Characterization of an N-Acetylgalactosamine-6-O-sulfotransferase from Human Respiratory Mucosa Active on Mucin Carbohydrate Chains*

Sophie Degroote‡, Jean-Marc Lo-Guidice‡, Gérard Streekers§, Marie-Paule Ducourouble‡, Philippe Roussel‡, and Geneviève Lamblin¶

A microsomal GlcNAc-6-O-sulfotransferase activity from human bronchial mucosa, able to transfer a sulfate group from adenosine 3′-phosphate 5′-phosphosulfate onto methyl-N-acetylgalactosaminides or terminal N-acetylgalactosamine residues of carbohydrate chains from human respiratory mucins, has been characterized. The reaction products containing a terminal HO3S-6GlcNAc were identified by high performance anion-exchange chromatography. Using methyl-β-N-acetylgalactosaminide as a substrate, the optimal activity was obtained with 0.1% Triton X-100, 30 mM NaF, 20 mM Mn2+, 5 mM AMP in a 30 mM MOPS (3-(N-morpholino)propanesulfonic acid) buffer at pH 6.7. The apparent K values for adenosine 3′-phosphate 5′-phosphosulfate and methyl-β-N-acetylgalactosaminide were observed at 9.1 × 10−6 M and 0.54 × 10−3 M, respectively. The enzyme had more affinity for carbohydrate chains with a terminal GlcNAc residue than for methyl-β-N-acetylgalactosaminide; it was unable to catalyze the transfer of sulfate to position 6 of the GlcNAc residue contained in a terminal Galβ1-4GlcNAc sequence. However, oligosaccharides with a nonreducing terminal HO3S-6GlcNAc were substrates for a β1-4 galactosyltransferase from human bronchial mucosa. These data point out that GlcNAc-6-O-sulfotransferase must act before β1-4 galactosylation in mucin-type oligosaccharide biosynthesis.

Human respiratory epithelium is covered by a gelatinous layer of mucus, situated on the top of the cilia, and continuously moved toward the pharynx where it is swallowed (1). Mucins are the main components of the respiratory mucus and are responsible to a large extent for the characteristic rheological properties of respiratory mucus, necessary for the efficiency of the mucociliary system.

Human respiratory mucins consist of a broad family of polydisperse and high molecular mass glycoproteins synthesized by the respiratory mucosa. The peptide diversity stems from the expression of several genes (2, 3); however, this mucin diversity has increased widely by post-translational phenomena, mostly O-glycosylation but also sulfation, which are responsible for 70–80% of the mass of the mucin molecule. These phenomena lead to a remarkable diversity of carbohydrate chains (4) allowing the recognition of inhaled microorganisms (for review, see Ref. 5) which are then eliminated by the activity of the mucociliary system. Thus mucins play an essential role in the defense of the respiratory mucosa. Any change in the glycosylation or sulfation of mucins may affect the rheological properties of respiratory mucus and the efficiency of the mucociliary system, leading to bronchial obstruction as observed in cystic fibrosis (CF).1

CF, a general exocrinopathy, is the most common severe genetic disease among Caucasians. In its most typical form, the severity of CF is the result of chronic lung infection and mucus hypersecretion. This infection is very peculiar and characterized by the predominance of Staphylococcus aureus in early life and, later on, of Pseudomonas aeruginosa which is almost impossible to eradicate and is responsible for most of the morbidity and mortality of the disease (6).

CF is caused by mutations in the gene encoding for cystic fibrosis transmembrane conductance regulator (CFTR) (7), a chloride channel of low conductance activated by protein kinase A (8–11). ∆F508 deletion is the most frequent mutation (about 70% of the CF chromosomes) and leads to a misfolding of CFTR which is retained in the endoplasmic reticulum and degraded (12). This mislocation of mutated CFTR may have consequences for other cell compartments. In CF cells, a defect in the acidification of endosomes, prelysosomes, or trans-Golgi/transport-Golgi network has been observed and could be responsible for abnormal glycosylation and sulfation processes (13–16).

