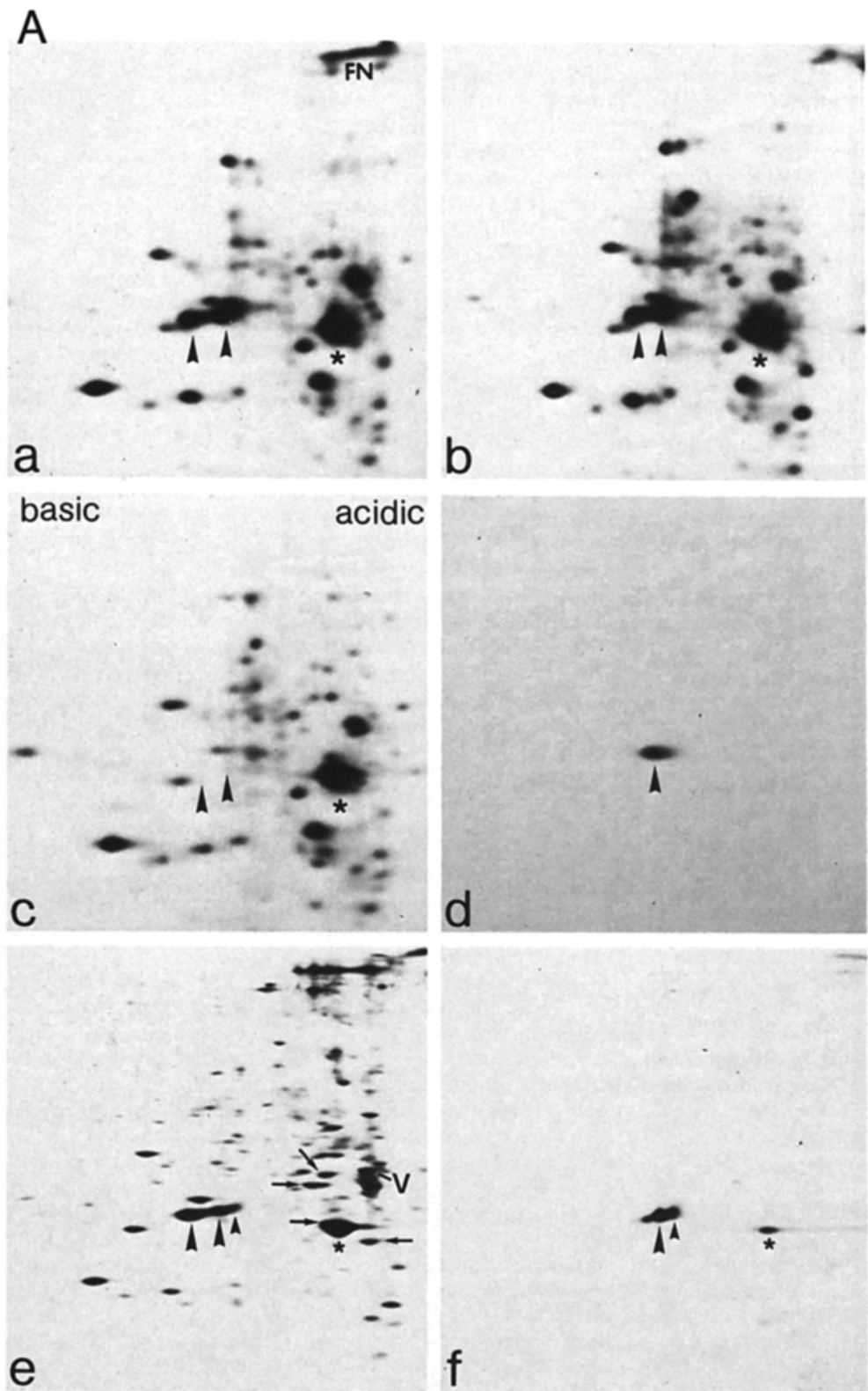
Sp46 Deposition Is Distinct from That of Fibronectin.

