Biosynthesis of Cell Surface Sulfated Glycoproteins by Cultured Vascular Endothelial Cells†

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ABSTRACT: Cultured vascular endothelial cells incorporate $^{35}SO_4$ into a class of oligosaccharides which are N-glycosidically linked to cell-surface or extracellular proteins. This type of sulfated oligosaccharide was not synthesized by smooth muscle cell cultures and may represent sulfation of vascular tissue glycoproteins which are unique to endothelial cells. These endothelial cell ^{35}S-labeled oligosaccharides are not released from the polypeptides under alkaline conditions that cleave O-glycosidically linked chains. The incorporation of both [^{3}H]hexosamine and $^{35}SO_4$ into these oligosaccharides is inhibited by tunicamycin, an antibiotic which inhibits protein N-glycosylation. ^{35}S-Labeled oligosaccharides, isolated as glycopeptides, were resistant to a variety of chemical and enzymatic treatments which degraded vascular cell sulfated glycosaminoglycans but appear to contain terminal sialic acid residues. Endothelial cell sulfated oligosaccharide chains could also be distinguished from sulfated glycosaminoglycans by the apparent size and charge. Endothelial cells released or secreted ^{35}S-labeled glycoproteins into the culture medium. These soluble ^{35}S-labeled glycoproteins were partially purified by DEAE-cellulose chromatography and separated from the ^{35}S-labeled proteoglycans, which are also released into the medium. The major ^{35}S-labeled glycoproteins released from the cells had apparent molecular weights of 24 000–66 000. The cell-associated ^{35}S-labeled glycoproteins, released with ethylenediaminetetraacetate or extracted with 0.5% octyl glucoside, were partially purified on DEAE-cellulose and had apparent molecular weights of 45 000–250 000.

The regulation of the glycoprotein biosynthesis of cells within blood vessels is important in controlling and maintaining vascular function. The intima media portion of many blood vessels is composed largely of smooth muscle cells and their extracellular matrix, which are separated from the vascular flow by a layer of endothelial cells. There is currently an increased interest concerning the effects that modifications of the carbohydrate side chains of glycoproteins and proteoglycans would have on vascular cell function. Sulfated glycosaminoglycans, the high molecular weight polysaccharide components of proteoglycans, have been reported as one class of cell-surface or extracellular molecules synthesized by both endothelial and smooth muscle cells (Gamse et al., 1978; Namiki et al., 1980). In addition to sulfated glycosaminoglycans, the majority of other sulfated glycoconjugates from a variety of cell types have been characterized as mucinlike chains that are O-glycosidically linked to protein (Roussel et al., 1975; Lombart & Winzler, 1974; Horowitz, 1977; Schwieger, 1978; Miller & Waechter, 1979; Sherblom & Carraway, 1980). Margolis & Margolis (1970) described sulfated sugar residues as part of mannose-containing brain glycopeptides. Parsons & Pierce (1980) identified *N*-acetylhexosamine sulfate as the terminal sugar residue of bovine lutropin *N*-asparaginyl-linked oligosaccharides. Recently the synthesis of sulfated oligosaccharide chains, which are presumably N-glycosidically linked to glycoproteins and are not keratan sulfate, has been demonstrated in sea urchin embryos (Heifetz & Lennarz, 1979), paramyxovirus SV5 infected cells (Nakamura & Compans, 1978; Prehm et al., 1979), and in embryonic liver and lung (Heifetz et al., 1980; Heifetz & Snyder, 1981). In order to investigate the functions of sulfated oligosaccharides, it was of interest to determine which specific cell types within tissues are able to synthesize these sulfated glycoproteins. This paper describes observations which suggest that a class of cell-surface or matrix-associated glycoproteins, synthesized by vascular endothelial cells in culture, contains sulfated oligosaccharide chains which are N-glycosidically linked to the protein moiety and that this class of sulfated glycoproteins does not contain sulfated glycosaminoglycan side chains. Vascular smooth muscle cells in culture, though active in synthesizing N-glycosidically linked glycoproteins and sulfated glycosaminoglycans, did not synthesize these N-linked sulfated oligosaccharide chains.

Experimental Procedures

Cell Cultures and Radioisotope Labeling. Bovine endothelial and smooth muscle cell cultures were obtained from fetal bovine thoracic aortas by the methods of Fensleau & Mello (1976) and were cultured in minimal essential medium (GIBCO) containing 10% fetal bovine serum (FBS).[1] Human endothelial cell cultures were obtained and cultured as described by Johnson & Erdos (1977) and Johnson (1980). Human endothelial cells were grown in medium 199 containing 20% FBS and 10% pooled human serum. Smooth muscle cells from aortas, pulmonary arteries and veins, and human umbilical cord arteries and veins were obtained from explant cultures and maintained as described by Coltoff-Schiller et al. (1976). Cell cultures were labeled with 50 μCi/mL D-[6-^{3}H]glucosamine (Amersham Searle) and 250 μCi/mL $H_2{}^{35}SO_4$ (New England Nuclear) in 5 mL of culture medium in the absence or presence of 0.2 μg/mL tunicamycin. Tunicamycin was a generous gift of Dr. G. Tamura, Tokyo. Alternately, cells were labeled with 50–100 μCi/mL $H_2{}^{35}SO_4$ in Ham's F-12 nutrient medium. The medium was removed and incubated with 10% trichloroacetic acid for 20 min at 4 °C. The acid-insoluble medium materials were recovered by centrifugation. Cell layers were rinsed with ice-cold medium and removed from the flask by scraping in 2 mL of methanol. Alternatively, cells were scraped from the flask in 0.1 M Tris

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; FBS, fetal bovine serum; Tris, tris(hydroxymethyl)aminomethane.