Previous works have shown that salivary and respiratory mucins (17–19) as well as glycoproteins secreted by CF nasal epithelial cells in culture (20) were oversulfated. More recently, Zhang et al. (21), using a model of human xenograft that eliminates the influence of inflammation and infection, observed an increased mucin sulfation that may correspond to a primary defect, varying according to the CF genotype. Mucin sulfation affords a strong negative charge to carbohydrate chains influencing the viscoelastic properties of bronchial mucus. Moreover, sulfated carbohydrate sequences, identical to those found in respiratory mucins, have been shown to be specific ligands for selectins (22, 23) and for microorganisms (24). The development of a protocol of purification based mainly on high performance anion-exchange chromatography (HPAEC) has allowed the structural determination of sulfated carbohydrate chains from CF and non-CF mucins in which sulfation occurs either on the C-3 of a terminal galactose (Gal)

1 The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; HPAEC, high performance anion-exchange chromatography; PAPS, adenosine 3′-phosphosulfate 5′-phosphosulfate; APS, adenosine 5′-phosphosulfate; sLe x, sialyl Lewis x; MOPS, 3-(N-morpholino)propanesulfonic acid; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; MES, 2-(N-morpholino)ethanesulfonic acid.
residue or on the C-6 of an N-acetylgalcosamine (GlcNAc) residue (25). These data suggest that sulfation of respiratory mucins involves at least two sulfotransferases.

Lo-Guidice et al. (26) have characterized recently a sulfotransferase from human airways responsible for the 3-O-sulfation of terminal galactose in N-acetyllactosamine-containing mucin carbohydrate chains. In this paper we report the characterization of a microsomal GlcNAc-6-O-sulfotransferase activity from human bronchial mucosa. This enzyme is able to transfer a sulfate group from PAPS to a terminal GlcNAc residue contained in a carbohydrate chain. The GlcNAc-6-O-sulfation is inhibited by the prior β1–4 galactosylation of the GlcNAc residue, indicating that 6-O-sulfation has to precede galactosylation during biosynthesis of sulfated mucin-type oligosaccharides. The main difference in the enzymatic properties of the 5-O-Gal- and the 6-O-GlcNAc-sulfotransferase is their optimum pH, 6.1 and 6.7, respectively.

EXPERIMENTAL PROCEDURES

Enzyme Preparation—Tissues collected in macroscopically healthy areas of the bronchial tree from patients undergoing surgery for bronchial carcinoma were placed in Leibovitz L15 medium (Life Technolo-

gies, Inc.) and transported immediately on ice to the laboratory and processed for mucosa isolation. Mucosa (2–3 cm²) pieces, and homogenates were prepared in 50 mM Tris-HCl buffer, pH 7.4, containing 25 mM potassium chloride, 250 mM saccharose, 5 mM β-mercaptoethanol, 5 mM magnesium acetate, using a glass-Teflon homogenizer (1,400 rpm, five strokes). The mixture obtained was submitted to dryness, and then evaporated to dryness. The sulfated GlcNAcβ1–O-Met was purified on a silica column (1.2 × 13 cm) (Florisil, Merek) eluted by a mixture of dichloromethane/methanol (5:3, v/v), and their structure was determined by 400 MHz 1H NMR spectroscopy on a two-dimensional homo-

nuclear COSY spectrum. The analysis proved the presence of two sulfated products: HO3S-6GlcNAcβ1–O-Met, which was the most synthesized product; and HO3S-3GlcNAcβ1–O-Met (90%/10%). The sul-

fated on the Nα1–C-6 was confirmed by the same methods as for fraction IIIc1 (25). The structures of 11 sul-

fated mucin-type oligosaccharides were described for the sulfotransferase assays, and the synthesized product was incubated with 0.5 μCi of [35S]PAPS (ENL Life Science Products, 2.48–2.50 Ci/mmol), 5 mM of methylglycosides (GlcNAcβ1–O-Met, NeuAcβ2–3Galβ1–4GlcNAcβ1–O-Met, NeuAcα2–3Galβ1–4GlcNAcβ1–O-Met, or sLeα1–O-Met), or 100 μCi of one of the oligosaccharide-alditols (OS1, OS2, OS3) in a 50 mM MOPS/NaOH buffer, pH 6.7, containing 0.1% (w/v) Triton X-100, 20 mM MnCl₂, 30 mM NaF, 5 mM AMP, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF). After incubation for 60 min at 30 °C, the reaction was stopped by the addition of 300 μl of ice-cold methanol. The resulting mixture was kept overnight at 4 °C, and the formed precipitates were eliminated by centrifugation at 10,000 × g for 20 min. The pellets were washed twice with ice-cold methanol and centrifuged. The supernatants were collected, evaporated to dryness, and then submitted to HPAEC.

