SECRETED GLYCOPROTEINS OF HUMAN KIDNEY TUMOUR CELLS CONTAIN SULPHATED COMPLEX-TYPE OLIGOSACCHARIDES

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SUMMARY

Human kidney tumour cells in culture incorporated [3H]glucosamine and 35SO4 into glycoprotein products, which were secreted into the culture medium. The effects of sodium butyrate, a known differentiation-inducing agent, on the production of these sulphated glycoproteins were studied. Cells were cultured in the absence or presence of butyrate (2 mM) in serum-containing medium, for various times, and the labelled glycoproteins were partially purified by DEAE-cellulose chromatography. Treatment of these cells with butyrate resulted in an increase in the synthesis of secreted [3H]glucosamine- and 35SO4-labelled glycoproteins over several days of culture. This same increase in levels of 35SO4 incorporation was not observed with B16 melanoma cells. Sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis revealed that five major glycoproteins labelled with [3H]glucosamine also were labelled with 35SO4. The major secreted glycoproteins from cells cultured in the absence or presence of butyrate over a 3-day period were similar by SDS/polyacrylamide gel electrophoresis. Analyses of Pronase-derived glycopeptides indicated that these secreted 3H/35S-labelled glycoproteins contained sulphated oligosaccharides with terminal sialic acid→Gal→GlcNAc residues similar to glycoproteins secreted by vascular endothelial cells.

INTRODUCTION

During the investigation of chemically induced differentiation by butyrate treatment of human kidney tumour cells (Heifetz & Prager, 1981), we observed an incorporation of 35SO4 into glycopeptides prepared from secreted glycoproteins. Reports concerning the incorporation of 35SO4 into a variety of glycoproteins secreted by epithelial and endothelial cells in culture have increased dramatically in recent years (Heifetz, Watson, Johnson & Roberts, 1982; Hogan, Taylor, Korkinen & Couchman, 1982, and references therein). Many of these glycoproteins seem to be distinct from mucin or proteoglycan glycoconjugates. However, only a few investigators (Prehm, Scheid & Choppin, 1979; Parsons & Pierce, 1980; Anumula & Bahl, 1983; Heifetz et al. 1982) have biochemically characterized the type of substituent into which the 35SO4 was incorporated. In these cases the sulphation site was an N-acetylhexosamine on an asparagine-linked oligosaccharide. It is possible that sulphation of ASN-linked oligosaccharides is common to a variety of secretory products. In this report we demonstrate that the major [3H]glucosamine-labelled secretory glycoproteins of human kidney tumour cells in culture contain SO4 incorporated into complex-type oligosaccharides and that the synthesis and secretion of these major
glycoproteins are markedly increased in cells cultured for several days in the presence of butyrate.

**MATERIALS AND METHODS**

Human kidney tumour cells were maintained in culture in Dulbecco's Minimal Essential Medium (Grand Island Biological Co.) containing 15 % (v/v) foetal bovine serum. Cells were treated with 2.0 mM-butyrate and labelled in Ham's F-12 nutrient medium containing foetal bovine serum (Heifetz & Prager, 1981).

Cell cultures were labelled in Ham's F-12 nutrient medium containing 25 μCi/ml of D-[6-3H]glucosamine (25 Ci/mmol, Pathfinder Labs), 250 μCi/ml of H235SO4 (43 Ci/mg), 50 μCi/ml of L-[35S]methionine (400 Ci/mmol, New England Nuclear), or 50 μCi/ml of L-[2,3,4,5-3H]proline (73 Ci/mmol, I.C.N.). The proteins secreted into the culture medium were treated with 1 mM-diisopropylfluorophosphate for 30 min at 4°C, and as described previously (Heifetz et al. 1982), partially purified by chromatography on DEAE-cellulose. Glycoproteins were solubilized in the presence of 40 mM-dithiothreitol and analysed by SDS/polyacrylamide gel electrophoresis on 5 % to 12.5 % (w/v) gradient slab gels according to the method of Laemmli (1970). Cellular DNA was measured by the method of Labarca & Paigen (1980) using a fluorescent DNA-binding dye (Hoechst 33258, Calbiochem) and salmon sperm DNA (Sigma) as a standard.