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buffer (pH 8.0) and homogenized, and aliquots were removed for protein (Lowry et al., 1951) and DNA (Burton, 1956) determinations. Cells, homogenates, or acid-insoluble materials were extracted to remove glycolipids after the solvent was adjusted to chloroform/methanol/water (10:10:3 v/v) (Heifetz & Snyder, 1981). After centrifugation, the solvent was removed, and the residual denatured glycoconjugate pellet was dried under N_2 and then digested with Pronase (Heifetz & Lennarz, 1979). The resulting glycopeptides were analyzed by Sephadex G-50 column chromatography in 0.1 M ammonium acetate.

Enzymatic Treatments. Glycopeptides and glycosaminoglycans were digested as previously described (Heifetz et al., 1980) with chondroitinase ABC, *Streptomyces* hyaluronidase, testicular hyaluronidase, *Clostridium* neuraminidase, jack bean α-mannosidase, *Streptomyces* endo-β-*N*-acetylglucosaminidase H, and endo-β-galactosidase. The endo-β-galactosidase, purified from *Escherichia fruendii* (Fukuda et al., 1979), was kindly supplied by Dr. M. N. Fukuda, Fred Hutchinson Cancer Center, University of Washington, Seattle. Glycopeptides and glycosaminoglycans were treated with nitrous acid and analyzed as previously described (Hart & Lennarz, 1978). This treatment selectively degrades N-sulfated glycosaminoglycans and is specific for *N*-sulfate removal (Cifonelli & King, 1973).

Glycosaminoglycan Analysis. Glycosaminoglycans were analyzed by sequential treatments with nitrous acid, *Streptomyces* hyaluronidase, chondroitinase ABC, and endo-β-galactosidase as previously described (Heifetz et al., 1980). Glycosaminoglycan products degraded by these treatments were separated from undegraded materials by Sephadex G-50 chromatography, and the undegraded material was then subjected to the next treatment and rechromatography.

DEAE-cellulose Chromatography. Glycopeptides in 0.01 M ammonium acetate were applied to columns (1 × 5 cm) of DEAE-cellulose (acetate form) equilibrated in 0.01 M ammonium acetate. Neutral glycopeptides were eluted with equilibration buffer, and negatively charged glycopeptides were eluted with increasing concentrations of ammonium acetate.

Glycoproteins from cell culture medium were isolated by adjusting the medium to 10% trichloroacetic acid at 4 °C. The acid-insoluble glycoproteins were recovered by centrifugation, and the glycoprotein pellet was redissolved in buffer A [0.05 M Tris-HCl (pH 8.0) containing 2 M urea, 5 mM phenylmethanesulfonyl fluoride, and 5 mM *N*-ethylmaleimide]. The glycoproteins were dialyzed against buffer A overnight at 4 °C. These glycoproteins were then applied to a column (1 × 10 cm) of DEAE-cellulose (chloride form) preequilibrated at 4 °C in buffer A. The column was washed with the same buffer. Glycoproteins were then eluted with increasing concentrations of NaCl in buffer A. The glycoproteins recovered were dialyzed against 0.05 M Tris-HCl (pH 8.0) and then against water. The resulting glycoproteins were lyophilized and stored frozen in 0.05 M Tris-HCl (pH 8.0) containing 5 mM phenylmethanesulfonyl fluoride and 5 mM *N*-ethylmaleimide.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Samples for electrophoresis were mixed with an equal volume of reducing sample buffer, heated to 100 °C for 5–10 min, and loaded on an $NaDodSO_4$–polyacrylamide slab gel with a 3% or 4% polyacrylamide stacking gel according to the method of Laemmli (1970). Electrophoresis was performed at constant voltage until the bromophenol blue marker dye was 1 cm from the bottom of the gel. Gels were removed and fixed in 10% trichloroacetic acid/10% acetic acid/30% methanol overnight.

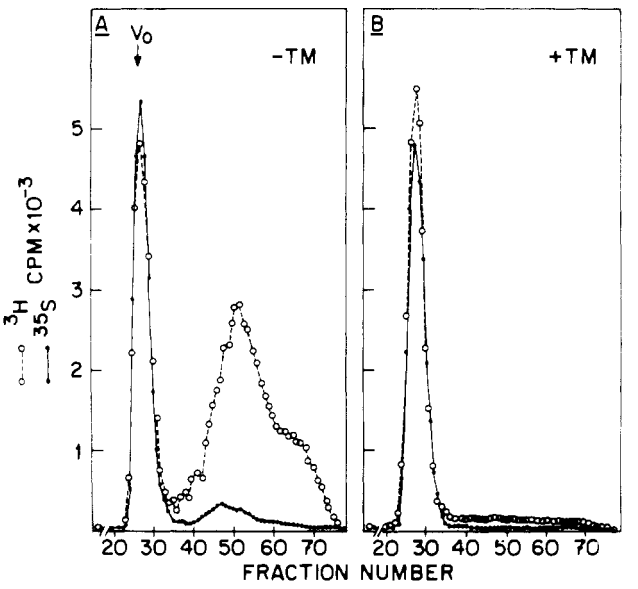

FIGURE 1: Sephadex G-50 column chromatography of glycoconjugates after digestion with Pronase. Bovine aortic endothelial cells (1.7×10^6 cells) were incubated with [^{3}H]glucosamine and $^{35}SO_4$ and then digested with Pronase as described under Experimental Procedures. Samples were applied to Sephadex G-50 columns (1.0 × 100 cm) equilibrated in 0.1 M ammonium acetate and then eluted with the same solvent. Panel A represents incubations in the absence of tunicamycin. Panel B represents incubations performed in the presence of 0.2 μg of tunicamycin per mL. The position of elution marker blue dextran (V_0) is indicated.