Action of Human Microsomal Galactosyltransferase on HO3S-6GlcNAcβ1–O-Met or on Oligosaccharide-alditol IVc-19 (OS2 with a Terminal HO3S-6GlcNAc)—After incubation of GlcNAcβ1–O-Met or OS2 with radiolabeled [35S]PAPS and microsomal preparation for 60 min at 30 °C as described above, UDP-Gal (Sigma) was added to the mixture to have a final concentration of 5 mM. The mixture was kept for 1 h at 30 °C, and the reaction was stopped as described for the sulfotransferase assays. The synthesized products were identified by HPAEC.

The sulfation of this galactosyltransferase was performed using GlcNAcβ1–O-Met/HO3S-6GlcNAcβ1–O-Met and OS2/IVc-19, each substrate (1 mM) was incubated with 1 μCi of radiolabeled UDP-6-3H2Gal (Sigma, 17.65 Ci/mmol) for 1 h at 30 °C in a 30 mM MOPS/NaOH buffer containing 5 mM AMP, 30 mM NaF, 20 mM MnCl₂, 1 mM AEBSF, and 0.1% Triton X-100. The reaction was stopped as described for the sulfotransferase assays, and the radiolabeled products were studied by HPAEC.

The sulfation of this galactosyltransferase was performed using GlcNAcβ1–O-Met/HO3S-6GlcNAcβ1–O-Met, chemically synthesized HO3S-6GlcNAcβ1–O-Met (5 mM) was incubated with 1 μCi of radiolabeled UDP-6-3H2Gal (Sigma, 17.65 Ci/mmol) and 150 milliunits of β1–4 galactosyltransferase from bovine milk (Sigma) in 50 μl of 100 mM sodium cacodylate, 154 mM NaCl buffer, pH 7.4, containing 1 mM AMP, 10 mM MnCl₂, 0.5% (w/v) Triton X-100 (32). After 1 h at 30 min at 30 °C, the reaction was stopped as described for the sulfotransferase assays, and the synthesized product was characterized by HPAEC.

Characterization of Labeled Products by HPAEC—Dry samples of sulfated or galactosylated products were dissolved directly on a CarboPac PA-100 column (4 × 250 mm) for HPAEC (Dionex Corp.). The elution of neosynthesized products was monitored both by pulsed amperometric detection (PAD 2 model, Dionex Corp.) and by radioactivity on line (high performance liquid chromatography radioactivity detector LB 506 C-1, EG & G, Berthold, Wildbad, Germany).

Elution of sulfated GlcNAcβ1–O-Met and sulfated products synth-
ized from methylglycosides (GlcNAcβ1-3Gal-O-Met, NeuAcO2-3Galβ1-4GlcNAc-O-Met, and sLeα-O-Met) was performed at alkaline pH at a flow rate of 1 ml/min in 0.05 M NaOH, 0.2 M sodium acetate with a linear gradient of sodium acetate to 0.05 M NaOH, 0.3 M sodium acetate at 22 min, to 0.05 M NaOH, 0.95 M sodium acetate at 24 min, and followed by isotonic elution with 0.05 M NaOH, 0.95 M sodium acetate for 10 min (gradient I). The standards used were HO3S-6GlcNAcβ1-O-Met and HO3S-6GlcNAcβ1-3Gal-O-Met for sulfated GlcNAcβ1-O-Met and sulfated GlcNAcβ1-3Gal-O-Met, respectively.

The same gradient I was used for elution of the 35S-sulfated and galactosylated products synthesized from GlcNAcβ1-O-Met. The standard used was Galβ1-4HO3S-6GlcNAc-O-Met (resulting from incubation of HO3S-β-GlcNAcβ1-O-Met with UDP-[6-3H]Gal and β1-4 galactosyltransferase from bovine milk).

Elimination of neosynthesized radiolabeled sulfated oligosaccharide-alditols was performed at alkaline pH at a flow rate of 1 ml/min in 0.05 M NaOH, 0.07 M sodium acetate at 16 min, to 0.1 M NaOH, 0.1 M sodium acetate at 30 min, to 0.1 M NaOH, 0.45 M sodium acetate at 80 min, to 0.1 M NaOH, 0.95 M sodium acetate at 82 min and followed by isotonic elution with 0.1 M NaOH, 0.95 M sodium acetate for 10 min (gradient II).

