Sulphation of N-linked oligosaccharides of vesicular stomatitis and influenza virus envelope glycoproteins: host cell specificity, subcellular localization and identification of substituted saccharides

Velislava K. KARAIVANOVA and Robert G. SPIRO[1]

Departments of Biological Chemistry and Medicine, Harvard Medical School, and Joslin Diabetes Center, One Joslin Place, Boston, MA 02215, U.S.A.

The presence of sulphate groups on various saccharide residues of N-linked carbohydrate units has now been observed in a number of glycoproteins. To explore the cell specificity of this post-translational modification, we evaluated sulphate incorporation into virus envelope glycoproteins by a variety of cells, since it is believed that assembly of their N-linked oligosaccharides is to a large extent dependent on the enzymic machinery of the host. Employing the vesicular stomatitis virus (VSV) envelope glycoprotein (G protein) as a model, we noted that the addition of [^{35}S]sulphate substituents into its complex carbohydrate units occurred in Madin–Darby canine kidney (MDCK), Madin–Darby bovine kidney, LLC-PK$_1$ and BHK-21 cell lines but was not detectable in BRL 3A, BW5147.3, Chinese hamster ovary, HepG2, NRK-49F, IEC-18, PtK1 or 3T3 cells. The sulphate groups were exclusively located on C-3 of galactose [Gal(3-SO$_4$)] and/or C-6 of *N*-acetylglucosamine [GlcNAc(6-SO$_4$)] residues in the *N*-acetyllactosamine sequence of the branch chains. Moreover, we observed that the pronounced host-cell-dependence of the terminal galactose sulphation was reflected by the 3′-phosphoadenosine 5′-phosphosulphate:Gal-3-*O*-sulphotransferase activity assayed *in vitro*. Comparative studies carried out on the haemagglutinin of the influenza virus envelope formed by MDCK and LLC-PK$_1$ cells indicated that sulphate in this glycoprotein was confined to its complex N-linked oligosaccharides where it occurred as Gal(3-SO$_4$) and GlcNAc(6-SO$_4$) on peripheral chains as well as on the mannose-substituted *N*-acetylglucosamine of the core. Since sulphation in both internal and peripheral locations of the virus glycoproteins was found to be arrested by the $\alpha 1 \rightarrow 2$ mannosidase inhibitor, kifunensine, as well as by the intracellular migration block imposed by brefeldin A, it was concluded that this modification is a late biosynthetic event which most likely takes place in the *trans*-Golgi network.

INTRODUCTION

The occurrence of sulphate substituents on the N-linked oligosaccharides of a variety of secreted [1–4] as well as membrane-associated cell surface [5,6] and virus envelope [7–10] glycoproteins is now well documented. Moreover, a beginning has been made in characterizing the sulphotransferases involved in attaching these anionic groups to specific positions on galactose [11], *N*-acetylgalactosamine [12] and *N*-acetylglucosamine [13] residues located in the branches of complex carbohydrate units. Although in mammalian cells the complex-type N-linked oligosaccharides have in common a $Man_3GlcNAc_2$ core, which is the result of extensive processing of a co-translationally transferred glucosylated polymannose precursor [14,15], they can achieve almost limitless diversity through the subsequent action of a large number of Golgi-situated saccharide and, in certain instances, sulphate-transfer enzymes.

It has become apparent that, to a large extent, the structure of the mature N-linked complex carbohydrate unit is dictated by the enzymic glycosylation machinery characteristic of the cell involved in its biosynthesis so that the oligosaccharides present even on an identical genomic polypeptide could differ among cell types (see [16] for a review). This cell-specific glycosylation should be clearly demonstrable from studies of virus envelope glycoproteins as the synthesis of their oligosaccharide chains is believed to be predominantly dependent on the enzymic equipment of the host cell, and indeed this has already been shown for several structural aspects of N-linked oligosaccharides [17–20].

The present study was undertaken to extend the examination of the host-cell–virus relationship to the sulphation of oligosaccharides occurring on envelope glycoproteins. Since addition of sulphate to N-linked carbohydrate units is a relatively uncommon phenomenon and occurs in a highly specific manner on a number of defined positions of several sugar constituents, we believed that this event could serve as a rewarding model for the exploration of cell-specificity. We chose the vesicular stomatitis virus (VSV) for these investigations as this pathogen has the capacity to infect and multiply readily in a large variety of cells and, moreover, produces a well-defined envelope glycoprotein (G protein) which bears two complex N-linked oligosaccharides the major structural features of which have been known for some time [21]. Indeed the selection of this virus for study proved to be fortunate as we observed that glycoprotein sulphation was clearly host-cell-dependent and occurred exclusively on the C-3 and C-6 positions of the galactose and *N*-acetylglucosamine residues respectively, present on the branch chains. Furthermore, the sulphation correlated well with measurement of sulphotransferase activity *in vitro*. Comparative studies carried out on

Abbreviations used: VSV, vesicular stomatitis virus; G protein, VSV glycoprotein; HA, haemagglutinin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MDBK, Madin-Darby bovine kidney; MDCK, Madin–Darby canine kidney; KIF, kifunesine; BFA, brefeldin A; endo H, endo-β-*N*-acetylglucosaminidase; PNGase, peptide:*N*-glycosidase; PAPS, 3′-phosphoadenosine 5′-phosphosulphate; Gal(3-SO$_4$), sulphate group located on C-3 of galactose; GlcNAc(6-SO$_4$), sulphate group located on C-6 of N-acetylglucosamine; AnManH$_2$, 2,5-anhydromannitol; AnMan(6-SO$_4$), sulphate group located on C-6 of AnManH$_2$; HIV, human immunodeficiency virus.

[1] To whom correspondence should be addressed.

the influenza virus haemagglutinin (HA) indicated that sulphation of its complex oligosaccharides occurred in these peripheral locations as well as in its core. Through the use of transport and processing inhibitors, it became apparent that these virus oligosaccharide sulphations are a late *trans*-Golgi-network-localized biosynthetic event.