Gels were then stained by the procedure of Fairbanks et al. (1971). Radiolabeled materials were detected on gels by fluorography of Enhance (New England Nuclear) impregnated gels by exposing Kodak X-Omat film to the dried gel at −80 °C.

Results

Endothelial Cell Sulfated Glycopeptides. Endothelial cells were incubated with [^{3}H]glucosamine and $^{35}SO_4$, and the total labeled glycoconjugates were isolated, digested with Pronase, and chromatographed on Sephadex G-50 as described under Experimental Procedures. As shown in Figure 1A, endothelial cells incorporated [^{3}H]hexosamine and $^{35}SO_4$ into two classes of glycopeptides, apparently differing by size. The larger ^{3}H,^{35}S-labeled glycopeptides eluting with the column's excluded volume (V_0) are composed of glycosaminoglycans while the smaller size ^{3}H,^{35}S-labeled glycopeptides eluting in the column's fractionation volume (fractions 40–70, Figure 1A) contain the oligosaccharide chains of glycoproteins (Heifetz et al., 1980). The glycosaminoglycan material, excluded from the column, was lyophilized and treated again with Pronase. After Sephadex G-50 column chromatography, all labeled components still eluted with the excluded volume (data not shown). This ^{3}H,^{35}S-labeled glycosaminoglycan material was analyzed as described under Experimental Procedures and found to consist of 77% [^{3}H,^{35}S]heparan sulfate and 13% [^{3}H]hyaluronic acid. The remainder of the ^{3}H,^{35}S-labeled glycosaminoglycan material was identified as [^{3}H,^{35}S]-chondroitin sulfate. As shown in Figure 1B, the incorporation of both [^{3}H]hexosamine and $^{35}SO_4$ into these glycosaminoglycans was not inhibited in cultures labeled in the presence of tunicamycin. However, the synthesis of ^{3}H,^{35}S-labeled glycopeptides (Figure 1B, fractions 40–70) was completely inhibited in the presence of tunicamycin. Tunicamycin produced minimal effects on protein synthesis, and glycosaminoglycan synthesis, which is sensitive to the inhibition of protein synthesis (Hart & Lennarz, 1978), was not affected.

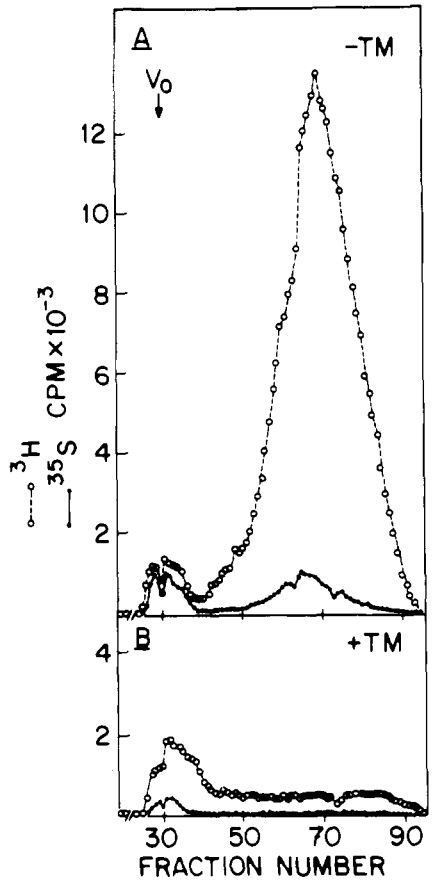

FIGURE 2: Sephadex G-50 column chromatography of total glycopeptides prepared after alkaline treatment of the total glycoproteins. ^3H- and ^{35}S-labeled endothelial cells were prepared as described under Experimental Procedures. The denatured proteins were washed with water and then incubated in 0.1 N NaOH at 37 °C. After 48 h, the sample was treated with 10 volumes of 10% trichloroacetic acid at 2 °C for 30 min. After centrifugation, the alkali-stable glycoproteins were washed twice with ice-cold 10% trichloroacetic acid and then twice with ethyl ether to remove excess trichloroacetic acid. The resulting residue was dried, resuspended in 0.2 M Tris-HCl (pH 8.0), and digested with Pronase. The resulting glycopeptides were analyzed by gel filtration on Sephadex G-50 as described in Figure 1A. The position of elution marker blue dextran (V_0) is indicated.