Fraction IVc containing 11 sulfated oligosaccharide-alditols was used as a standard (25). For elution of both 35S-sulfated and galactosylated products synthesized from OS2, we also used gradient II and fraction IVc as a control.

**Protein Determination**—The protein content of the microsomal fractions was determined by BCA Protein Assay (Pierce) (33).

**RESULTS**

**Sulfation of Methyl-N-acetylglucosaminides**

Methyl-N-acetylglucosaminides were first used to test the N-acetylglucosamine-sulfotransferase activity since it is difficult to obtain enough oligosaccharide-alditols from human respiratory mucins to characterize the enzyme completely. After incubation of methyl-β-N-acetylglucosaminide (GlcNAcβ1-O-Met) with microsomes and [35S]PAPS and separation of the radiolabeled products by HPAEC, two peaks were observed: a peak at 11 min 30 s corresponding to free [35S]sulfate (this peak is still present when incubation is performed without any carbohydrate acceptor) and another peak at 9 min 30 s, absent when there is no GlcNAcβ1-O-Met in the incubation mixture, corresponding to a sulfated GlcNAcβ1-O-Met (Fig. 1a). Unreacted [35S]PAPS or [35S]PAPS, which are very acidic compounds, are eluted with high concentrations of sodium acetate, and so they are not visualized on the elution profile.

To find out the position of the sulfate group on the GlcNAc residue, the radiolabeled products were chromatographed with a mixture (90%/10%) of HO3S-6GlcNAcβ1-O-Met and HO3S-3GlcNAcβ1-O-Met that was chemically synthesized. The sulfated GlcNAcβ1-O-Met synthesized during incubation with microsomes and [35S]PAPS coeluted with the standard HO3S-6GlcNAcβ1-O-Met. This showed that the sulfotransferase was able to transfer a sulfate group from PAPS to the C-6 of GlcNAcβ1-O-Met (Fig. 1b).

A very low sulfotransferase activity was found when methyl-α-N-acetylglucosaminide (GlcNAcα1-O-Met) was used as a substrate acceptor, showing that the enzyme was more active on the β anomers than on the α anomers. The activity was about 120-fold higher for GlcNAcβ1-O-Met (17.27 pmol/mg of protein/min) than for GlcNAcα1-O-Met (0.14 pmol/mg of protein/min) using the same conditions of incubation.

**Properties of the GlcNAc-6-O-sulfotransferase**

We first looked at the effect of pH on the transfer of sulfate group from PAPS to GlcNAcβ1-O-Met, using MES buffer (pH 5.4–6.4) and MOPS buffer (pH 6.1–7.9). The optimal conditions for sulfation of GlcNAcβ1-O-Met were obtained with 30 mM MOPS/NaOH buffer at pH 6.7 (data not shown).

The influence of divalent cations on the activity of this GlcNAc-6-O-sulfotransferase from respiratory mucosa was also studied. Mn2+ and Mg2+ had a stimulatory effect on the GlcNAc-6-O-sulfotransferase with an optimal activity at 20 mM Mn2+. The sulfotransferase activity was 4-fold higher with 20 mM Mn2+ than without this cation. Ca2+ had an inhibitory effect even at very low concentrations (data not shown).

We also studied the influence of AMP, ATP, and NaF on the transfer of [35S]sulfate to GlcNAcβ1-O-Met. Both AMP and ATP had a stimulatory effect at a concentration of 1 mM. Above this concentration, ATP abolished the sulfotransferase activity, whereas AMP had an increased stimulatory effect up to a 5 mM concentration. The presence of NaF in the incubation mixture stimulated the sulfotransferase activity, with a maximal effect at 30 mM NaF (Fig. 2).

These three compounds had an inhibitory effect on the liberation of free [35S]sulfate from [35S]PAPS during the incubations, allowing an increase of sulfotransferase activity. There was a free [35S]sulfate decrease, 10, 85, and 80%, when the incubations were performed with 30 mM NaF, 5 mM AMP, and 10 mM ATP, respectively. Concerning ATP, the stimulatory
effect was only observed at low concentrations (1 mM). Above this concentration, ATP had an inhibitory effect. This compound, which can be considered as a structural analog of PAPS has already been shown to inhibit sulfotransferase activities (34). At high concentrations, the impact on PAPS degradation would be lower than the inhibitory effect.