Glycoproteins were denatured in 0.2 ml Tris buffer (pH 8.0) by heating at 100°C for 10 min, then digested with 2.4 mg Pronase CB (Calbiochem-Behring), which had been preincubated for 30 min at 50°C in 0.2 M-Tris-HCl (pH 8.0) containing CaCl2 (2 mM). Pronase digestions contained 2 mM-CaCl2, 10 mM-NaF, 50 mM-Na2SO4, 4 % (v/v) ethanol and 1 drop of toluene. Fresh preincubated Pronase was added at 24 h. After 48 h the digestes were lyophilized and stored at −20°C. The resulting glycopeptides were then analysed by Sephadex G-50 column chromatography (Heifetz et al. 1982). Glycopeptides were digested with neuraminidase (Clostridium perfringens, affinity-purified, Sigma), β-galactosidase (jackbean, Sigma), β-N-acetylglucosaminidase (jackbean, Sigma), α-mannosidase (jackbean, Sigma) and peptide:N-glycosidase as previously described (Heifetz et al. 1982). Peptide:N-glycosidase was purified from almond emulsin (Sigma) by the method of Tarentino & Plummer (1982).

**RESULTS**

**Secreted glycoproteins**

Human kidney tumour cells cultured in the absence or presence of 2 mM-butyrate for 24 h were incubated with [3H]glucosamine and 35SO4 for 18 h. The labelling medium was used to isolate the glycoproteins synthesized and secreted by these cells. The labelled glycoproteins were concentrated by precipitation by adding an equal volume of 20 % (v/v) trichloroacetic acid at 4°C followed by centrifugation at 10,000 g for 30 min. The glycoproteins were redissolved in 0.05 M-Tris (pH 8.0), and chromatographed on DEAE-cellulose. As shown in Fig. 1a, the 3H/35S-labelled glycoproteins (GP) were eluted as a broad peak with about 0.2 M-NaCl whereas the 3H/35S-labelled proteoglycans (PG) were eluted with salt concentrations greater than 0.3 M. Fig. 1a shows a similar separation of 3H/35S-labelled glycoproteins isolated from cells cultured in the presence of butyrate. Butyrate treatment decreased the level of 3H/35S-labelled proteoglycans released from the cell layer and recovered in the medium, and increased the synthesis 3H/35S-labelled glycoproteins.

As shown in Fig. 2, which represents five separate experiments whose results differed by less than 10 %, culture of cells in the presence of butyrate over a period
Fig. 1. DEAE-cellulose chromatography of $^3$H/$^{35}$S-labelled glycoconjugates isolated from cell culture medium. The $^3$H/$^{35}$S-labelled glycoconjugates in the medium of cell cultures labelled with $[^3$H]glucosamine (O—O) and $^{35}$SO$_4$ (●—●) were isolated by precipitation in 10% trichloroacetic acid. These labelled glycoconjugates were dialysed and then chromatographed on a 1.5 cm x 10 cm column of DEAE-cellulose (chloride form) in 0.05 M Tris buffer (pH 8.0). The glycoconjugates were eluted with a 200 ml linear gradient of 0 to 0.5 M NaCl. After the radioactivity in each 2 ml fraction was determined, materials in the glycoprotein (GP) fractions and proteoglycan (PG) fractions were pooled.

A. $^3$H/$^{35}$S-labelled glycoconjugates from cell cultures.
B. $^3$H/$^{35}$S-labelled glycoconjugates from cell cultures treated with butyrate.
Fig. 2. Effect of butyrate on the incorporation of radioactivity into glycoproteins. The incorporation of \[^{3}H\]\textit{glucosamine} (○–○, □–□) and \[^{35}S\]O\textsubscript{4} (●–●, ■–■) in glycoproteins was determined. Cell cultures were maintained in the absence (○–○, ■–■) or presence (□–□, ■–■) of 2 mM butyrate for the indicated times and then labelled for 18 h in the continued absence or presence of butyrate.

Table 1. Increase in levels of \[^{35}S\]O\textsubscript{4} incorporated into glycoproteins by cells cultured in the presence of butyrate

<table>
<thead>
<tr>
<th>Cells</th>
<th>Butyrate addition</th>
<th>Days in culture</th>
<th>Glycoproteins secreted into medium ((\text{c.p.m.} \times 10^{-3}/\text{dish}))^†</th>
<th>Ratio [^{3}H : {^{35}S}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HKT*</td>
<td>–</td>
<td>2</td>
<td>34-3, 1-2</td>
<td>29</td>
</tr>
<tr>
<td>HKT</td>
<td>+</td>
<td>2</td>
<td>53-1, 2-5</td>
<td>22</td>
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<tr>
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<td>–</td>
<td>2</td>
<td>35-5, 2-3</td>
<td>16</td>
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<tr>
<td>HKT</td>
<td>+</td>
<td>2</td>
<td>45-0, 3-7</td>
<td>12</td>
</tr>
<tr>
<td>B16*</td>
<td>–</td>
<td>2</td>
<td>10-6, 0-4</td>
<td>27</td>
</tr>
<tr>
<td>B16</td>
<td>+</td>
<td>2</td>
<td>19-0, 0-4</td>
<td>48</td>
</tr>
<tr>
<td>HKT</td>
<td>–</td>
<td>4</td>
<td>48, 5-1</td>
<td>9</td>
</tr>
<tr>
<td>HKT</td>
<td>+</td>
<td>4</td>
<td>42, 11-6</td>
<td>4</td>
</tr>
</tbody>
</table>