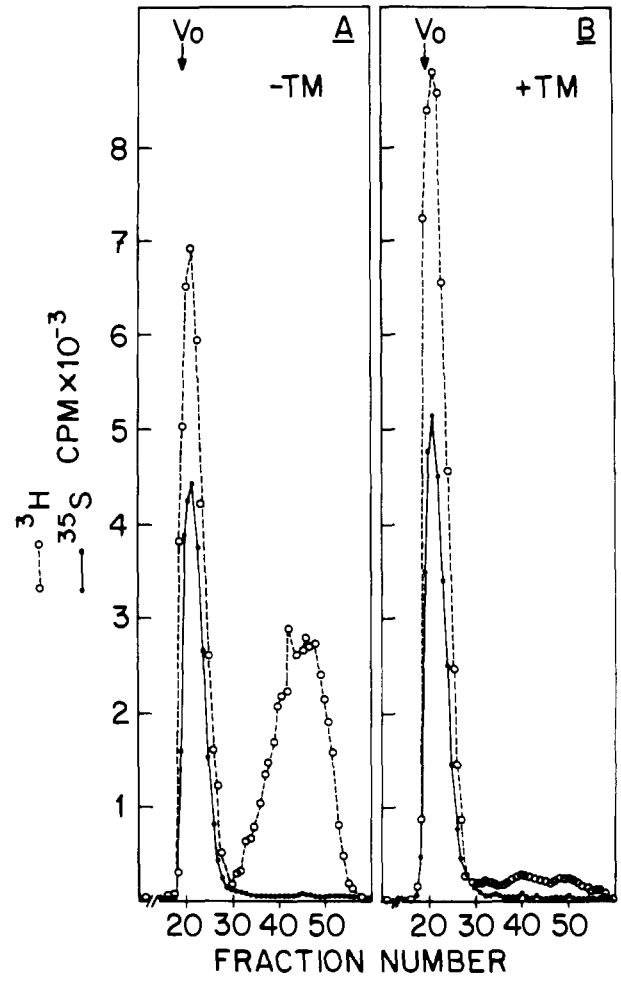

FIGURE 3: Sephadex G-50 column chromatography of total glycoproteins after digestion with Pronase. Bovine aortic smooth muscle cells (1.5×10^6 cells) were labeled, and glycopeptides were prepared as described in the text and then analyzed as described in Figure 1. Panel A represents incubations in the absence of tunicamycin. Panel B represents incubations performed in the presence of 0.2 μg of tunicamycin per mL. The position of elution marker blue dextran (V_0) is indicated.

This indicates that the incorporation of both [^{3}H]hexosamine and $^{35}SO_4$ into these glycopeptides was probably dependent upon the formation of oligosaccharide lipids, which are intermediates in the biosynthesis of N-glycosidically linked glycoproteins and whose biosynthesis is the target of tunicamycin action [see Tkacz & Lampen (1975) and Struck & Lennarz (1980) and references cited therein].

The ^{3}H,^{35}S-labeled glycopeptides isolated by Sephadex G-50 chromatography were resistant to treatments with nitrous acid, *Streptomyces* hyaluronidase, and chondroitinase ABC which degraded the ^{3}H,^{35}S-labeled glycosaminoglycans. These endothelial cell ^{3}H,^{35}S-labeled glycopeptides were also resistant to treatments with testicular hyaluronidase, β-glucuronidase, aryl sulfatase, and endo-β-galactosidase. These latter enzymes were all active in degrading their authentic biological substrates (Heifetz & Snyder, 1981). Since these ^{3}H,^{35}S-labeled glycopeptides are not degraded by endo-β-galactosidase, it is unlikely that they represent unusually low molecular weight keratan sulfate I chains, the only sulfated glycosaminoglycan which is known to be N-glycosidically linked to protein and whose synthesis is inhibited by tunicamycin (Hart & Lennarz, 1978).

For further support of the conclusion that the tunicamycin-sensitive oligosaccharides were not linked to protein via an alkali-labile O-glycosidic bond, labeled cells were extracted to remove glycolipids, and the glycoprotein residue was then treated with 0.1 N NaOH (pH 13.0) at 37 °C for 48 h. The alkali-stable glycoproteins were isolated by precipitation with cold 10% trichloroacetic acid and then digested with Pronase. As shown in Figure 2A, when the alkali-stable glycopeptides were analyzed by gel filtration on Sephadex G-50, greater than 90% of the ^{3}H,^{35}S-labeled glycopeptides (see Figure 1A for comparison) are recovered in the partially included volume whereas less than 10% of the ^{3}H,^{35}S-labeled glycosaminoglycans (V_0) are recovered after alkali treatment. Figure 2B shows that the ^{3}H,^{35}S-labeled glycopeptides that are stable to alkali are not synthesized when the cells are labeled in the presence of tunicamycin.

For comparison, smooth muscle cell cultures were also incubated with [^{3}H]glucosamine and $^{35}SO_4$, and the labeled glycoconjugates were isolated and digested with Pronase. Chromatography of the digests on Sephadex G-50 (Figure 3A) revealed that while smooth muscle cells synthesized sulfated glycosaminoglycans (V_0), smaller molecular weight glycopeptides (fractions 30–80) were not labeled with $^{35}SO_4$, even though the biosynthesis of these components was inhibited by tunicamycin (Figure 3B). Similar results have been observed in all cultures of vascular endothelial and smooth muscle cells tested from human umbilical cord arteries and veins, human