The optimal sulfotransferase activity was obtained with 0.1% (w/v) Triton X-100, 30 mM NaF, 20 mM MnCl₂, and 5 mM AMP in a 30 mM MOPS/NaOH buffer at pH 6.7.

When using these conditions, the sulfotransferase activity increased linearly up to 360 min, in the range of 2–96 μg of microsomal proteins (data not shown).

Kinetic measurements of GlcNAc-6-O-sulfotransferase activity with different concentrations of [35S]PAPS (Fig. 3a) and GlcNAcβ1-O-Met (Fig. 3b) allowed the determination of Kₘ values from Lineweaver-Burk plots for these two components, 9.1 μM and 0.54 mM, respectively.

**Sulfation of Oligosaccharidic Substrates**

*Sulfation of Different Oligosaccharide-alditols—*The sulfotransferase activity was measured on different oligosaccharide-alditols: OS1, OS2, OS3 (Table I), and fraction IIIc1 (sialylated oligosaccharide-alditols from human bronchial mucins (25)) with or without prior treatment with β-galactosidase. These oligosaccharides were incubated with microsomal preparation and [35S]PAPS in conditions described under “Experimental Procedures.” The 35S-radiolabeled products were analyzed by HPAEC using a CarboPac PA-100 column and gradient II and compared with a mixture of 11 sulfated oligosaccharide-alditols whose structures have been determined previously (IVc) (25). Three of them were particularly interesting. The oligosaccharide-alditol IVc-10 had the same structure as OS1 with a sulfate group on the C-3 of the terminal galactose residue; IVc-12 had the same structure as OS1 with a sulfate group on the C-6 of the internal GlcNAc residue, and IVc-19 corresponded to OS2 with a sulfate group on the C-6 of the terminal GlcNAc residue. The structure of the oligosaccharide-alditol IVc-2 was also very useful for this study (Table II).

After sulfation of OS1, two radiolabeled peaks were obtained (Fig. 4a). The first peak was eluted at 45 min 24 s and was also present when the incubation mixture did not contain any acceptors; it corresponded to free [35S]sulfate. The second peak was eluted at 46 min 36 s; it corresponded to the product synthesized from OS1. When this product was injected with a mixture of sulfated oligosaccharides (fraction IVc), it coeluted with oligosaccharide-alditol IVc-10, which corresponds to OS1 with a sulfate group on the C-3 of the terminal galactose residue. No radiolabeled peak had the same retention time as oligosaccharide-alditol IVc-12, which corresponds to OS1 with a sulfate group on the internal GlcNAc residue (Table II). Thus the GlcNAc-6-O-sulfotransferase activity from human bronchial mucosa was not directly active on the internal GlcNAc residue of OS1 (Scheme 1).

OS1 was then submitted to the action of β-galactosidase from *S. pneumoniae* and led to OS2 (Table I), which was incubated under the same conditions as OS1. The radiolabeled products were separated by HPAEC in two peaks (Fig. 4b). The first peak was eluted at 45 min 24 s and corresponded to free [35S]sulfate; the second peak was eluted at 56 min 6 s and
coeluted with oligosaccharide-alditol IVc-19 from human bronchial mucins, which is sulfated on the C-6 of the terminal GlcNAc residue (Fig. 4b). Thus this sulfotransferase from human bronchial mucosa is able to transfer a sulfate residue on the C-6 of a terminal GlcNAc residue contained in mucin carbohydrate chains (Scheme 1).

For OS3 (Table I), no radiolabeled peak was observed except the free [35S]sulfate peak at 45 min 24 s (data not shown).

A pool of sialylated oligosaccharide-alditols from human bronchial mucins (fraction IIIc1) was also used as substrate acceptors for the GlcNAc-6-O-sulfotransferase. The structures of the main components of this fraction have been identified by Lo-Guidice et al. (25). One oligosaccharide-alditol of this fraction has the same structure as OS1. Coinjection on the CarboPac PA-100 column of fraction IVc and the neosynthesized products obtained from fraction IIIc1 showed the presence of three main labeled peaks. Two peaks correspond to oligosaccharides having a 3-O-sulfated terminal galactose. One was eluted at 34 min 30 s and had the same retention time as oligosaccharide-alditol IVc-2 (Table II). The other one was eluted at 46 min 36 s and had the same retention time as oligosaccharide-alditol IVc-10 (Table II). The peak that was eluted at 45 min 24 s corresponded to free [35S]sulfate (Fig. 5a).