EXPERIMENTAL

Cell culture

All cell lines were purchased from A.T.C.C. (Rockville, MD, U.S.A.). The cells were grown in the following media: Dulbecco's modified Eagle's medium (DMEM) containing 1.0 g/l glucose and supplemented with 10% (v/v) fetal bovine serum (FBS) for Madin–Darby bovine kidney (MDBK) cells and 3T3-Swiss albino cells; DMEM containing 4.5 g/l glucose and supplemented with 5% (v/v) FBS for Madin–Darby canine kidney (MDCK) cells; Medium 199 (GIBCO) supplemented with 3% (v/v) FBS for LLC-PK_1 pig kidney cells; DMEM containing 4.5 g/l glucose and supplemented with 10% tryptose phosphate broth (DIFCO) and 10% (v/v) FBS for BHK-21 baby hamster kidney cells; DMEM containing 4.5 g/l glucose and supplemented with 5% (v/v) FBS for NRK-49F normal rat kidney fibroblast cells; RPMI-1640 (GIBCO) supplemented with 10% (v/v) FBS for HepG2 cells; Ham's F-12 (GIBCO) supplemented with 10% (v/v) FBS for Chinese hamster ovary cells (CHO); DMEM containing 4.5 g/l glucose and 0.1 unit/ml insulin supplemented with 5% (v/v) FBS for IEC-18 rat ileum epithelial cells; DMEM containing Earle's balanced salt solution and supplemented with 10% (v/v) FBS for PtK1 kangaroo rat kidney cells; DMEM containing 4.5 g/l glucose and supplemented with 10% (v/v) FBS for BW5147.3 mouse lymphoma cells; and Coon's modified F12 medium (Sigma) supplemented with 5% (v/v) FBS for BRL 3A buffalo rat liver cells. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were included in all cell cultures which were carried out at 37 °C in an atmosphere of 95% air and 5% CO_2 on 60- or 100-mm dishes (Falcon), with the exception of BW5147.3 cells, which were grown in suspension.

Virus infection and radioactive labelling of cells

VSV, Indiana strain, was obtained from A.T.C.C. and propagated in BHK-21 cells; the stock virus (3×10^9 plaque forming units/ml) was stored at −80 °C. Influenza virus, PR8/MS, (2.4×10^{11} plaque forming units/ml) was generously given by Dr. Karl S. Matlin (Massachusetts General Hospital, Boston, MA, U.S.A.). The cultured cells were infected with either virus at a multiplicity of infection of 20–40 plaque forming units/cell.

Cell monolayers (90% confluent) on 60-mm or 100-mm dishes or in suspension (1×10^7 BW5147.3 cells on 60-mm dishes) were infected with VSV or influenza virus and after 3 h or 5 h, respectively, incubated with methionine or sulphate-free DMEM containing 3% (v/v) dialysed FBS for 30 min. Fresh carrier-free medium was then added for an additional 30 min incubation with or without specified inhibitors which included 0.18 mM kifunensine (KIF; Toronto Research Chemicals), 1 μg/ml tunicamycin (Sigma) or 5 μg/ml brefeldin A (BFA; Epicentre Technologies). Subsequently the infected cells were metabolically radioactively labelled with 50–75 μCi [^{35}S]methionine (1110 Ci/mmol; DuPont-New England Nuclear) or 400–750 μCi [^{35}S]sulphate (carrier-free; DuPont-New England Nuclear) for 16–18 h. The [^{35}S]methionine labelling was carried out on 60-mm plates (1 ml medium) while the incubations with [^{35}S]sulphate were performed on 100-mm plates (2 ml medium), except for BW5147.3 cells which were suspended on 60-mm plates (1 ml medium). Throughout the radiolabelling period the media were carrier-free and the inhibitors were present. The only exceptions were experiments with BFA in which, after incubation with the radioactive label for 45 min, a 2 h chase with medium containing unlabelled methionine (2 mM) or sulphate (0.8 mM) and including the inhibitor was carried out.

Free virus was harvested from the medium at the end of the incubations by low speed centrifugation (500 ***g*** for 15 min) to remove free cells followed by ultracentrifugation (105000 ***g*** for 120 min). Solubilization of viral pellet proteins was achieved by the addition of 0.2 ml of lysis buffer [100 mM Tris/HCl (pH 7.6)/400 mM NaCl/2% (v/v) Triton X-100 containing a mixture of protease inhibitors] at 4 °C [22]. After centrifugation at 14000 ***g*** for 20 min (Eppendorf, 5415C) the clear supernatant was used for further study.

For the examination of intracellularly-located virus the host cells were scraped from the plates, briefly centrifuged (500 ***g*** for 15 min) and the pellets were disrupted at 4 °C with lysis buffer. Further centrifugation (Eppendorf) yielded a clear supernatant.

Isolation of radioactively-labelled viral glycoproteins and preparation of glycopeptides

Radioactively-labelled G protein and influenza virus HA, were resolved by SDS/PAGE and the bands, revealed by fluorography, were excised from the dried gel and digested with Pronase for 72 h with several additions of the enzyme as described previously [23]. The Pronase-digested virus glycoproteins (10000–200000 d.p.m.) were subsequently applied to Bio-Gel P-2 (1.5 $\times$ 35 cm, 200–400 mesh) columns equilibrated with 0.1 M pyridine acetate, pH 5.0, and were eluted with the same buffer (22 ml/h). Fractions (3 ml) were collected and aliquots of these were monitored for radioactivity by scintillation counting. The fractions containing the [^{35}S]sulphate-labelled glycopeptides from the virus proteins were pooled and freeze-dried to await further analysis.

Hydrazine/nitrous acid fragmentation

The [^{35}S]sulphate-labelled glycopeptides were treated with hydrazine (28 h at 100 °C) followed by treatment with nitrous acid and $NaBH_4$ reduction as described previously [1,24]. After passage of the samples through Dowex 50 (H^+ form) and removal of boric acid as methyl borate, the released radioactively labelled saccharides were examined by TLC. The well characterized ^{3}H-labelled standards were prepared as described previously [1,11,24]. Smith periodate degradation of [^{35}S]sulphate-labelled fragments was carried out as defined previously [1,11].