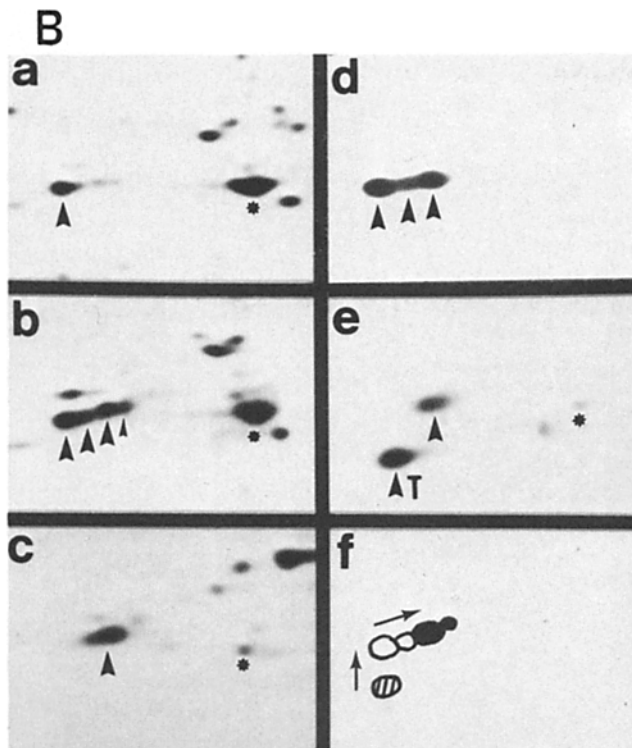
We compared the distribution of fibronectin with that of Sp46 in mesothelial cell cultures by double-label indirect immunofluorescence (Fig. 5, g and h). Little fibronectin staining was observed in sparse cultures, but as cells approached confluency in the presence of EGF they deposited fibronectin in great abundance as fibers and fibrous blobs. Most of the fibronectin was cell, rather than substratum, associated. "Optical sectioning" by examining cultures at different focal planes revealed that the fibronectin fibers were primarily on the dorsal surface of the cells, rather than beneath the cells as was Sp46. The smooth, even deposition of Sp46 also contrasted with the particulate and fibrous deposits of fibronectin. When cultures were fixed in 3.7% formaldehyde and permeabilized with 0.5% Triton X-100, the patterns of Sp46 and fibronectin deposition were the same as observed in methanol-fixed cultures (data not shown). Thus Sp46 does not appear to have a special affinity for fibronectin.

Cell Type Specificity of Sp46 Synthesis and Secretion.

We compared Sp46 synthesis by a variety of normal human cell types in culture with that of mesothelial cells in three ways: (a) [³⁵S]methionine incorporation into Sp46 relative to total cell protein synthesis as analyzed in 2D gels of pulse-labeled cell extracts; (b) the amount of labeled Sp46 present in the culture medium relative to that in total cell protein 18 h

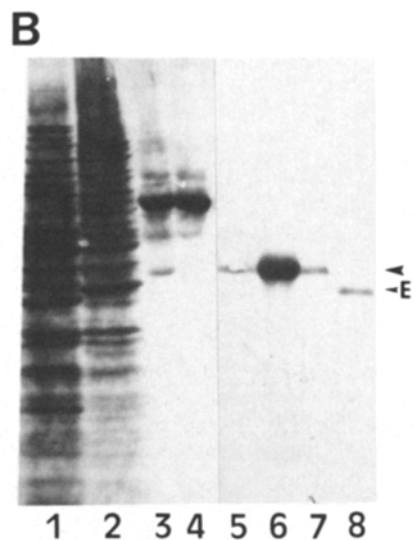
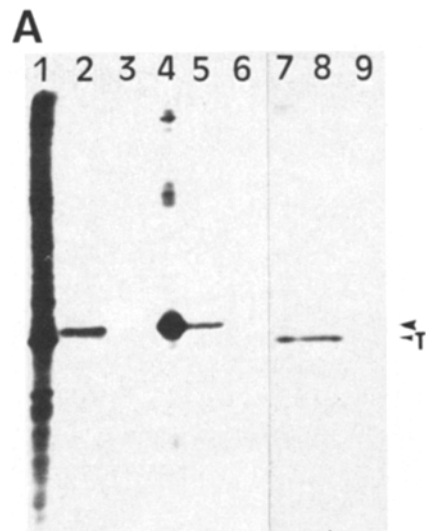
Figure 3. Synthesis, processing, and secretion of Sp46 by growing and quiescent cells. LP-9 cultures, either subconfluent and rapidly growing in the presence of EGF, or confluent and quiescent in the absence of EGF, were labeled with [³⁵S]methionine. They then were either lysed in THS immediately (pulse cultures) or at various times after refeeding with unlabeled medium (chase cultures). The medium from chase cultures was also harvested. Proteins were separated on 2D NEPHGE gels and autoradiographed. (NEPHGE was run from right to left toward the negative electrode. Basic proteins are on the left and more acidic proteins are on the right.) (A) Panels *a-d* show THS-soluble proteins from 1-h pulse cultures (*a*) with EGF, pre-confluent; *b*) without EGF, post-confluent) and from a 1-h pulse/16-h chase culture (*c*) with EGF, pre-confluent), and CM from a 1-h pulse/16-h chase culture (*d*) without EGF, postconfluent). Panels *e* and *f* are gels of a different experiment showing total protein from a 1-h pulse-labeled confluent culture (*e*), and CM from a similar pulse/chased culture (*f*). The gel in panel *f* is overloaded to show the minor, more acidic form of Sp46 and the position of the small amount of actin present in CM. (B [right page]). The *M_r* 35–60-kD/pI 5.3–7.8 regions of 2D NEPHGE separations. THS-soluble fraction from (*a*) a 20-min pulse culture and (*b*) a 1-h pulse culture; (*c*) THS-insoluble fraction from a 20-min pulse/2-h chase culture; (*d*) immunoprecipitate of 1-h pulse-labeled extract using rabbit anti-Sp46 antiserum; (*e*) medium as in *d* except that the culture had been treated with 2 μg/ml tunicamycin for 2 h before and during the 1 h of labeling; (*f*) idealized diagram of Sp46 synthesis, glycosylation, and processing before secretion. (*Hatched oval*) The unglycosylated form of Sp46, which only occurs in the presence of tunicamycin. (*Solid oval*) The normally secreted form of Sp46. After translation and the initial *N*-glycosylation, additional processing, including terminal sialation, occurs in mesothelial cells during the next 1–2 h to make Sp46 apparently 1 kD larger (as estimated by SDS PAGE mobility) and ~0.3 pH units more acidic before it is secreted. *Arrowheads* indicate the several intracellular forms of Sp46; *T* indicates the form of Sp46 synthesized and secreted in the presence of tunicamycin; *V* indicates vimentin (*M_r* 57 kD/pI 5.2); *arrows* indicate the four keratins (nos. 7, 8, 18, and 19) of *M_r* 55, 52, 44, and 40 kD synthesized by mesothelial cells; *asterisks* are below β-actin (*M_r* 43 kD/pI 5.4); *FN* is below fibronectin.