Treatment of fraction IIIc1 with β-galactosidase followed by incubation with microsomes and [35S]PAPS and fractionation of the neosynthesized products by HPAEC led to the elution of three main radiolabeled peaks: the free [35S]sulfate peak at 45 min 24 s, one radiolabeled peak that coeluted with oligosaccharide-alditol IVc-19 (Table II) (retention time: 56 min 6 s), and another peak that was eluted at 50 min 42 s and could not be identified (Fig. 5b).

Sulfation of Methylglycosides—Three different methylglycosides containing GlcNAc residues were used as sulfate acceptors as described under “Experimental Procedures.” The neosynthesized products were analyzed by HPAEC on a CarboPac PA-100 column using gradient I.

When using GlcNAcβ1–3Gal-O-Met, two radiolabeled peaks were found on the elution profile: the free [35S]sulfate peak at 11 min 30 s and another peak that was eluted at 3 min 54 s and corresponded to the radiolabeled oligosaccharide synthesized from GlcNAcβ1–3Gal-O-Met. This peak coeluted with HO3S-6GlcNAcβ1–3Gal-O-Met, showing that the sulfate group was transferred on the C-6 of the terminal GlcNAc residue by a microsomal sulfotransferase (data not shown). No sulfation occurred on sLex-O-Met or on NeuAcα2–3Galβ1–4GlcNAc-O-Met.

These data argued that a sulfotransferase from human bronchial mucosa is active on GlcNAcβ1–3Gal-O-Met (bearing a terminal nonreducing GlcNAc residue) but not on the two substrates with an internal GlcNAc residue.

Competition Experiments

To determine whether the same sulfotransferase was responsible for the 6-O-sulfation of GlcNAcβ1-O-Met and OS2 and for the 3-O-sulfation of OS1, it was necessary to perform competition experiments.

For this study, we used the oligosaccharide-alditols OS1 and

---

**Table II**

**Structures of oligosaccharide-alditols IVc-2, IVc-10, IVc-12, and IVc-19 from human respiratory mucins**

| Oligosaccharide-alditol | Structure |
|-------------------------|-----------|
| IVc-2                   | HO$_3$S  Fuc(α1–3) Gal(β1–3) GalNAc-ol |
| IVc-10                  | HO$_3$S  Gal(β1–4)GlcNAc(β1–6) GalNAc-ol |
| IVc-12                  | HO$_3$S  Gal(β1–4)GlcNAc(β1–6) GalNAc-ol |
| IVc-19                  | HO$_3$S  Gal(β1–4)GlcNAc(β1–6) GalNAc-ol |

---

**Fig. 4.** HPAEC elution profile on a CarboPac PA-100 column (4 x 250 mm) of a mixture containing [35S]-labeled products enzymatically synthesized from OS1 (panel a) or OS2 (panel b) and sulfated oligosaccharide-alditols (fraction IVc) isolated from human respiratory mucins. Elution was performed with gradient II described under “Experimental Procedures.” Peaks were detected by pulsed amperometry (dashed line) and by radioactivity (solid line).
OS2 at a molar concentration of about 1 mM. GlcNAc\textsubscript{b}1-O-Met had an important inhibitory effect on the sulfation of OS2 but no effect on the sulfation of OS1 (Fig. 6). The sulfation of OS2 was 50–66% lower when 1–33 mM GlcNAc\textsubscript{b}1-O-Met was added in the incubation mixture. These data proved that the same enzyme was responsible for the 6-O-sulfation of GlcNAc\textsubscript{b}1-O-Met and OS2. The 3-O-sulfation of OS1 was not affected by the presence of GlcNAc\textsubscript{b}1-O-Met. These results prove that the GlcNAc-6-O-sulfotransferase characterized in the present work is different from the Gal-3-O-sulfotransferase described by LoGuidice et al. (26). This GlcNAc-6-O-sulfotransferase has more affinity for carbohydrate chains with a terminal GlcNAc residue than for GlcNAc\textsubscript{b}1-O-Met since oligosaccharide sulfation is not inhibited completely by high concentrations of GlcNAc\textsubscript{b}1-O-Met.