Glycosidase digestions

Digestion of viral glycopeptides with 4 m-units of endo-β-*N*-acetylglucosaminidase (endo H; Genzyme) and 1 unit of peptide-*N*-glycosidase F (PNGase; Oxford GlycoSystems) was carried out as described previously [25] for 48 h and 24 h, respectively, at 37 °C before SDS/PAGE. Incubation of [^{35}S]sulphate-labelled core oligosaccharide with jack-bean α-mannosidase (1.2 units; Sigma) was at 37 °C for 24 h in 200 μl of 0.15 M sodium acetate buffer, pH 5.0, in the presence of toluene. Digestions with snail β-mannosidase (0.1 unit; Sigma) were carried out under similar conditions. The incubations were terminated by heating at 100 °C for 3 min and the samples were desalted and deproteinized by application to a column (1.0 $\times$ 0.3 cm) of charcoal-Celite (Darco G-60-Celite 535; 1:1, by weight) [26] and following a water wash (12 ml), the oligosaccharides were eluted with 12 ml of 25% ethanol. Radioactively labelled proteins were digested with 0.1

unit of *Clostridium perfringens* neuraminidase (Type V) for 24 h followed by SDS/PAGE.

Assay of sulphotransferase activity

For the determination of 3′-phosphoadenosine 5′-phosphosulphate (PAPS):galactosyl-3-*O*-sulphotransferase activity, cultured cells were harvested by centrifugation (600 ***g*** for 5 min) in ice-cold PBS and, after three washes in PBS, were suspended in 4 vol. of 50 mM Tris/acetate (pH 7.0)/25 mM potassium acetate. Cell disruption was carried out in an ice bath and was achieved by 3×10 s bursts of a Branson sonicator (setting no. 1) equipped with a microprobe. The homogenates were then centrifuged (600 ***g*** for 10 min) and the postnuclear supernatants were further centrifuged at 160000 ***g*** for 60 min to obtain membrane pellets. The pellets were washed and suspended in the homogenization buffer at a protein concentration of about 15 mg/ml. Aliquots of the membranes (75–150 μg of protein) were incubated with 0.3 μCi [^{35}S]PAPS (1.6–2.4 Ci/mmol, DuPont-New England Nuclear) and 80 nmol of *N*-acetyllactosamine as described previously [11]. The products were separated by TLC and revealed by fluorography. The radioactively labelled sulphated product, i.e. Gal(3-SO$_4$)β1-4 → GlcNAc, was quantified by scintillation counting [11].

Immunoprecipitation procedure

To confirm the identity of the radioactively labelled G protein and the HA component of the influenza virus, immunoprecipitations of the lysed viruses were carried out with antisera and Protein A–Sepharose as described previously [27] and subjected to SDS/PAGE. For the VSV studies, rabbit polyclonal serum (5 μl) (generously provided by Dr. John K. Rose, Yale University, New Haven, CT, U.S.A.) was used and HA was immunoprecipitated with a monoclonal antibody (5 μl) (a gift of Dr. Karl S. Matlin, Massachusetts General Hospital, Boston, MA, U.S.A.); in the latter case, incubation with rabbit anti-mouse IgG (Sigma) for 2 h preceded the addition of the Protein A–Sepharose.

SDS/PAGE

Gel electrophoresis was carried out on 10 % polyacrylamide gels (1.5 mm thick) overlaid with a 2.5 % stacking gel in SDS under reducing conditions, according to the procedure of Laemmli [28]; the radioactive components were detected by fluorography.

TLC

Resolution of desalted saccharides was achieved on plastic sheets precoated with cellulose (0.1 mm thickness; Merck) in pyridine/ethyl acetate/water/acetic acid (5:5:3:1, by vol.). The chromatography was performed with a wick of Whatman 3MM paper clamped to the top of the thin layer plates. The components were detected by fluorography and, where indicated, were eluted with water for scintillation counting or for further study as described previously [11].

Protein determination

Protein was quantified using the Bio-Rad dye-binding assay [29] with BSA as a standard.

Radioactivity measurements

Liquid scintillation counting (Beckman LS 7500) was performed in Ultrafluor. Radioactive components on thin layer plates were detected with X-Omatic AR film (Eastman Kodak) after spraying with a scintillation mixture containing 2-methylnaphthalene [30]. Radioactive components on electrophoresis gels were monitored by fluorography at −80 °C, after treatment with ENHANCE (DuPont-New England Nuclear), using X-Omatic AR film and were quantified (within the linear range) by laser densitometry (model 300A, Molecular Dynamics).

RESULTS

Evaluation of the capacity of various cell lines to introduce sulphate into G protein

Since VSV has the facility to infect a wide range of cell lines, we determined the radioactivity incorporated into the G envelope glycoprotein of this virus after incubation of various infected cells with [^{35}S]sulphate and compared it with that observed after labelling with [^{35}S]methionine. Densitometry of the G protein, after resolution from other VSV constituents by SDS/PAGE, revealed that it was sulphated by only a limited number of host cells, all of which were of renal origin and included most prominently the MDCK, MDBK and LLC-PK$_1$ cell lines (Figure 1). As shown in the electrophoretograms (Figure 2) of the virus produced by the latter two cell lines, sulphate