after a 1-h pulse label; and (c) the amount of substratum-associated Sp46 near cells in sparse cultures as detected by immunofluorescence microscopy. These three methods yielded the same results for comparative Sp46 production among the cell types examined, but the level of labeled Sp46 appearing in CM was the easiest method to quantitate. A sample experiment is shown in Fig. 6 A, and the results of all analyses are summarized in Table I. Umbilical vein endothelial cells expressed Sp46 at levels similar to that of mesothelial cells. Type II renal cortex epithelial cells (Rheinwald and O'Connell, 1985) expressed about half as much, and Type I renal cortex epithelial cells about one-fifth as much as mesothelial

Figure 4. Immunoprecipitation and immunoblot analysis of Sp46. (A) Immunoprecipitation of Sp46 from [³⁵S]methionine-labeled LP-9 cells and CM. Lane 1: the THS-soluble cell fraction, and the proteins precipitated by rabbit anti-Sp46 antiserum (lane 2) or by preimmune serum (lane 3). Lane 4: labeled (pulse-chased) CM, and the proteins precipitated from it by rabbit anti-Sp46 antiserum (lane 5) or by preimmune serum (lane 6). Lane 7: labeled (pulse-chased) CM from tunicamycin-treated cells, and the proteins precipitated from it by rabbit anti-Sp46 antiserum (lane 8) or by preimmune serum (lane 9). Arrowhead indicates Sp46; T indicates the smaller (40 kD) form of Sp46 synthesized and secreted in the presence of tunicamycin. (B) Immunoblot detection of Sp46. Upper panel: Fast Green-stained proteins electrotransferred to nitrocellulose paper from a gel of 1D SDS PAGE. Lower panel: the same blot after incubating with rabbit anti-Sp46 serum and developing with avidin-biotin complex peroxidase and diaminobenzidine (a blue filter was used during photography of the lower panel to block out the Fast Green color). (Note: an identical blot treated with pre-immune rabbit serum antibody was absolutely unstained by immunoperoxidase). Lane 1, SDS-soluble proteins from bladder urothelial cell strain HB1-4; lane 2, SDS-soluble proteins from



mesothelial cell strain LP-9; lane 3, concentrated LP-9 CM; lane 4, concentrated, serum-containing medium that had been incubated without cells for 3 d; lane 5, SDS-soluble proteins remaining on the dish after the mesothelial cells from a confluent culture had been detached by incubation with EDTA; lane 6, purified Sp46; lane 7, purified Sp46 incubated as in lane 8 but without endoglycosidase F; lane 8, purified Sp46 after incubation with endoglycosidase F. Arrowheads indicate Sp46; E indicates the 40-kD form of Sp46 resulting from treatment with endoglycosidase F.