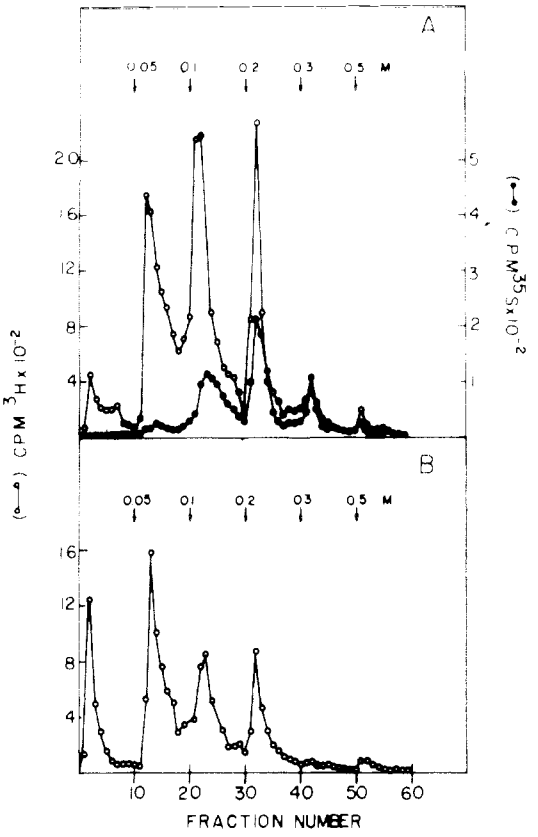

FIGURE 4: DEAE-cellulose column chromatography of [^{3}H]glucosamine and $^{35}SO_4$-labeled glycopeptides. Labeled glycopeptides were applied to a column (1 × 5 cm) of DEAE-cellulose (acetate form). The glycopeptides were eluted with 10 mL of solutions of increasing ammonium acetate concentrations as indicated. Fractions (1 mL) were collected, and the radioactivity in ^{3}H (○) and ^{35}S (●) was determined. (A) ^{3}H,^{35}S-Labeled glycopeptides from endothelial cells prepared as described in Figure 1A, fractions 40–70. (B) ^{3}H-Labeled glycopeptides from smooth muscle cells prepared as described in Figure 3A, fractions 30–60.

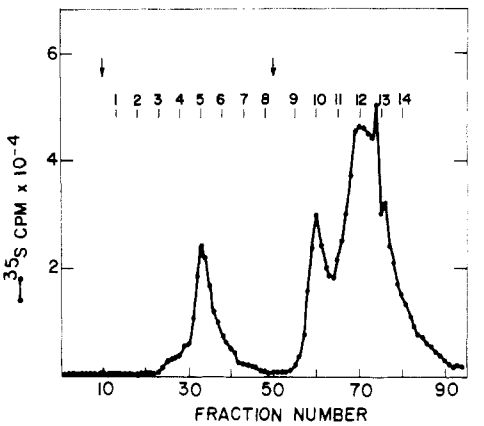

FIGURE 5: DEAE-cellulose chromatography of ^{35}S-labeled glycoconjugates isolated from cell culture medium. The ^{35}S-labeled glycoconjugates in the medium of cell cultures labeled with $^{35}SO_4$ were isolated by precipitation with 10% trichloroacetic acid. These ^{35}S-labeled glycoconjugates were dialyzed and then chromatographed on DEAE-cellulose (chloride form) in 0.05 M Tris buffer, pH 8.0, containing 2 M urea. The ^{35}S-labeled glycoproteins were eluted with a linear gradient of 0–0.25 M NaCl (beginning at the first arrow), and ^{35}S-labeled proteoglycans were eluted with a gradient of 0.25–0.5 M NaCl (beginning at the second arrow). After the radioactivity in each fraction was determined, aliquots of representative fractions (numbers above peaks) were removed for further analysis.

pulmonary arteries, and bovine aortas. ^{35}S-Labeled glycopeptides usually represented from 5 to 15% of the total ^{35}S-labeled glycoconjugates synthesized in these endothelial cells. The labeling medium from cell cultures was also analyzed as described above, and ^{3}H,^{35}S-labeled glycopeptides were found in the medium from endothelial cell incubations. Thus, endothelial cells in culture appear to incorporate sulfate oligosaccharide chains which are N-glycosidically linked to protein, whereas smooth muscle cell cultures from matching vascular sources incorporate little or no $^{35}SO_4$ into N-glycosidically linked oligosaccharide chains.

The labeled glycopeptides synthesized by endothelial cells and smooth muscle cells were each chromatographed on DEAE-cellulose (acetate form) and eluted with increasing concentrations of ammonium acetate as shown in Figure 4. The results indicate that a greater proportion of the ^{3}H-labeled glycopeptides from endothelial cells (Figure 4A) are eluted at higher salt concentrations compared to the ^{3}H-labeled glycopeptides from smooth muscle cells (Figure 4B). This may be a reflection of the increased sulfation of endothelial cell oligosaccharides as indicated by $^{35}SO_4$ labeling experiments, or both increased sulfation and sialylation. In contrast, endothelial cell ^{3}H,^{35}S-labeled glycosaminoglycans eluted from similar columns of DEAE-cellulose with concentrations of ammonium acetate greater than 0.5 M. These ^{3}H,^{35}S-labeled glycosaminoglycans were also quantitatively precipitated with cetylpyridinium chloride whereas the ^{3}H,^{35}S-labeled glycopeptides could not be precipitated with cetylpyridinium chloride. These data indicate that endothelial cell sulfated oligosaccharides are much less charged and have a lower degree of sulfation than most endothelial cell sulfated glycosaminoglycans.