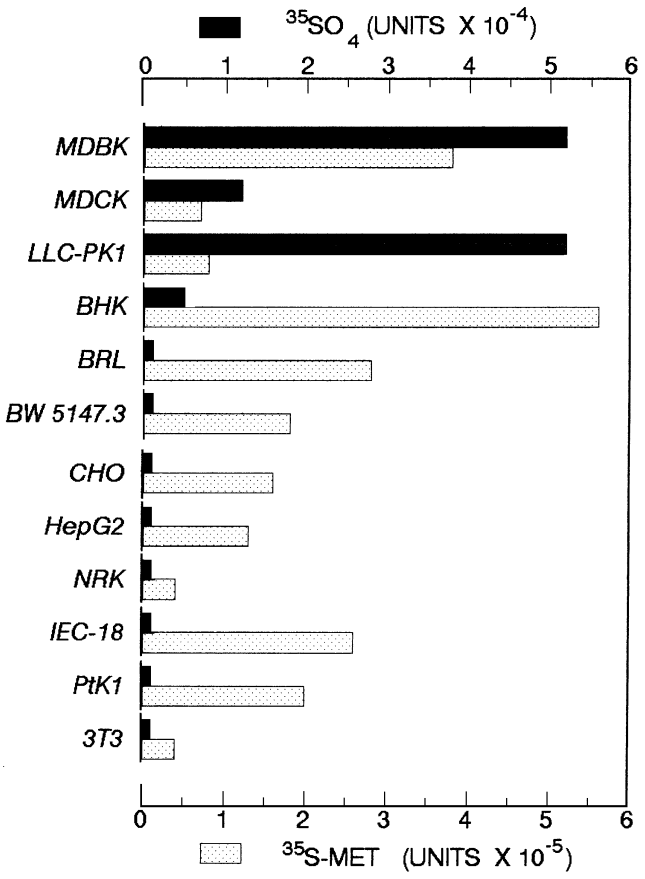

Figure 1 Extent of sulphation of the G protein by various cell lines

The designated infected host cells were incubated with [^{35}S]sulphate (400 μCi) or [^{35}S]methionine (60 μCi) for 16 h as described in the Experimental section. The free virus in the medium was then subjected to SDS/PAGE and after fluorography the radioactivity incorporated into G protein was determined by densitometric scanning. The values for [^{35}S]sulphate (solid bars) and [^{35}S]methionine (stippled bars) represent densitometric units/10^7 infected host cells. See the Experimental section for key to host cell abbreviations.

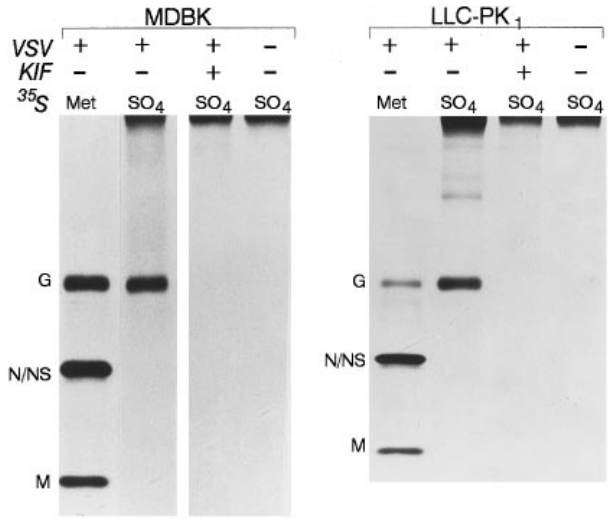

Figure 2 Demonstration that the G protein is sulphated by infected MDBK and LLC-PK1 cells and that this event does not take place during inhibition of N-linked polymannose processing

Infected (+) and uninfected (−) cells were incubated with 500 μCi [^{35}S]sulphate (SO_4) or 75 μCi [^{35}S]methionine (Met) in the absence (−) or presence (+) of KIF (0.18 mM) for 16 h as described in the Experimental section. Subsequently the free virus was harvested from the medium and subjected to SDS/PAGE. The components were visualized by fluorography and the position of the G protein (G) and of the other major VSV constituents are indicated on the left of the gels. The position of the G protein was confirmed by immunoprecipitation as described in the Experimental section. A similar electrophoretic pattern was obtained with MDCK cells.

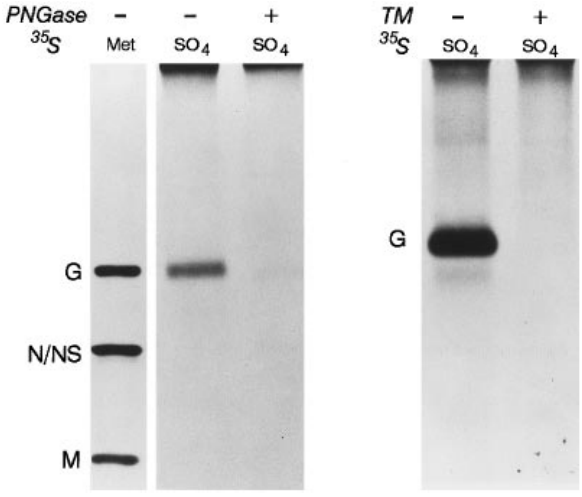

Figure 3 Action of PNGase on the [^{35}S]sulphated G protein produced by MDBK cells and the effect of tunicamycin on the formation of this component

VSV-infected MDBK cells were incubated (18 h) with 400 μCi [^{35}S]sulphate (SO_4) and, after incubation with (+) or without (−) PNGase (left panel), the lysed virus harvested from the medium was subjected to SDS/PAGE as described in the Experimental section. Using a similar procedure, MDBK cells were labelled with [^{35}S]sulphate in the absence (−) or presence (+) of 1 μg/ml tunicamycin (TM) before electrophoretic examination of the virus (right panel). The position of the G protein (G) and other viral components was determined by radiolabelling with [^{35}S]methionine (Met). The bands were revealed by fluorography.

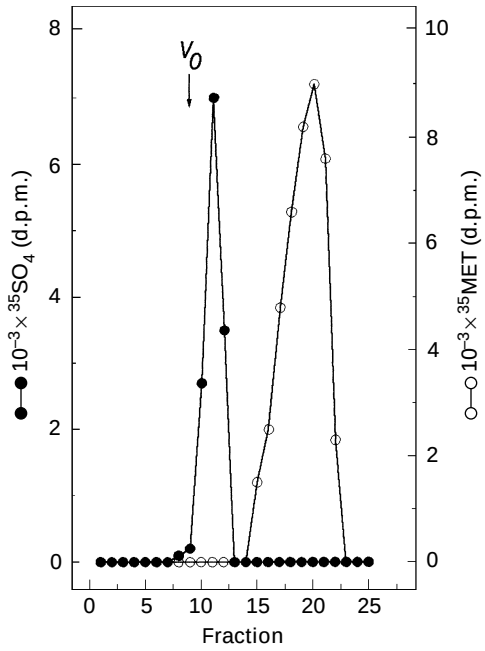

Figure 4 Filtration of Pronase-digested [^{35}S]sulphate- and [^{35}S]methionine-labelled G protein on Bio-Gel P-2