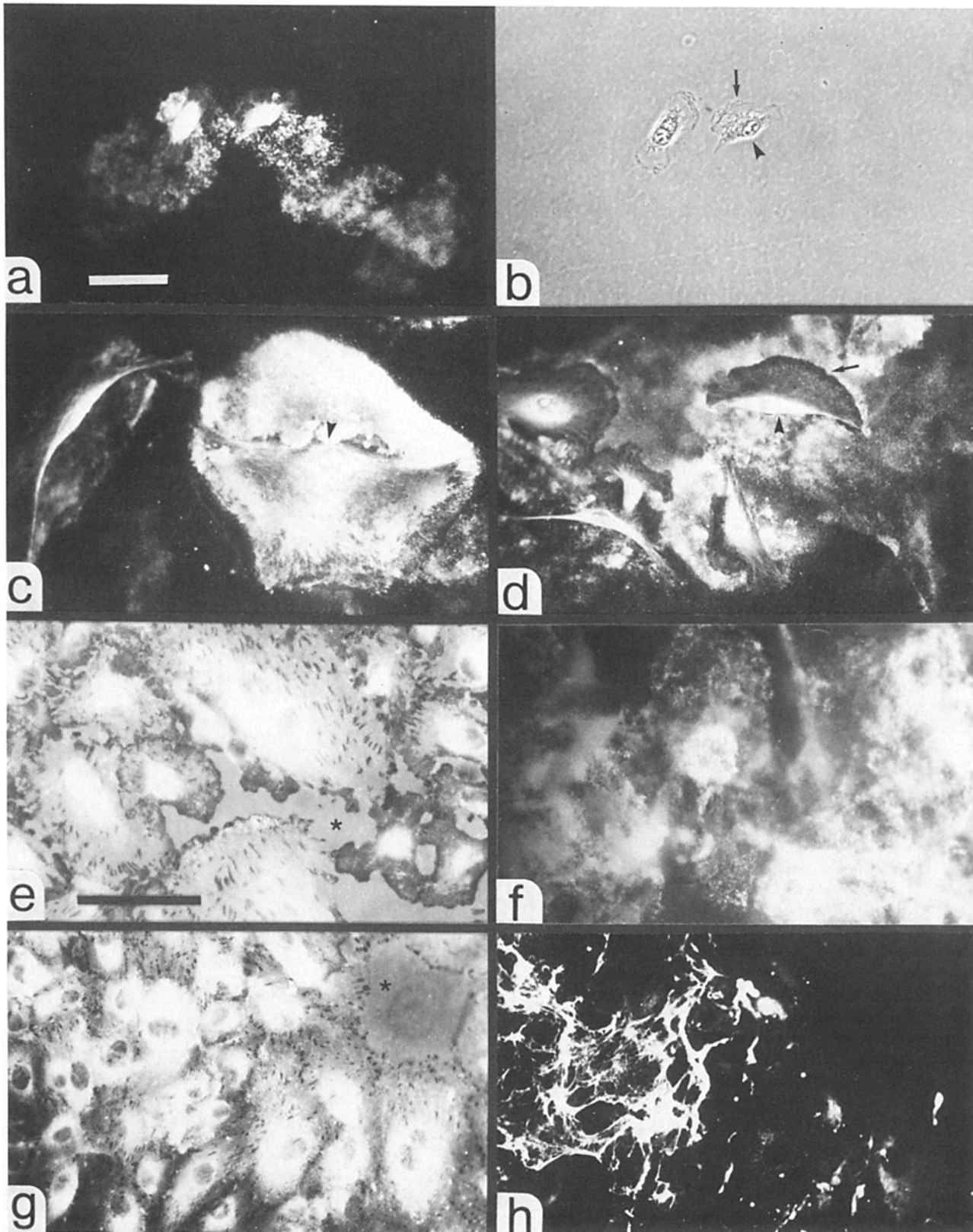


Figure 5. Immunofluorescence localization of Sp46 in mesothelial cultures. Panels *a*, *c*, *d*, *e*, and *f* show staining with the J1 rabbit antiserum. Panel *b* is phase-contrast illumination of the field shown in panel *a*. Panels *a-d* are preconfluent cultures, with EGF. Panels *e* and *f* are confluent cultures, without EGF; the culture in panel *f* was treated with EDTA to release the cells before fixation. Panels *g* and *h* are the same field of a confluent culture, with EGF, stained with M3 mouse anti-Sp46 antiserum and rhodamine secondary antibody (*g*), and rabbit anti-human fibronectin antiserum (Cappel Laboratories, Inc., Malvern, PA) and fluorescein secondary antibody (*h*). Arrows indicate the leading edge and arrowheads the trailing edge of motile cells in panels *a-d*. Asterisks in panels *e* and *g* indicate an area of the dish unoccupied by cells at the time of fixation. Bar in panel *a* indicates 50 μ m, showing magnification for all panels except panel *e*, which contains its own 50- μ m standard.