*HKT (human kidney tumour cells; B16, B16 melanoma cells.
†Cell cultures in sets plated at the same cell density (20% confluent, 50 μg cellular DNA/flask) were grown in the absence or presence of butyrate and labelled under identical conditions in each experiment. The level of radioisotope precursor varied.
of 3 days markedly increases the synthesis and sulphation of secreted kidney tumour
glycoproteins per μg of cellular DNA. Table 1 indicates that in addition to increased
synthesis, the relative level of $^{35}$SO$_4$ to $[^3]$Hglucosamine incorporated into
glycoproteins also increased in these cells. Similar effects of butyrate upon sulphation
were not observed in B16 melanoma cells (Table 1) or in human vascular smooth
muscle cells or human foreskin fibroblasts (data not shown).

Glycoprotein analysis

Glycoproteins labelled with either $[^3]$Hglucosamine or $^{35}$SO$_4$ were isolated and
partially purified by DEAE-cellulose chromatography. The glycoproteins were then

![Fluorogram](image)

Fig. 3. Fluorogram of reducing SDS/polyacrylamide slab gel electrophoresis. 
(a) Glycoproteins labelled with $[^3]$Hglucosamine (lane 1) and $^{35}$SO$_4$ (lane 2) from butyrate-
treated cells analysed on a 7.5% to 12.5% gel. (b) Glycoproteins labelled with $[^3]$H-
glucosamine (lanes 3–6) or $^{35}$SO$_4$ (lanes 7, 8) from cells grown in the absence (lanes 3, 5, 7) or presence (lanes 4, 6, 8) of butyrate for 2 days (lanes 3, 4, 8) or 3 days (lanes 5–7) prior
to labelling for 20 h. Glycoproteins were analysed on a 5% to 12.5% gel. Labelled bands
were detected by fluorography. Numbers at the right of the figure indicate molecular
weight values ($\times10^{-3}$) of protein standards.
Fig. 4. Effect of butyrate upon secreted proteins. Cell cultures were grown in the absence (lanes 1, 3, 5, 7) or presence (lanes 2, 4, 6, 8) of butyrate for 3 days and then labelled for 18 h with [35S]methionine (lanes 1, 2), [3H]proline (lanes 3, 4), 35SO4 (lanes 5, 6) or [3H]glucosamine (lanes 7, 8). The secreted glycoproteins were isolated and analysed on a SDS/5% to 12-5% gradient polyacrylamide gel after reduction with 10% mercaptoethanol. Protein bands were detected by fluorography. The molecular weights (x 10^-3) of protein standards (●) are indicated. Arrowheads indicate positions of major 35SO4-labelled glycoproteins also seen in Fig. 3.

dialysed against water, lyophilized, and analysed by reducing SDS/polyacrylamide (Fig. 3a, 7-5% to 12% gradient) gel electrophoresis. As shown in Fig. 3a, the major 3H-labelled glycoproteins secreted by human kidney tumour cells (lane 1) appear to be also sulphated (lane 2). Comparison (Fig. 3b, 5% to 12-5% gradient gel) of [3H]glucosamine-labelled glycoproteins synthesized and secreted by cells cultured for up to 4 days in butyrate (Fig. 3b, lanes 3–6) indicated that butyrate (Fig. 3b, lanes 4, 6) markedly increased the synthesis of secretory glycoproteins of similar subunit sizes compared to glycoproteins produced by cells grown in the absence of butyrate (Fig. 3b, lanes 3, 5). In addition, the same major glycoproteins (Fig. 3b, lanes 7 and 8) are sulphated with longer times of culture. Additional analysis of total secretory
proteins (Fig. 4) revealed that these sulphated glycoproteins corresponded to several major secretory protein products labelled with $^{35}\text{S}\text{[methionine, having apparent}$

molecular weights of 100, 90, 65 and 50($\times 10^3$), respectively.