Subsequent to radioactive labelling of MDBK cells with [^{35}S]sulphate or [^{35}S]methionine, the G protein was harvested from the culture medium and isolated by preparative SDS/PAGE. The radioactive bands were excised from the gels and digested with Pronase as described in the Experimental section. The digests from the [^{35}S]sulphate- and [^{35}S]methionine-labelled protein (13 000 and 43 000 d.p.m. respectively) were each applied to a Bio-Gel P-2 column (1.5 × 35 cm) equilibrated with 0.1 M pyridine/acetate buffer, pH 5.0, and eluted with the same buffer at a flow rate of 22 ml/h; 3.0 ml fractions were collected. Aliquots were taken for scintillation counting and the radioactivity of each fraction was plotted. The void volume (V_0) of the column is indicated.

labelling was limited to the G protein and was completely abolished in the presence of KIF, which is known to be a potent inhibitor of $\alpha 1 \rightarrow 2$-mannosidase processing of N-linked oligosaccharides [31,32]. The action of KIF was specific as [^{35}S]methionine-labelling of the glycoprotein remained unchanged in the presence of this reagent (results not shown). The radioactively labelled material observed at the origin of the electrophoretograms has been noted previously in virus-infected cells [33] and is believed to be sulphated proteoglycan associated with fragments of the cell, which are shed into the medium and which co-sediment with the free virus.

Demonstration that sulphation occurs on the N-linked oligosaccharides of the G protein

A number of observations, in addition to the inhibitory effect of KIF already noted (Figure 2), indicated that the sulphate residues were present on N-linked oligosaccharides of the VSV glycoprotein. When VSV-infected MDBK cells were incubated in the presence of tunicamycin, incorporation of sulphate into the G protein was abolished (Figure 3, right panel). Moreover, PNGase treatment of the [^{35}S]sulphated envelope glycoprotein produced under non-inhibitory conditions resulted in essentially complete release of the radioactive label (Figure 3, left panel). On the other hand, digestion with endo H did not result in a decrease in the [^{35}S]sulphate label (results not shown), which would be

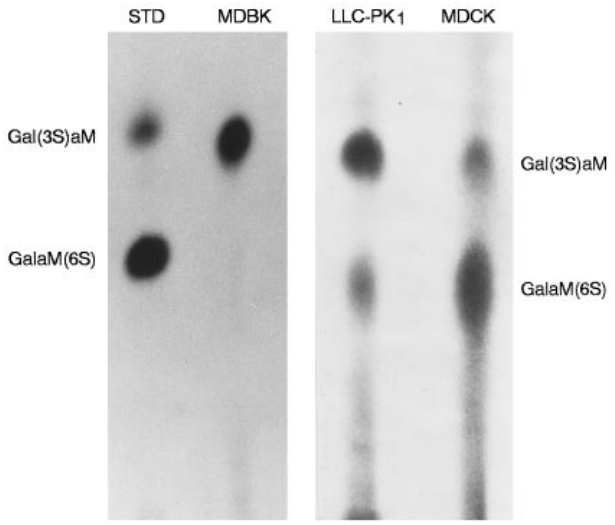

Figure 5 Identification by TLC of the radioactively-labelled saccharides obtained by hydrazine/nitrous acid/$NaBH_4$ treatment of [^{35}S]sulphate-labelled glycopeptides from G protein produced by several cell lines

VSV-infected MDBK, LLC-PK_1 and MDCK cells were labelled with 700 μCi [^{35}S]sulphate for 18 h, the SDS/PAGE-purified G protein was digested with Pronase and the glycopeptide fraction, obtained by Bio-Gel P-2 filtration, was subjected to hydrazine/nitrous acid/$NaBH_4$ fragmentation as described in the Experimental section. An aliquot ($2–5 \times 10^3$ d.p.m.) of the products from this treatment of each cell type was applied to a cellulose-coated TLC plate and was developed for 20 h in pyridine/ethyl acetate/water/acetic acid (5:5:3:1, by vol.). The separated components were visualized by fluorography. The position of Gal(3-SO_4)β(1 → 4)AnManH_2 [Gal(3S)aM] and Galβ(1 → 4)AnMan(6-SO_4) [GalaM(6S)] was determined by co-chromatography of ^{3}H-labelled standards (STD) prepared by hydrazine/nitrous acid/$NaBT_4$ treatment of porcine thyroglobulin glycopeptides.

consistent with its presence in the complex N-linked oligosaccharides of the mature G protein. Similar findings were made in MDCK and LLC-PK_1 cells (results not shown).

Following isolation by preparative SDS/PAGE, the Pronase-digested [^{35}S]sulphate-labelled G protein yielded a single radioactive peak, on Bio-Gel P-2 filtration, which emerged in the void volume of the column and was clearly separated from the peptides resulting from a digest of the protein labelled with [^{35}S]methionine (Figure 4). Similar elution patterns were observed for the G protein from MDBK, MDCK and LLC-PK_1 cells.

Location of sulphate residues on the N-linked oligosaccharides of the G protein

Treatment of the [^{35}S]sulphate-labelled glycopeptides from the G protein by the hydrazine/nitrous acid/$NaBH_4$ procedure yielded a radioactive disaccharide in the three cell types examined, which migrated to the position of Gal(3-SO_4)β(1 → 4)-AnManH_2 and would be derived from the Gal(3-SO_4)β(1 → 4)-GlcNAc sequence. Furthermore, a slower component identified as Galβ(1 → 4)AnMan(6-SO_4), which originates from Galβ(1 → 4)GlcNAc(6-SO_4), was identified in the G protein from LLC-PK_1 and MDCK cells (Figure 5); the ratios of Gal(3-SO_4)β(1 → 4)AnManH_2/Galβ(1 → 4)AnMan(6-SO_4) in these two cell lines were 2.3 and 0.7 respectively. Examination of the hydrazine/nitrous acid fragmentation product from BHK cells revealed solely the Galβ(1 → 4)AnMan(6-SO_4) component (results not shown).