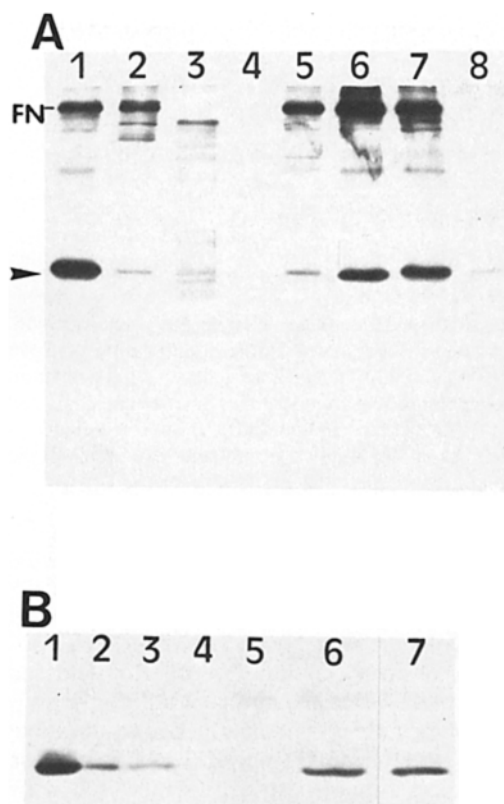


Figure 6. Cell type specificity of Sp46 synthesis. (A) Near-confluent, exponentially growing cells were labeled for 1 h with [35 S]methionine and refed with unlabeled medium. 18 h later, the CM was harvested and the cells were dissolved in Laemmli lysis buffer. Samples of labeled CM from each cell type (volumes loaded were adjusted to normalize for differences among the cell types in incorporation of label into total cell protein) were then separated by 1D SDS PAGE and autoradiographed on preflashed film. Lane 1, mesothelial strain LP-9; lane 2, dermal fibroblast strain R₂-F; lane 3, epidermal keratinocyte strain N; lane 4, bladder urothelial strain HB1-4; lane 5, kidney cortex type I epithelial strain HKI-12 clone D; lane 6, kidney cortex type II epithelial strain HKI-12 clone 2.1; lane 7, kidney cortex type II epithelial strain HKI-14 clone 8; lane 8, a 1:16 dilution of the LP-9 CM sample shown in lane 1. Arrowhead indicates Sp46. FN indicates fibronectin, which is synthesized at relatively high rates by mesothelial cells when they are growing rapidly in the presence of EGF, as in this experiment, but at much lower rates by cells that are quiescent in the absence of EGF. (B) Immunoblot detection and quantitation of Sp46 in CM from human and orangutan cells. 3-d CM was harvested from cultures containing $3-6 \times 10^6$ cells per 100-mm dish. Cells were grown in the presence of 0.4 μ g/ml HC. The CM was concentrated 50-fold and samples were separated by SDS PAGE, electrotransferred to nitrocellulose paper, and reacted with rabbit anti-Sp46 antiserum and avidin-biotin complex peroxidase. Lane 1, 5 μ l of 50-fold concentrated LP-9; lanes 2-4, serial threefold dilutions of the LP-9 CM sample of lane 1; lane 5, 5 μ l of 50-fold concentrated unconditioned medium; lane 6, 10 μ l of 50-fold concentrated CM from human fibroblast strain R2-F; lane 7, 10 μ l of 50-fold concentrated CM from orangutan fibroblast strain OrangF-1.

cells. Connective tissue fibroblasts secreted about 1/10th as much as mesothelial cells. Sp46 synthesis by fibroblasts, but not by mesothelial cells, was dependent upon HC: in the absence of HC, fibroblasts reduced their Sp46 production about threefold.

Table I. Relative Rates of Sp46 Synthesis by Normal Human Cell Types in Culture

| Cell type (number of strains tested) | Relative Sp46 synthesis |
|--------------------------------------|-------------------------|
| Mesothelial (3) | 80-120 |
| Vein endothelial (2) | 80-100 |
| Kidney tubule, type II (2) | 40-50 |
| Kidney tubule, type I (1) | 20 |
| Dermal fibroblast (3) | 8-15 |
| Epidermal keratinocyte (2) | <3 |
| Bladder urothelial (2) | <3 |
| Conjunctival epithelial (1) | <3 |
| Mammary epithelial (2) | <3 |

Data were compiled from experiments as described in legend to Fig. 6. Each strain was examined both by 1D SDS PAGE of pulse-chased CM and by 2D NEPHGE of pulse-labeled whole-cell extracts.

Epidermal keratinocytes, conjunctival epithelial cells, bladder urothelial cells, and mammary epithelial cells did not produce Sp46 at detectable levels (<3% that produced by mesothelial cells). Except for the effect of HC on fibroblasts, culturing cells in different media did not markedly affect the level of Sp46 synthesis: fibroblasts synthesized Sp46 at the same rate when growing in DME/F12 as in M199/MCDB202 medium, and mammary epithelial cells failed to produce detectable amounts of Sp46 when growing either in the 3T3 feeder system or in standard medium plus EGF. Thus epithelial cells and epithelial-like cells (i.e., endothelial cells) of embryonic mesodermal origin synthesize and secrete large amounts of Sp46 but other epithelial cell types do not. Immunoblot analysis failed to detect Sp46 in ascites fluid of the peritoneal cavity, in pleural effusion fluid, or in homogenates of mesotheliomas or of normal human fetal kidney. Sp46 was not detected immunohistologically in cryostat or paraffin sections of normal kidney or of mesotheliomas.

We did not detect metabolically labeled Sp46 in the presumptive rat mesothelial cell line RM-4 (ATCC No. CCL216) (Aronson and Cristofalo, 1981), in a presumptive bovine mesothelial cell strain (AG4907 of the Coriell Institute for Medical Research), in the bovine endothelial cell line ABAE (Gospodarowicz et al., 1976), in the kangaroo rat kidney epithelial cell line PtK2, in the canine kidney epithelial cell line MDCK, in the Swiss mouse fibroblast line 3T3 (Todaro and Green, 1963), or in early passage cultures of normal rabbit mesothelial or kidney cortex epithelial cells. Nor were we able to detect immunologically cross-reacting protein in Western blots of concentrated CM harvested from these cultures. In contrast, normal orangutan fibroblasts synthesized and secreted a protein of identical size, charge, and immunoreactivity as human Sp46 and at levels similar to that of human fibroblasts (Fig. 6 B). Normal owl monkey and lemur catta fibroblasts also secreted a protein identifiable electrophoretically as Sp46.