Endothelial Cell Sulfated Glycoproteins. Vascular endothelial cells derived from human umbilical cord veins (Johnson, 1980) were incubated with $^{35}SO_4$ for 18 h, and the labeling medium was used as a source of soluble ^{35}S-labeled glycoproteins. The ^{35}S-labeled glycoproteins were separated from the ^{35}S-labeled proteoglycans by chromatography on DEAE-cellulose (chloride form) at 4 °C. All of the ^{35}S-labeled glycoproteins bound to DEAE-cellulose. As shown in Figure 5, the ^{35}S-labeled glycoproteins were eluted with a gradient of 0–0.25 M NaCl, whereas some ^{35}S-labeled proteoglycans were eluted with concentrations of NaCl between 0.25 and 0.5 M. Additional ^{35}S-labeled proteoglycans (not shown) were eluted with 1 M NaCl. When the ^{35}S-labeled glycoproteins (Figure 5) were further analyzed by reducing $NaDodSO_4$ gel electrophoresis, the major ^{35}S-labeled glycoproteins detected from peak areas 5–7 (Figure 5) had apparent molecular weights of between 24 000 and 66 000, although several bands with apparent molecular weights of higher than 70 000 were usually detected (data not shown). It is not known presently if the lower molecular weight ^{35}S-labeled glycoproteins in the culture medium are degradation products of higher molecular weight materials or if they represent intact secreted ^{35}S-labeled glycoproteins. However, ^{35}S-labeled glycoproteins with similar molecular weights were detected in the culture medium of endothelial cells labeled in the presence or absence of serum and aprotinin (100 units/mL). Labeled proteoglycan materials from peak areas 10–14 (Figure 5) entered a 7.5% polyacrylamide gel but ran as broad smears.

Endothelial cells were labeled in the presence of [^{3}H]-glucosamine and $^{35}SO_4$, and the ^{3}H,^{35}S-labeled glycoproteins released into the medium were partially purified by chromatography on DEAE-cellulose as described above. The ^{3}H,^{35}S-labeled glycoproteins were eluted batchwise with 0.2 M NaCl, and the ^{3}H,^{35}S-labeled proteoglycans were eluted batchwise with 2.0 M NaCl. These materials were dialyzed against water, lyophilized, and extracted with chloroform/

methanol/water (10:10:3 v/v). The residual glycoconjugates were then digested with Pronase and analyzed by Sephadex G-50 column chromatography. The ^3H,^{35}S-labeled glycoproteins eluting from DEAE-cellulose with 0.2 M NaCl yielded only ^3H,^{35}S-labeled glycopeptides of approximately 2000–4000 molecular weight. A total of 95% of these glycopeptides were also recovered if the intact ^3H,^{35}S-labeled glycoprotein residue was first treated with 0.1 N NaOH to remove O-glycosidically linked oligosaccharide chains. No ^3H,^{35}S-labeled glycosaminoglycan containing materials were found in these partially purified ^3H,^{35}S-labeled glycoprotein preparations, and no free $^{35}SO_4$ was released from the ^3H,^{35}S-labeled glycopeptides during the Pronase digestion. The ^3H,^{35}S-labeled proteoglycans yielded, after Pronase digestion, ^3H,^{35}S-labeled glycosaminoglycans and ^3H-labeled glycopeptides. The ^3H,^{35}S-labeled glycosaminoglycans contained [^3H,^{35}S]heparan sulfate (16% ^3H, 20% ^{35}S), [^3H]hyaluronic acid (72% ^3H), and [^3H,^{35}S]chondroitin sulfate (12% ^3H, 80% ^{35}S). Thus, most endothelial cell ^{35}S-labeled glycoproteins do not appear to be covalently linked to proteoglycans. However, it is not known if the ^3H-labeled glycopeptide-containing materials, eluted from DEAE-cellulose with 2.0 M NaCl, are part of a proteoglycan complex. The ^3H,^{35}S-labeled glycoproteins, partially purified by DEAE-cellulose chromatography, were also chromatographed on concanavalin A–agarose. A total of 21% of the ^{35}S-labeled glycoproteins and 23% of the ^3H-labeled glycoproteins bound to concanavalin A and were eluted with 1% methyl α-mannoside. When the total ^3H,^{35}S-labeled glycopeptides, following Pronase digestion, were chromatographed on concanavalin A–agarose, 34% of the ^3H-labeled glycopeptides and only 1% of the ^{35}S-labeled glycopeptides were bound by the lectin. The ^{35}S-labeled glycopeptides displayed altered mobility upon Bio-Gel P-4 column (1 × 200 cm) chromatography after digestions with neuraminidase but not after treatment with α-mannosidase or endoglycosidase H (data not shown). Mild acid hydrolysis followed by gel filtration chromatography and high-voltage electrophoresis (Heifetz et al., 1980) demonstrated the presence of GlcNAc-$^{35}SO_4$. Thus, endothelial ^{35}S-labeled glycoproteins appear to contain sulfated complex-type oligosaccharide chains.

In order to examine if the ^{35}S-labeled glycoproteins synthesized by endothelial cells are surface glycoproteins, cells were incubated in the presence [^3H]glucosamine and $^{35}SO_4$ for 18 h. The labeled cell layers were washed with phosphate-buffered saline, and the cells were released from the plastic flask by incubation with 10 mM EDTA in phosphate-buffered saline at 20 °C. The released cells were collected by centrifugation, washed, and then treated with 1 mg/mL chymotrypsin in phosphate-buffered saline for 5 min at 20 °C. The cells were again collected by centrifugation, and 99% of these treated cells were found to still exclude trypan blue. These cells were then incubated twice with 1.25 mg/mL trypsin for 5 and 10 min, respectively. In each case, the enzyme-released material and the final cell residue were incubated at 100 °C for 10 min to stop the reaction. These materials were subsequently treated with Pronase and analyzed by Sephadex G-50 chromatography as described under Experimental Procedures. As seen in Table I, these analyses indicated that all of the ^{35}S-labeled glycoproteins recovered on the cells were on the cell exterior and susceptible to chymotrypsin treatment. However, the more dramatic results of these analyses were that most of the ^{35}S-labeled glycoprotein material was removed from the cell layer and recovered in the EDTA-containing washes of the chelator-released cells. The

Table I: Cell-Surface Localization of ^{35}S-Labeled Glycoproteins

	% [^3H]glucosamine- and $^{35}SO_4$-released glycoconjugates			
	glycopeptides		glycosaminoglycans	
	^3H	^{35}S	^3H	^{35}S
cell[a] materials sequentially released by				
chymotrypsin (5 min)	32	8	18	47
then trypsin (5 min)	10	0	31	14
then trypsin (10 min)	5	0	2	1
remaining residue	12	0	20	5
EDTA-released materials[b]	41	92	29	33
total	100	100	100	100

[a] Cells were isolated by removing the cells from the culture flask with an EDTA treatment and harvesting by low-speed centrifugation. [b] Material remained in the supernatant after harvesting cells with an EDTA treatment.