Table 1 Distribution of PAPS:galactosyl-3-*O*-sulphotransferase among various cell lines

The assay of PAPS:galactosyl-3-*O*-sulphotransferase activity was carried out on cultured cell membranes as described in the Experimental section. In BHK-21 cells no activity above background was detected; the sulphation of G protein in BHK cells *in vivo* was shown to occur exclusively in the Galβ1 → 4GlcNAc-6-SO_4 sequence.

Cell type	Enzyme activity (10^{-3} × d.p.m./mg of protein)
MDBK	135
MDCK	108
LLC-PK_1	165
BHK-21	0
BRL	2
BW5147.3	1
NRK-49F	0
HepG2	0
CHO	0

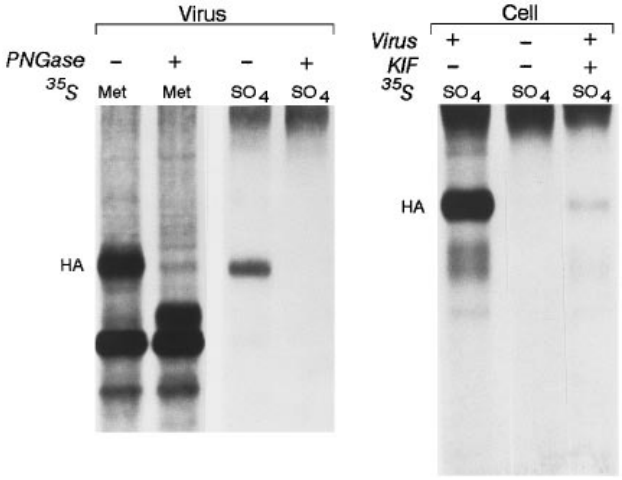

Figure 6 Sulphation of influenza virus HA by MDCK cells and the effects of PNGase on the [^{35}S]sulphated glycoprotein and of KIF on its production

Influenza-virus-infected (+) and non-infected (−) MDCK cells were incubated with 700 μCi [^{35}S]sulphate (SO_4) or 70 μCi [^{35}S]methionine (Met) for 16 h. The virus (Virus) harvested from the medium was subjected to SDS/PAGE after incubation with (+) or without (−) PNGase (left panel), as described in the Experimental section, and lysed cells (Cell) were electrophoresed directly (right panel). The components were visualized by fluorography and the position of HA was determined by immunoprecipitation of the [^{35}S]methionine-labelled virus as described in the Experimental section. The HA component of the virus from the medium was not [^{35}S]sulphate-labelled in the presence of KIF.

Distribution of PAPS:galactosyl-3-*O*-sulphotransferase among various cell lines

Assay of the 3-*O*-sulphotransferase characterized previously [11] in the various host cells indicated that the activity of this enzyme correlated with their capacity for sulphation of the G protein *in vivo* (Table 1). The sulphotransferase was not detectable in the cell lines which failed to incorporate [^{35}S]sulphate into this envelope glycoprotein (compare Table 1 and Figure 1). The only exception was the BHK cell line, which, as already indi-

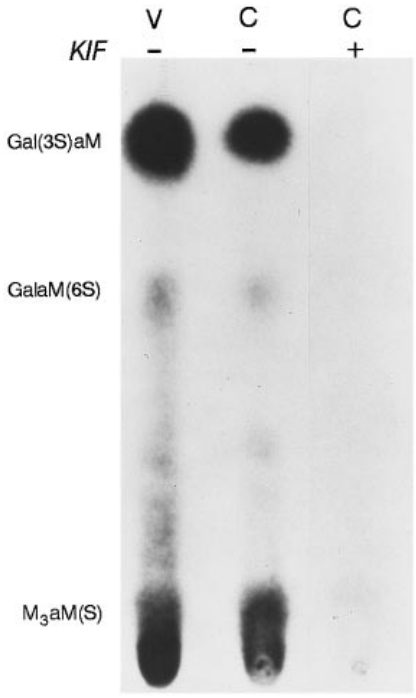

Figure 7 TLC of components obtained by hydrazine/nitrous acid/$NaBH_4$ treatment of [^{35}S]sulphate-labelled glycopeptides from influenza virus HA produced by LLC-PK_1 cells and the effect of KIF on their formation

After LLC-PK_1 influenza-virus-infected cells were labelled with [^{35}S]sulphate (750 μCi) for 18 h in the absence (−) and presence (+) of KIF (0.18 mM), the glycopeptide fraction of the SDS/PAGE-purified HA from the medium virus (V) and the cell lysate (C) was fragmented with hydrazine/nitrous acid/$NaBH_4$ as described in the Experimental section. An aliquot of the products was applied to a cellulose-coated TLC plate and developed in pyridine/ethyl acetate/water/acetic acid (5:5:3:1, by vol.); the components were visualized by fluorography. The positions of Gal(3-SO_4)β(1 → 4)AnManH_2 [Gal(3S)aM] and Galβ(1 → 4)AnMan(6-SO_4) [GalaM(6S)] were determined by co-chromatography of standards. The component designated as Man_3AnManH_2(SO_4) [M_3aM(S)] is believed to be the sulphated core of the complex N-linked oligosaccharides.

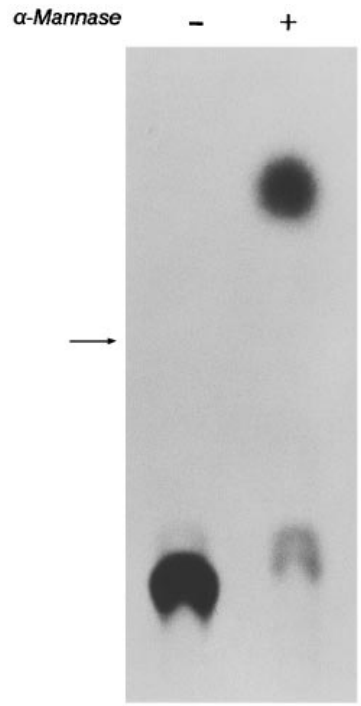

Figure 8 Effect of α-mannosidase on the sulphate core oligosaccharide from the HA N-linked carbohydrate unit

The component, designated as M_3aM(S), which was obtained by hydrazine/nitrous acid/$NaBH_4$ treatment of [^{35}S]sulphate-labelled glycopeptides from influenza virus HA produced by MDCK cells (see Figure 7), was isolated by preparative TLC and incubated with (+) and without (−) jack-bean α-mannosidase as described in the Experimental section. The desalted samples were applied to a cellulose-coated TLC plate and developed for 28 h in pyridine/ethyl acetate/water/acetic acid (5:5:3:1, by vol.). The components were visualized by fluorography; the position of the lactitol standard is indicated by the arrow. Similar results were obtained when M_3aM(S) from LLC-PK_1 cells was treated with α-mannosidase.

cated, introduced sulphate solely on to *N*-acetylglucosamine residues.