Amino Acid Analysis and Amino-terminal Sequencing of Sp46. To concentrate and increase the purity of Sp46 before preparative electrophoretic isolation, concentrated human mesothelial cell CM was bound to Con A-Sepharose beads and eluted with α -methylmannoside, as described in Fig. 2. The eluted proteins could be loaded onto slab gels such that 20 μ g of Sp46 separated cleanly from other proteins in each lane. 75 μ g of electroeluted Sp46 was used for amino acid analysis and 75 μ g for amino-terminal sequenc-

Table II. Amino Acid Composition of Sp46

| | *mol% | *Approximate number of residues per molecule |
|---------|-------|--|
| Glu/Gln | 11.5 | 42 |
| Asp/Asn | 8.9 | 32 |
| Ser | 8.3 | 30 |
| Thr | 8.3 | 30 |
| Tyr | 1.4 | 5 |
| Gly | 6.5 | 24 |
| Pro | 2.1 | 8 |
| Val | 9.0 | 33 |
| Leu | 8.9 | 32 |
| Ile | 4.1 | 15 |
| Ala | 5.8 | 21 |
| Phe | 5.7 | 21 |
| Met | 1.1 | 4 |
| His | 4.1 | 15 |
| Lys | 4.4 | 16 |
| Arg | 8.1 | 30 |
| Trp | ND† | ? |
| Cys | ND | ? |

* The contents of Trp and Cys were each assumed to be 1% for calculating moles% and residues per molecule of the other amino acids.

† ND, not determined.

ing, performed by Dr. David Andrews and Dr. William Lane of the protein sequencing facility at the Harvard Biological Laboratories. Sp46 was found to contain only 6.5 mol% glycine, corresponding to approximately 24 residues per 40-kD polypeptide (Table II). Thus Sp46 cannot have a lengthy (Gly-X-Y)_n repeat sequence as collagens possess (see review by Martin et al., 1985), and is therefore unlikely to be a collagenlike protein. The mobility of Sp46 was identical under reducing or nonreducing conditions (with and without 2-mercaptoethanol) in SDS PAGE; thus the protein is unlikely to have extensive intrachain disulfide bonds (see Sage et al., 1984) nor does it exist in the medium as larger disulfide-bonded aggregates.

The NH₂-terminal sequence of Sp46 was determined for the first 40 amino acids by automated Edman degradation (Fig. 7). The repetitive yield during the sequencing was ≥93%. The sequence was compared with all protein sequences in the National Data Base by F. Temple Smith of this institute, and was not identical or closely related to any sequence in that library. About half of the Sp46 molecules in the sample, which was purified from 3-d CM, lacked the first two amino acid residues of the NH₂-terminal end. We do not know whether there are alternate cleavage sites for the intracellular removal of a signal sequence, or whether the protein is subject to proteolysis after secretion.

Discussion

Sp46 is the predominant protein secreted by human mesoderm-derived epithelial and endothelial cells in culture, and we therefore propose for it the name "mesosecrin." Except for β-actin, mesosecrin is synthesized at a higher rate than any other protein by mesothelial cells, representing several percent of total protein synthesis.

Mesosecrin is a glycoprotein, containing two or three N-glycan units. These oligosaccharides are processed before secretion, with the addition of terminal sialic acid residues

val-ala-(val)-his-his-pro-pro-ser-tyr-
 val-ala-his-leu-ala-ser-asp-phe-gly-
 val-arg-val-phe-gln-gln-val-ala-gln-
 ala-ser-lys-asp-arg-asn-val-val-phe-
 (?) -pro-tyr-(gly)-val-ala-

Figure 7. Amino acid sequence of the NH₂-terminal end of Sp46. Sequence determined by automated Edman degradation of Sp46 purified from medium conditioned by human mesothelial cell strain LP-9. Residues in parentheses are uncertain. The residue indicated by (?) could not be determined. About half the Sp46 molecules in the sample were missing the first two amino acid residues from the NH₂-terminal end.

resulting in the protein becoming more acidic and exhibiting a slightly decreased (~1 kD) mobility in SDS PAGE. Many N-glycans undergo such terminal sialation when they are converted in the Golgi apparatus from the initial "high mannose" form to the ultimate "complex" form (Kornfeld and Kornfeld, 1985). Mesosecrin apparently is not O-glycosylated, nor is the polypeptide portion modified in any way that would affect its charge or mobility in SDS PAGE, because the N-glycan-deficient form of mesosecrin synthesized in the presence of tunicamycin does not change in size or charge from the time it is translated until it is secreted. Mesosecrin in mesothelial cells would be an excellent system for studying the regulation of glycoprotein synthesis, N-glycan processing, and secretion because mesosecrin is synthesized at very high levels by these cells, all forms of mesosecrin are well resolved from other major cell proteins in 2D NEPHGE gels, and a polypeptide-specific antiserum is available.