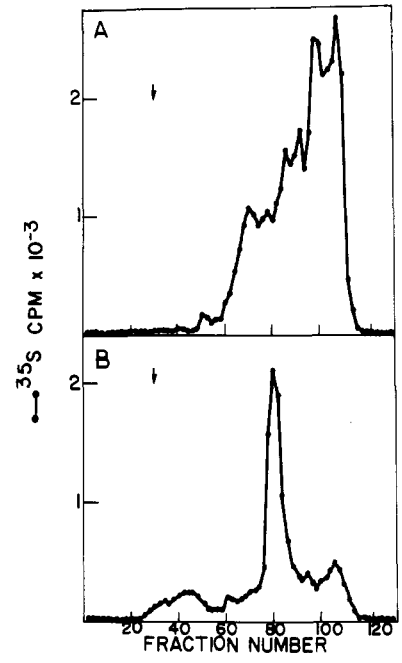

FIGURE 6: DEAE-cellulose chromatography of cell layer ^{35}S-labeled glycoproteins. ^{35}S-Labeled glycoproteins from the octyl glucoside–urea extracts of cells removed from culture flasks with EDTA treatment (A) and the ^{35}S-labeled glycoproteins released into the EDTA supernatant (B) were dialyzed and chromatographed on DEAE-cellulose (chloride form) in 0.05 M Tris buffer containing 2 M urea. The labeled glycoproteins were eluted with a linear gradient of 0–0.25 M NaCl (beginning at the arrow).

distribution of ^{35}S-labeled glycoproteins between the cells and EDTA supernatant was variable, depending on the type of plastic culture flask and cell growth conditions used, but usually at least 50% of these cell-surface ^{35}S-labeled glycoproteins were released into the EDTA-containing supernatant. When the plastic-associated endothelial cell matrix was examined after EDTA-mediated cell release, no ^{35}S-labeled glycoproteins were found although this matrix still contained 20–30% of the total ^{35}S-labeled glycosaminoglycans synthesized by the cell culture.

The cell-surface ^{35}S-labeled glycoproteins could be extracted from EDTA-treated cells with 0.05% octyl glucoside and partially purified by DEAE-cellulose (chloride form) chromatography as shown in Figure 6A. Many ^{35}S-labeled glycoprotein peaks are eluted by salt concentrations up to 0.2 M NaCl. Figure 6B shows the comparative behavior on

DEAE-cellulose of the ^{35}S-labeled glycoproteins released from the cells by EDTA. When the ^{35}S-labeled glycoproteins recovered from DEAE-cellulose were dialyzed, lyophilized, and subjected to analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis (data not shown), several major ^{35}S-labeled glycoproteins from the cell surface were observed with apparent molecular weights of between 45 000 and 200 000. Negligible ^{35}S-labeled glycoproteins remained associated with the cell matrix after endothelial cells were released with EDTA treatment. The EDTA-released class of ^{35}S-labeled glycoproteins is composed of many of the same electrophoretic species as are on the cells, plus some additional components. When similar samples were analyzed by Sephadex G-50 column chromatography following lipid extraction and Pronase digestion, ^{35}S-labeled glycopeptides devoid of any larger ^{35}S-labeled glycosaminoglycans were observed.

Discussion

Vascular endothelial cells from both human and bovine sources incorporated [^3H]glucosamine and $^{35}SO_4$ into the carbohydrate side chains of glycoproteins and proteoglycans. Tunicamycin, an antibiotic which prevents the N-glycosylation of proteins (Struck & Lennarz, 1980), inhibited the incorporation of [^3H]glucosamine and $^{35}SO_4$ into the oligosaccharide chains of glycoproteins, measured as glycopeptides, but had little effect on the synthesis of the ^3H,^{35}S-labeled glycosaminoglycan side chains of proteoglycans. The same ^{35}S-labeled oligosaccharides whose synthesis is tunicamycin sensitive are also stable to cleavage with mild alkali treatment and are, therefore, probably N-glycosidically linked to protein. These endothelial cell sulfated glycoproteins can be isolated both from cultured cells and from the materials which they release or secrete into the culture medium. The sulfated glycoproteins from the culture medium were partially purified by DEAE-cellulose chromatography. The major ^{35}S-labeled glycoproteins from the medium were found to have apparent molecular weights of 24 000–66 000. In contrast, the ^{35}S-labeled glycoproteins from endothelial cell layers were mostly of higher apparent molecular weight (45 000–200 000) and could be extracted from cells and their extracellular matrixes with nonionic detergents.