Sulphation of N-linked oligosaccharides of influenza virus HA

Since influenza virus has a much narrower range of host cell infectivity, our studies were limited to the MDCK and LLC-PK_1 cell lines. Incubation of influenza-virus-infected MDCK cells with [^{35}S]sulphate resulted in the exclusive labelling of HA from the free virus and, indeed, this envelope glycoprotein constituted the predominant radioactive component of the cell lysate (Figure 6). Incubation with PNGase resulted in the total release of the sulphate label from HA while the [^{35}S]methionine-labelled HA was converted into a component of reduced molecular mass, as would be anticipated from the excision of N-linked complex carbohydrate units (Figure 6). Endo H treatment, however, did not bring about a decrease in the [^{35}S]sulphate associated with HA (results not shown). Inhibition of α1 → 2-mannosidase action by KIF abolished sulphation of HA, indicating that some processing of the polymannose chains had to precede the introduction of sulphate into this glycoprotein (Figure 6). ^{35}S-labelling of influenza-virus-infected LLC-PK_1 cells produced similar results (not shown).

Localization of sulphate residues on peripheral and internal saccharide residues of HA

Fragmentation by hydrazine/nitrous acid of [^{35}S]sulphate-labelled glycopeptides obtained by gel filtration of Pronase-digested HA derived from LLC-PK_1 yielded Gal(3-SO_4)β(1 → 4)AnManH_2 and Galβ(1 → 4)AnMan(6-SO_4) after $NaBH_4$ reduction (Figure 7). Moreover, a slow migrating component was evident (Figure 7) which was believed to be derived from the sulphated core of the N-linked oligosaccharide by cleavage of the di-*N*-acetylchitobiose sequence to yield Man_3AnManH_2(SO_4). Application of the hydrazine/nitrous acid/$NaBH_4$ procedure to [^{35}S]sulphate-labelled glycopeptides from the HA of MDCK cells yielded similar results (results not shown).

When the core oligosaccharide, after isolation by preparative TLC, was subjected to jack-bean α-mannosidase digestion it was converted from a component which migrated with a chromatographic mobility relative to lactitol of 0.28 to one which moved with a chromatographic mobility relative to lactitol of 1.50. (Figure 8). This α-mannosidase product is believed to be Manβ(1 → 4)AnManH_2(SO_4), and represents the Manβ(1 → 4)GlcNAc(SO_4) sequence of the native oligosaccharide, as it migrated in a similar manner to the disaccharide Galβ(1 → 4)AnManH_2(6-SO_4), which has been shown to have a chromatographic mobility relative to lactitol of 1.41 [1]. Not unexpectedly, in view of its sulphate substitution, this component was found to

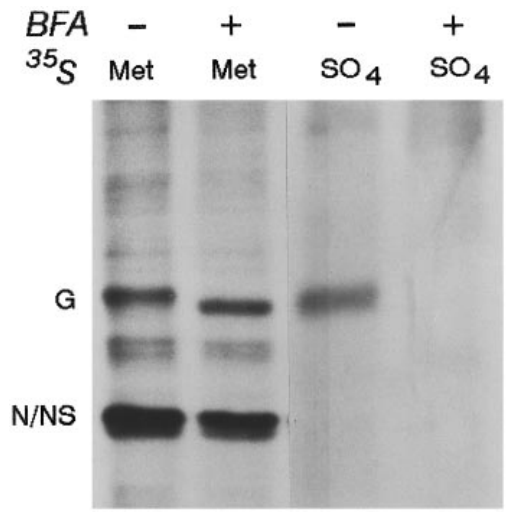

Figure 9 Effect of BFA on sulphation of G protein by LLC-PK$_1$ cells

Infected cells were incubated with 700 μCi [^{35}S]sulphate (SO_4) or 75 μCi [^{35}S]methionine (Met) for 45 min in the presence (+) or absence (−) of BFA (5 μg/ml) followed by a 2 h chase with unlabelled substrate, as described in the Experimental section. The lysed cells were subjected to SDS/PAGE and the components were visualized by fluorography. The position of the G protein (G) and N/NS proteins are indicated.

be resistant to β-mannosidase cleavage. The disaccharide was destroyed by Smith periodate oxidation and yielded a radioactive component which was identified as AnMan(6-SO_4) by TLC (results not shown), which excluded the possibility that the sulphate was located on the mannoside residue.

Evaluation of the effect of BFA on the sulphation of N-linked oligosaccharides of virus glycoproteins

To assess in which Golgi compartment the sulphation of the VSV and influenza virus envelope glycoprotein takes place, virus-infected LLC-PK$_1$ cells were pulsed and chased in the presence of BFA. In the VSV-infected cells, sulphation of the G protein was completely prevented (Figure 9), although we found no inhibition of [2-^{3}H]mannose incorporation into this glycoprotein (results not shown). In the presence of BFA radioactive labelling of the G protein by [^{35}S]methionine was not impaired but the migration of this component on electrophoresis was more rapid (Figure 9). This reduction in molecular mass can be attributed to the known block in sialylation, in addition to its effect on sulphation, which BFA has on N-linked oligosaccharides [34,35] and, indeed, this was confirmed by the finding that G protein from BFA-treated cells was resistant to neuraminidase digestion in contrast to control cells. Abolition of sulphation of HA was likewise noted in BFA-treated influenza-virus-infected LLC-PK$_1$ cells (results not shown).

DISCUSSION

From the studies presented in this report, it is apparent that the VSV envelope glycoprotein can become sulphated in a limited number of cell lines. This biosynthetic event appears to be host-cell dependent to a pronounced extent as it could be detected in only four of the twelve cell types examined.