For the 6-O-sulfation of GlcNAc\textsubscript{b}1-3Gal-O-Met, we had a standard that was a gift from K. L. Matta. Using this standard, we could show that sulfation of GlcNAc\textsubscript{b}1-3Gal-O-Met occurred on the C-6 of the terminal GlcNAc residue, resulting from the action of a GlcNAc-6-O-sulfotransferase. GlcNAc\textsubscript{b}1-O-Met also inhibited the 6-O-sulfation of this component (data not shown), suggesting that the same enzyme was responsible for the sulfation of GlcNAc\textsubscript{b}1-O-Met and GlcNAc\textsubscript{b}1-3Gal-O-Met.

Galactosylation of HO\textsubscript{b}S-6GlcNAc\textsubscript{b}1-O-Met and IVc-19 by Human Microsomal Galactosyltransferase

As HO\textsubscript{b}S-6GlcNAc may be a component of a sulfated N-acetyllactosamine unit, it was interesting to determine whether this sulfated sugar could be an acceptor for a microsomal galactosyltransferase. When GlcNAc\textsubscript{b}1-O-Met was incubated first with [\textsuperscript{35}S]PAPS and microsomal preparation and then with UDP-Gal, the neosynthesized products analyzed by HPAEC in conditions described under “Experimental Procedures” using gradient I showed three main radiolabeled peaks (Fig. 7a). One was eluted at 9 min 30 s and corresponded to [\textsuperscript{35}S]-labeled HO\textsubscript{b}S-6GlcNAc\textsubscript{b}1-O-Met. The free [\textsuperscript{35}S]sulfate peak was eluted at 11 min 30 s. The third peak was eluted at 4 min 36 s and probably resulted from the action of a galactosyltransferase on HO\textsubscript{b}S-6GlcNAc\textsubscript{b}1-O-Met.

To identify this product, we synthesized radiolabeled Gal\textsubscript{b}1-4(HO\textsubscript{b}S-6)GlcNAc\textsubscript{b}1-O-Met (synthesized according to Van Kuik et al. (30)) with UDP-[6-\textsuperscript{3}H]Gal in conditions described under “Experimental Procedures.” The neosynthesized products were studied by HPAEC using gradient I (Fig. 7b). Two radiolabeled peaks were obtained. One peak was eluted at 3 min 18 s and was also present when the incubation mixture did not contain HO\textsubscript{b}S-6GlcNAc\textsubscript{b}1-O-Met. It corresponded to UDP-[6-\textsuperscript{3}H]Gal. The
other peak was eluted at 4 min 36 s and corresponded to Gal$\beta_1$-4(HO$_3$S-6)GlcNAc$\beta_1$-O-Met synthesized by the action of $\beta_1$-4 galactosyltransferase from bovine milk on HO$_3$S-6GlcNAc$\beta_1$-O-Met. These results prove that a galactosyltransferase from human bronchial mucosa can transfer galactose from UDP-Gal on HO$_3$S-6GlcNAc$\beta_1$-O-Met in a manner similar to the $\beta_1$-4 galactosyltransferase from bovine milk.

These two galactosyltransferases were also active in a similar manner on GlcNAc$\beta_1$-O-Met (data not shown).

We also incubated OS2 (Table I) with $[35S]$PAPS and microsomal preparation and then with UDP-Gal as explained under "Experimental Procedures" and analyzed the neosynthesized products by HPAEC using gradient II. Two main radiolabeled peaks were obtained. The first one was eluted at 45 min 24 s and corresponded to free $[35S]$sulfate. The other peak was eluted at 49 min 30 s and coeluted with oligosaccharide IVc-12 (Table II) when the radiolabeled products were injected simultaneously with fraction IVc (Fig. 8). This indicates that a microsomal bronchial $\beta_1$-4 galactosyltransferase is active on a terminal HO$_3$S-6GlcNAc residue from human mucin carbohydrate chains. No other neosynthesized peak but IVc-12 appeared on the elution profile, indicating that this oligosaccharide-alditol IVc-12 has not been 3-$\text{O}$-sulfated further on the terminal Gal residue.