Glycopeptide analysis

Since the presence of $^{35}\text{SO}_4$ was common to many major glycoproteins (Figs 3, 4) secreted by these cells, it was important to characterize the moiety that was sulphated. Cells were labelled in the presence of $^{3}\text{H}\text{glucosamine and }^{35}\text{SO}_4$ and

Fig. 5. Sephadex G-50 gel filtration chromatography of $^3\text{H}/^3\text{S}-\text{labelled glycopeptides.}$ $^3\text{H}\text{glucosamine (O- - O) and }^{35}\text{SO}_4$ (● ●) labelled glycopeptides from butyrate-treated cell cultures were prepared and chromatographed on a 1 cm X 110 cm column of Sephadex G-50 and 1-ml fractions were collected. A. The $^3\text{H}/^3\text{S}-\text{labelled glycopeptides in each case were recovered and treated sequentially with neuraminidase (s), $\beta$- galactosidase+ $\beta$-N-acetylglucosaminidase (c). The }^3\text{H}/^{35}\text{S}-\text{labelled glycopeptides (c, fractions 62–86) were then treated with almond peptide N'-glycosidase (d). The elution position of blue dextran (V o) is indicated.
the $^3$H/$^{35}$S-labelled glycoproteins secreted into the medium were partially purified by chromatography on DEAE-cellulose as described above. The glycoproteins were then digested with Pronase and the resulting $^3$H/$^{35}$S-labelled glycopeptides were partially purified by Sephadex G-50 column chromatography (Heifetz et al. 1982). No $^{35}$SO$_4$ was found associated with non-glycosylated peptides by either ion-exchange or high-voltage electrophoretic analysis (Heifetz, Kinsey & Lennarz, 1980). The $^3$H/$^{35}$S-labelled glycopeptides were composed of mild alkali-stable (pH 13-0, 37°C, 24 h) oligosaccharide chains (Heifetz & Prager, 1981). The partially purified $^3$H/$^{35}$S-labelled glycopeptides were reanalysed by Sephadex G-50 chromatography as shown in Fig. 5. These $^3$H/$^{35}$S-labelled glycopeptides did not bind to Concanavalin A/Sepharose and were not degraded by treatment with endoglycosidase H. In order to characterize these $^3$H/$^{35}$S-labelled glycopeptides generally, the partially purified $^3$H/$^{35}$S-labelled glycopeptides (Fig. 5A) were subjected to treatment with neuraminidase to remove bound sialic acid and then were rechromatographed (Fig. 5B). The release of 13-17% of the label as free $[^3]$H sialic acid was accompanied by a decrease in size of the $^3$H/$^{35}$S-labelled glycopeptides. The identity of the neuraminidase-released $^3$H-labelled compound as sialic acid was confirmed by gel filtration chromatography on Biogel P-2, ion-exchange chromatography on DEAE-cellulose (acetate form), and by high-voltage paper electrophoresis (115 V/cm) in

![Diagram](image)

**Fig. 6.** Gel filtration of $^3$H/$^{35}$S-labelled glycopeptides secreted from control and butyrate-treated cells cultures. Glycopeptides from $[^3]$H]glucosamine- (O--O) and $^{35}$SO$_4$- (●-●) labelled secreted glycoproteins were analysed by Sephadex G-50 column chromatography. Glycopeptides are from cell cultures grown in the absence (a, c, e) or presence (b, d, f) of butyrate for 1 day (a, b) or 3 days (c, d, e, f) prior to labelling. The glycopeptides from c and d were recovered and digested with neuraminidase+β-galactosidase+β-N-acetylglucosaminidase+α-mannosidase for 48 h and reanalysed (e, f). The materials indicated by e and f were recovered for further analyses.
Secretory glycoproteins 129

0.05 M-ammonium acetate buffer (pH 5.0) using authentic sialic acid standards (Heifetz et al. 1980). Similar treatment of these $^{3}H/^{35}S$-labelled asialoglycopeptides with $\beta$-galactosidase plus $\beta$-$N$-acetylglucosaminidase (Fig. 5c), but not with $\beta$-$N$-acetylglucosaminidase alone, resulted in the release of $[^{3}H]$GlcNAc and smaller size $^{3}H/^{35}S$-labelled glycopeptides (Fig. 5c, fractions 62–86) without the release of $^{35}SO_{4}$. Treatment of the resistant glycopeptides with peptide:N-glycosidase further reduced the size of some $^{3}H/^{35}S$-labelled materials, indicating the presence of a susceptible GlcNAc-Asn linkage.

As shown in Fig. 6, butyrate treatment produced not only an increased incorporation of labelled precursors into glycopeptides but also produced a change in the relative proportions of the $^{3}H/^{35}S$-labelled glycopeptide species recovered after 1 day (Fig. 6a versus b) or 3 days (Fig. 6c versus d) of culture in the presence of butyrate before labelling. Fig. 6 also shows the results of combined enzymic digestions of $^{3}H/^{35}S$-labelled glycopeptides synthesized by cells grown in the absence (Fig. 6e) or presence (Fig. 6f) of butyrate. The results indicate that under both conditions oligosaccharides appear to contain sulphate incorporated into internal core residues not released with exoglycosidases, which degrade complex-type oligosaccharide structures terminating in sialic acid, galactose and $N$-acetylglucosamine residues.