Endothelial cells and smooth muscle cells synthesized [^3H,^{35}S]heparan sulfate, [^3H]hyaluronic acid, and [^3H,^{35}S]-chondroitin sulfates. The relative synthesis of these components varied with the tissue and species from which the cells were derived as well as with the density at which cultured cells were labeled. Although endothelial and smooth muscle cells both synthesized the various glycosaminoglycans, only endothelial cells incorporated $^{35}SO_4$ into the oligosaccharide side chains of glycoproteins. Glycoproteins and proteoglycans probably significantly contribute to the properties of endothelial cells and to their vascular surface properties. Novel modifications of the carbohydrate side chains of glycoproteins and proteoglycans may have a role in the unique function of vascular endothelial cells.

This report demonstrates that cells of the vascular endothelium have the ability to sulfate a population of N-glycosidically linked glycoproteins. It is known that endothelial cells in culture synthesize and secrete components of a subendothelial extracellular matrix or basal lamina, including fibronectin (Jaffe et al., 1976, 1978) as well as other glycoprotein materials (Johnson & Erdos, 1977). Dunham & Hynes (1978) described the incorporation of $^{35}SO_4$ into the large, external, transformation-sensitive (LETS) glycoprotein in cultures of NIL8 hamster fibroblasts. These authors then suggested the possibility that the sulfate may be attached to a glycoprotein core. It is not known if LETS glycoprotein (fibronectin) is among the ^{35}S-labeled glycoproteins synthesized by endothelial cells, although no major ^{35}S-labeled glycoproteins of approximately 200 000 daltons were seen. In a study using human kidney tumor cells in culture (Heifetz & Prager, 1981), butyrate-induced changes in cell spreading and morphological differentiation were accompanied by a markedly increased synthesis of tumor cell sulfated glycoproteins. When $^{35}SO_4$-labeled human pulmonary arterial endothelial cell cultures were analyzed (Heifetz & Johnson, 1981), 30–60% of the cellular ^{35}S-labeled glycopeptides were associated with components which appeared to be deposited on the basal side of the cell layer and anchored to the plastic substratum. Whether endothelial cell sulfated glycoproteins function as adhesion-mediating molecules is not clear at this time. Carlin et al. (1981) have isolated a $^{35}SO_4$-labeled basement membrane glycoprotein, entactin, from endodermal embryonal carcinoma cells and have also found this sulfated glycoprotein in rat kidney glomeruli. Lemkin & Farquhar (1981) have isolated $^{35}SO_4$-labeled rat kidneys and reported that one-third of the ^{35}S-labeled glycoconjugates from isolated cortex, glomeruli, and glomerular basement membranes are composed of ^{35}S-labeled glycopeptides which are smaller in size than the kidney ^{35}S-labeled glycosaminoglycans when analyzed by Sephadex G-50 chromatography. Investigations are in progress to further identify the endothelial cell glycoproteins that are sulfated.

The inability of smooth muscle cell cultures to sulfate a population of N-linked glycoproteins may be related to their state of differentiation in culture (Chamley et al., 1977); however, freshly explanted intima media tissues did not incorporate $^{35}SO_4$ into oligosaccharides even though these tissues were active in synthesizing ^{35}S-labeled glycosaminoglycans. It is possible that sulfation of oligosaccharide chains may be a part of a processing scheme (Kornfeld et al., 1978; Zilberstein et al., 1980) for some cell-surface extracellular glycoprotein products of vascular endothelial cells. It is not currently known if sulfated carbohydrates on glycoproteins made via the oligosaccharide–lipid pathway serve as recognition signals in cell-surface or intracellular interactions, as do terminal galactosyl or phosphorylated mannosyl residues (Neufeld & Ashwell, 1980; Freeze & Miller, 1980), or whether sulfation plays a role in the regulation of the turnover (Mian et al., 1979) of these endothelial cell glycoproteins. The biosynthesis of sulfated glycoproteins by vascular endothelial cells but not by smooth muscle cells may be an important difference relating to the thombogenicity pattern observed in these two cell types.

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O-Phosphoserine Content of Intermediate Filament Subunits†

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ABSTRACT: Purified subunits of intermediate filaments obtained from a variety of tissues and cell types contain *O*-phosphoserine and, in some cases, smaller amounts of *O*-phosphothreonine. The *O*-phosphoserine content was estimated by reaction of performic acid oxidized subunits with methylamine in NaOH. Decamin of BHK-21 and CHO fibroblasts contained about 1 mol/mol. Avian and mammalian desmin consists of two subunits, an acidic (α) subunit which contained 2 mol/mol and a more basic (β) nonphosphorylated subunit. The principal (M_r ~60 000) subunit of squid brain neurofilaments contained 5 mol/mol. Most mouse and bovine keratin subunits contained 3–6 mol/mol, although certain bovine subunits of higher molecular weight contained none. The *O*-phosphoserine contents of keratin subunits purified from the viable and stratum corneum layers were the same. The *O*-phosphoserine was located in non-α-helical regions of the subunits which presumably project out from the α-helical wall of the intermediate filaments. Most subunits could be partially dephosphorylated in vitro with alkaline phosphatase. It was found that the capacity of such partially dephosphorylated subunits for assembly into native-type filaments in vitro was independent of their phosphate content.

Intermediate filaments, microfilaments, and microtubules and their associated proteins constitute the three major classes of cytoskeletal proteins of eukaryote cells. Of these three classes, the intermediate filaments of different cells are the least conserved in terms of their subunit complexity and properties (Goldman et al., 1979; Lazarides, 1980; Zackroff et al., 1981). Nevertheless, intermediate filaments appear to be structurally homologous fibrous proteins (Steinert et al., 1978, 1980a).

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