Mesothelial cells modulate their synthesis and content of intermediate filament proteins over an extraordinarily wide range as a function of their proliferative state (Connell and Rheinwald, 1983). Mesothelial cells have a high rate of synthesis and acquire a very high content of the four simple epithelial keratins when they become replicatively quiescent in the absence of their essential growth factor EGF. When they are rapidly dividing in the presence of EGF, they synthesize and acquire high levels of vimentin, and greatly reduce their rate of synthesis and consequently their content of keratins. In this study we found that fibronectin synthesis and secretion were also modulated, being much greater during rapid growth in the presence of EGF than during quiescence in the absence of EGF. Because of their differential expression among cell types, mesosecrin synthesis and secretion also can be classified as differentiated functions. However, they do not vary in mesothelial cells as a function of the EGF-dependent proliferative state. Fibroblasts make some mesosecrin, and it is perhaps for this reason that mesosecrin synthesis does not become suppressed, as keratin synthesis does, when mesothelial cells and type II kidney epithelial cells are in a dedifferentiated, "fibroblastoid" state.

Some pulse-labeled mesosecrin is found in the THS-insoluble protein fraction along with some of the total cellular fibronectin, actin, and all of the intermediate filament protein of the mesothelial cells. In an earlier communication reporting our characterization of the four simple epithelial

keratins, we erroneously concluded that mesosecrin was also a keratin (Wu et al., 1982). In that early investigation we did not use protease inhibitors; in retrospect it seems certain that the 46-kD mesothelial protein that had keratin properties observed in experiments employing 1D gels was the prominent 46-kD breakdown product of the 52-kD keratin no. 8 (Schiller and Franke, 1983), which results from the action of an endogenous Ca^{2+} -dependent protease (Nelson and Traub, 1981).

Several secreted glycoproteins have been described recently that possess several *N*-glycan units and migrate in SDS-PAGE as molecules of M_r 40–50 kD. We have been able to rule out an identity of these proteins and mesosecrin. The 47-kD glycoprotein colligin (Kurkinen et al., 1983; Taylor et al., 1985) is much more basic than mesosecrin, is cell surface-associated, and binds to gelatin. The 43-kD secreted, acidic, cysteine-rich glycoprotein SPARC produced by bovine aortic endothelial cells (Sage et al., 1984) and by mouse parietal endoderm cells (Mason et al., 1986) has an amino acid composition and sequence very different from mesosecrin. The 43-kD protease-nexin (Baker et al., 1980) secreted by human fibroblasts has an amino acid composition and NH_2 -terminal sequence (Scott et al., 1985) very different from mesosecrin. Endothelial cells produce a 50-kD plasminogen-activator inhibitor (van Mourik et al., 1984; Erickson et al., 1985), but its *pI* is 4.5–5.0, which is very different from mesosecrin. MEP, a 39-kD major excreted protein of transformed NIH 3T3 cells (Gal et al., 1985) is much smaller than Sp46 and does not appear to associate with the substratum.

We did not detect mesosecrin as a protein secreted in amounts detectable autoradiographically by several mesothelial, endothelial, kidney epithelial, and fibroblast cell lines of rodent, rabbit, canine, or bovine origin. Mesosecrin was expressed, however, by orangutan fibroblasts. An additional factor might be required to induce mesosecrin expression in culture by cells of the nonprimate species examined. Alternatively, mesosecrin may have appeared rather late in evolution and may be restricted to primates, such as has been found for involucrin, the major structural protein of the epidermal cornified envelope (Banks-Schlegel and Green, 1980; Rice and Thacher, 1986).

The cell type specificity of mesosecrin synthesis is interesting. It groups several true epithelial (i.e., keratin-containing) cell types with the large vessel endothelial cell, which adopts an epithelioid morphology but contains vimentin rather than keratin filaments. Before the application of anti-intermediate filament immunohistochemistry and our own cell culture studies, many medical histologists considered mesothelial and endothelial cells to be virtually indistinguishable cell types (for example, see Reith and Ross, 1977; Leeson and Leeson, 1979), which serve the similar functions of presenting nonthrombogenic, nonadhesive surfaces to the blood and serous fluids, respectively. Cell culture studies have clearly demonstrated, however, that normal human endothelial and mesothelial cells differ greatly in their hormone and growth factor requirements (Thornton et al., 1983; Connell and Rheinwald, 1983), in addition to their differences in intermediate filament expression. Nevertheless, the abundant secretion of mesosecrin tends to group these two functionally similar cell types together again.