Combined treatment with neuraminidase, $\beta$-galactosidase, $\beta$-$N$-acetylglucosaminidase and $\alpha$-mannosidase released 36% of the radioactivity from glycopeptides labelled in the absence of butyrate (Fig. 6e) and 46% of the radioactivity from glycopeptides labelled in the presence of butyrate (Fig. 6f). Analyses of the resistant glycopeptide-core materials (Fig. 6e versus f) indicated that the relative $^{3}H$: $^{35}S$ ratios were 9.7 for control and 2.9 for butyrate-treated samples. Thus the inner-core sugar residues of glycopeptides from butyrate-treated cells display a three- to fourfold enrichment of incorporated $^{35}SO_{4}$.

**DISCUSSION**

Sodium butyrate has been used recently to induce the synthesis of various proteins in tumour cell lines, although the exact mechanism of butyrate action has not been determined. Our studies on the effect of sodium butyrate upon human renal carcinoma cells indicated that butyrate markedly increased the level of incorporation of both $[^{3}H]$glucosamine and $^{35}SO_{4}$ into secreted glycoproteins. Cell cultures treated with 2 mM-butyrate grew at 80–90% of the rate of cells cultured in the absence of butyrate and incorporated relatively less $^{35}SO_{4}$ into proteoglycans. Therefore, butyrate does not appear to increase $^{35}SO_{4}$ incorporation generally into all classes of glycoconjugates but appears to increase the synthesis of glycoprotein products.

Sodium butyrate has been reported to change the relative synthetic rate of specific proteins in HeLa cells (Tallman, Smith & Henneberry, 1977), and of both cellular and secreted carcinoembryonic antigen in human colorectal tumour cells (Tsao, Shi, Wong & Kim, 1983). Recently, Morrow, Weintraub & Rosen (1983) reported that 2 mM-butyrate was synergistic with low glucose concentration in reversing the effects of 'glucose-starvation' upon protein glycosylation and increased the total amount of
α-subunit of the glycoprotein hormones secreted in Chang liver cells. Via, Sramek, Larriba & Steiner (1980) reported that butyrate-treated murine sarcoma virus-transformed newborn rat kidney cells did not have significantly changed hexose uptake rates compared to those of the non-treated transformed cells. Our studies indicated that the levels of glucose remaining in the culture medium (3 mm) were the same for cells cultured for 2 days in the absence or presence of butyrate. Thus butyrate does not appear to alter glucosamine incorporation by markedly altering hexose pool sizes.

The human kidney tumour cells used in this study assumed a more flattened and spread morphology with more clearly defined cell borders after butyrate treatment (Heifetz & Prager, 1981). Many of the major proteins secreted by these cells in culture were glycoproteins and higher levels of these same glycoprotein products were secreted by butyrate-treated cells. The secreted glycoproteins contained asparagine-linked, complex-type, sulphated oligosaccharide chains. These oligosaccharides appear to be sulphated on the inner-core residues, similar to sulphated oligosaccharides previously characterized from human vascular endothelial cells (Heifetz et al. 1982). Recently, a major secreted protein of transformed mouse fibroblasts was found to contain phosphorylated, high-mannose-type, oligosaccharides (Sahagian & Gottesman, 1982). Thus phosphorylated and sulphated oligosaccharides may contribute to the properties of tumour cell secretory glycoproteins used as neoplastic indicators. Hsu & Kingsbury (1982) have implicated differential oligosaccharide sulphation along with sialylation as causes of vesicular stomatitis virus charge heterogeneity. Synthesis of sulphated glycoproteins has now been reported for a variety of tissues (Yonekura et al. 1982; Heifetz et al. 1980; and references therein) and cell types (Hogan et al. 1982; Anumula & Bahl, 1983; Heifetz et al. 1982). Butyrate treatment of human kidney tumour cells produced a three- to fourfold increase in 35SO4 incorporation and stimulated the synthesis of a sulphated glycopeptide that was intermediate in size as found by gel filtration analysis. Similar changes in fucose-labelled glycopeptides were observed after butyrate treatment of transformed rat cells (Via et al. 1980). Thus butyrate is a useful agent in the study the cellular regulation of various post-translational modifications such as oligosaccharide sulphation.

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REFERENCES


Secretory glycoproteins


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