Our investigations clearly indicated that the sulphate groups are introduced solely into the N-linked oligosaccharides, which constitute the two carbohydrate units of the G protein. The use of the hydrazine/nitrous acid/$NaBH_4$ fragmentation procedure, moreover, permitted us to locate the sulphate substituents in the *N*-acetyllactosamine sequence of the complex oligosaccharides in the form of Gal(3-SO_4) and/or GlcNAc(6-SO_4) residues. These sulphation sites have been identified previously in the N-linked carbohydrate units of a number of glycoproteins and specific sulphotransferases involved in their formation have been characterized [11,13]. The Galβ(1 → 4)GlcNAc(6-SO_4) sequence has been observed in thyroglobulins from several species [1,2], glycoproteins from various cell surfaces [5,6], and most recently in gp120 from the human immunodeficiency virus (HIV) envelope [10]. Gal(3-SO_4) in β(1 → 4) linkage to *N*-acetylglucosamine has been found to occur on thyroglobulins [1,2] and the Tamm-Horsfall glycoprotein [3]. The terminal C-3-sulphated galactose residues appear to occur as alternatives to sialyl capping groups [1] and, in the case of VSV, most likely contribute to the charge heterogeneity which the G protein exhibits [36]. The sulphation of galactose at the C-3 position is known to occur exclusively on terminal residues [1] and accordingly it can be inferred that, in the host cells in which the introduction of this anionic group on to the G protein was observed, the sulphotransferase must compete effectively with sialyltransferases and perhaps also α(1 → 3)-galactosyltransferase. Indeed, our measurements of PAPS : galactose-3-*O*-sulphotransferase activity of the various cell lines (Table 1) correlated well with their capacity to sulphate the G protein and, thereby, provided a clear example of cell-specified oligosaccharide assembly. The most complete structural formation of the two complex-type N-linked carbohydrate units of the G protein did not include sulphate substituents [21] but this can be attributed, most likely, to the observation that the BHK cell line, in which the virus of the aforementioned study was propagated, permits only modest sulphation of the G protein and is limited exclusively to *N*-acetylglucosamine residues. These GlcNAc(6-SO_4) substitutents might readily escape detection without [^{35}S]sulphate labelling.

The cell-dependent variation in the sulphation of N-linked oligosaccharides of virus envelope glycoproteins, noted in this study, is in accord with previous observations which have shown that the structure of N-linked oligosaccharides is dictated to a large extent by the post-translational enzymic machinery of the host cell [16–20].

Our observation that the HA glycoprotein of the influenza virus envelope is extensively sulphated is in agreement with previous reports [9,33]. Studies on the HA of the influenza virus [37] and the HA and F components of the closely related paramyxovirus [7] have located the sulphate on an internal *N*-acetylglucosamine residue of the complex oligosaccharides. Our investigations indeed indicate that the mannose-substituted *N*-acetylglucosamine of the di-*N*-acetylchitobiose core is substituted by this anionic group; this would differentiate HA sulphation from that observed in VSV. However, we found also that, in common with the G protein, Gal(3-SO_4) and GlcNAc(6-SO_4) groups occur in peripheral locations on the branches of the HA carbohydrate units. The possibility of introducing Gal(3-SO_4) capping groups into HA should be favoured by the absence of sialic acid residues in this glycoprotein [8,33,38]. The major competing enzyme for terminal sulphation would be α(1 → 3)-galactosyltransferase [39], which has been observed to be distributed in a species-dependent manner [40,41]. A recent report indicates that terminal α-galactosyl residues occur on HA from virus grown in bovine cells [38]. Since LLC-PK$_1$ and MDCK cells carried out core sulphation of the HA but not of G protein, it would appear that the sulphotransferase acting on the internal

N-acetylglucosamine may be influenced by the polypeptide chain. Although polymannose oligosaccharides have been identified among the multiple N-linked carbohydrate units of HA [8,38], it is unlikely that they are sulphate substituted as we were unable to release [^{35}S]sulphate from this protein by endo H action in contrast to its facile removal by PNGase.

The finding that KIF, a potent inhibitor α1-2-mannosidase processing, inhibited internal as well as the anticipated peripheral sulphations indicated that at least the outer residues of the polymannose chains need to be trimmed before any sulphate addition can occur. Studies with BFA further indicated that these sulphations were a late biosynthetic event as we found that this fungal metabolite, which is known to fuse the endoplasmic reticulum with the *cis*-, *medial*- and *trans*-Golgi compartments but not the *trans*-Golgi network [42], resulted in a complete cessation of sulphate additions. Sulphation of N-linked complex oligosaccharides, and indeed other modification such as sialylation and fucosylation, have been attributed previously to this most distal compartment [34,35,43].

Since it is known that VSV replicates efficiently in almost all mammalian cells [44] and in our study only four of the 12 cell types examined incorporated sulphate into the glycoprotein, it would appear that the presence of this substituent on the virus envelope glycoprotein is not essential for infectivity. Indeed, the biological role of sulphate on N-linked oligosaccharides in general, along with a large number of other modifications which these carbohydrate units undergo [16,45], still remains to a large extent elusive. The recognition of sulphate in HIV envelope glycoprotein [9,10] has focused attention on studies which had indicated that sulphate-containing oligosaccharides inhibit cell-binding, replication and syncytium formation by this virus [46–48]. The presence of sulphate substituents on virus envelope glycoproteins, particularly in terminal position as the Gal(3-SO_4) capping groups, present potential recognition sites in interactions with cell surfaces. The peripheral GalN(4-SO_4) residues of certain pituitary hormones have been implicated in specifying their uptake by hepatic reticuloendothelial cells [49], and a recent study has suggested that sulphated oligosialic acid units on sea urchin egg glycoproteins are involved in fertilization [50]. Furthermore, sulphation of the virus envelope glycoproteins by the cell-specific post-translational enzymic machinery may modify their antigenicity. It has been suggested [16] that the formation of viral surface oligosaccharides which closely resemble those of the host cell could result in a blunting of antiviral immune response by the infected host. Certainly, further studies will be required to define the biological *raison d'être* for the sulphation machinery which is distributed among a variety of cells.

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