Mesosecrin is also expressed at high levels by kidney tu-

bule epithelial cells, which are very different structurally and functionally from mesothelial and endothelial cells *in vivo*, but which have a similar embryonic origin. When the nephrons form during embryogenesis, the nephrogenic cells in a region of the mesoderm change from a fibroblastoid cell type, which had expressed vimentin and fibronectin, to an epithelioid cell type, which thenceforth expresses keratins and laminin (Holthofer et al., 1984; reviewed by Ekblom, 1984). Two morphologically distinctive cell types grow from explants of either fetal or adult kidney cortex: a high keratin/low vimentin epithelioid cell (type I), which forms tightly adherent colonies, and a low-to-moderate keratin/high vimentin cell (type II), which forms dispersed colonies of morphology similar to that of mesothelial cells (Rheinwald and O'Connell, 1985; Rheinwald et al., 1984). Type II kidney cells are less dependent upon exogenous growth factors and a complex nutrient medium than are mesothelial cells, but they still express high levels of vimentin during growth in culture and modulate their expression of keratins somewhat as a function of growth. We have found here that they also resemble mesothelial cells in the high level synthesis of mesosecrin.

The initial substratum association of mesosecrin is consistent with its being an extracellular matrix protein located at the basal surface of cells that synthesize it. The exclusive deposition onto the substratum, without any of the secreted protein escaping directly into the medium, is remarkable. It occurs even in sparse cultures of migrating cells, which would not be expected to have a polarized secretory apparatus, and it occurs on both positively and negatively charged substrata. Virtually all of the mesosecrin synthesized ends up in solution in the medium, but this is also the fate of much of the fibronectin and collagen synthesized by cells growing on tissue culture plastic. *In vivo* localization data will be required, of course, to determine the significance of the initial substratum-association in culture. The smooth, even deposition of mesosecrin on the culture substratum contrasts with the fibrous, supracellular organization of fibronectin, and indicates that mesosecrin neither self-assembles nor combines with fibronectin to form higher-order structures detectable by light microscopy. Its lack of binding to gelatin-Sepharose indicates that mesosecrin does not have a binding site for type I collagen similar to that of fibronectin (Hahn and Yamada, 1979), colligin (Kurkinen et al., 1984), or the glycine-rich gelatin-binding protein of porcine plasma (Isemura et al., 1982), all of which recognize denatured collagen.

Because of the present lack of an easily accessible animal model, we have no data as yet on the distribution of mesosecrin *in vivo*. We may have unmasked by growth in culture a potential of mesoderm-derived cells for extraordinarily high rates of mesosecrin synthesis, which usually does not occur under normal steady-state conditions *in vivo*. We are not yet in a position even to guess at its function. Mesothelial cells attach and spread very rapidly after plating on tissue culture plastic, presumably before much mesosecrin could be deposited. Preliminary experiments indicate that anti-mesosecrin antiserum does not inhibit the initial attachment and spreading of subcultured mesothelial cells, but causes them to round up and detach within a day after plating (K. Plummer and J. Rheinwald, unpublished observations). We do not know whether this result has significance beyond the general inability of cells to adhere to antibody molecules that have

bound to a surface coated with antigen (Giaever and Ward, 1978). Considering the types of cells that express mesosecrin, it would be interesting to determine whether the protein plays a role in cell-substratum adhesion, enhancement or inhibition of blood clotting, or growth regulation. Nilsen-Hamilton and Holley (1983) have reported that the African green monkey kidney cell line BSC-1 cultured in low serum medium secretes a 48-kD protein in response to treatment with transforming growth factor- β . We have found an M_r 45-kD/pI 7.2 protein secreted by BSC-1 which may cross-react with our anti-human mesosecrin antiserum, but we do not know whether its synthesis is subject to regulation by transforming growth factor- β .

Regardless of its function, mesosecrin may have an immediate practical application. Five of the six human mesothelioma cell lines that we have tested all secreted moderate-to-high levels of mesosecrin in culture, whereas four squamous cell carcinomas of oral and epidermal origin, HeLa cells, and MCF-7 cells did not (J. Rheinwald and A. Terpstra, manuscript in preparation). Thus mesosecrin may serve as a diagnostic marker to aid in identifying the cell type of origin of certain malignancies.

We thank Dr. Alan Schwartz for helpful discussions on characterizing glycoproteins, Dr. Tung-Tien Sun for advice on immunoblotting techniques, and Dr. Marcia Simon for help with electroelution of proteins. We are very grateful to Drs. William Lane and David Andrews for the amino acid analysis and NH_2 -terminal sequencing of mesosecrin, and to Dr. F. Temple Smith for computer analysis of the sequence. We also wish to thank Dr. Jordan Pober for providing us with human endothelial cell cultures, Dr. Robert Rice for nonhuman primate fibroblasts, Dr. Bruce Zetter for the bovine endothelial cell line, and Dr. Martha Stampfer for sending us collagenase-digested human mammary gland cell clusters. Ms. Marjorie Stack expertly prepared the manuscript. The portion of this work describing the characterization of mesosecrin as a secreted glycoprotein was done by Mr. Jorgensen as an undergraduate thesis in the Biochemical Sciences program at Harvard University.

These investigations were supported by the National Institute on Aging (grant ROI-AG02048), by the National Foundation for Cancer Research, and by an American Cancer Society Faculty Research Award to Dr. Rheinwald.

Received for publication 21 May 1986, and in revised form 28 October 1986.

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