The action of the $\beta_1$-4 galactosyltransferase from human bronchial mucosa was compared on the substrates GlcNAc$\beta_1$-O-Met/HO$_3$S-6GlcNAc$\beta_1$-O-Met and OS2/IVc-19 (Table III) to check the influence of the 6-$\text{O}$-sulfation of GlcNAc on its further galactosylation. These four substrates, having a terminal non-reducing GlcNAc residue, 6-$\text{O}$-sulfated or not, were galactosylated. The activity of the galactosyltransferase was slightly lower when the terminal GlcNAc residue was 6-$\text{O}$-sulfated.

**DISCUSSION**

In a previous work, we have developed a performant protocol of purification of acidic carbohydrate chains from CF and non-CF mucins, based mainly on HPAEC, which has proved to be a suitable and reliable method to separate and to identify sulfated oligosaccharides (25). These data have shown that sulfation may occur either on the C-3 of a terminal galactose part of an N-acetyllactosamine chain or on the C-6 of an N-acetylgalactosamine residue (25, 35). Therefore sulfation of human respiratory mucins involves at least two sulfotransferases. Recently, Lo-Guidice et al. (26) have characterized a galactose 3-$\text{O}$-sulfotransferase from human airways. We report here the characterization of a second sulfotransferase activity from human respiratory mucosa, responsible for the transfer of a sulfate group from PAPS to the C-6 of an N-acetylgalactosamine.

![Fig. 6. Competition for sulfation between GlcNac$\beta_1$-O-Met and oligosaccharide-alditols OS1 or OS2. The molar concentration of OS1 or OS2 used in the competition experiments was roughly estimated as 1 mM. These results show the residual sulfotransferase activity on oligosaccharide-alditols OS1 (●) or OS2 (○). The composition of the incubation mixture was the same as described under standard assay conditions, except that different concentrations of GlcNac$\beta_1$-O-Met (1–33 mM) were used.](image)

![Fig. 7. HPAEC elution profile on a CarboPac PA-100 column (4 × 250 mm) of (panel a) labeled products synthesized from GlcNac$\beta_1$-O-Met, [35S]PAPS, and UDP-Gal by microsomal sulfotransferase and galactosyltransferase and of (panel b) 3H-labeled products synthesized from HO$_3$S-6GlcNac$\beta_1$-O-Met and UDP-[6-3H]Gal by $\beta_1$-4 galactosyltransferase from bovine milk. Elution was performed with gradient I described under "Experimental Procedures." Peaks were detected by radioactivity.](image)

![Fig. 8. HPAEC elution profile on a CarboPac PA-100 column (4 × 250 mm) of a mixture containing $[35S]$-labeled products enzymatically synthesized from OS2 and sulfated oligosaccharide-alditols from human bronchial mucins (fraction IVc). Elution was performed with gradient II described under "Experimental Procedures." Peaks were detected by pulsed amperometry (dashed line) and by radioactivity (solid line).](image)
residue.

The activity and the properties of this sulfotransferase were determined as described by Lo-Guidice et al. (26). The chemical synthesis of a standard of 6-O-sulfated methyl-β-N-acetylglucosaminide (30) allowed us to demonstrate that the bronchial microsomal preparation contained a sulfotransferase capable of transferring a sulfate group from PAPS to the C-6 of a terminal GlcNAc residue.

The presence of free [35S]sulfate in the reaction mixture was also observed in the previous work on the galactose 3-O-sulfotransferase (26) and in several other tissue extracts (36–38). The addition of NaF and AMP in sulfotransferase assays protects the PAPS from degradation, allowing an increase in the sulfotransferase activity (26, 39). The GlcNAc-6-sulfotransferase is stimulated significantly by Mg²⁺ and more particularly by Mn²⁺.

Using methyl-β-N-acetylglucosaminide as an acceptor, the optimal activity of the GlcNAc-6-O-sulfotransferase was obtained with 0.1% Triton X-100, 30 mM NaF, 20 mM Mn²⁺, 5 mM AMP in a 30 mM MOPS/NaOH buffer at pH 6.7. The main difference in the enzymatic properties of the Gal-3-O- and GlcNAc-6-O-sulfotransferase is their optimum pH.

Most of the sulfated or sialylated and sulfated oligosaccharide-alditols described previously have a core type 2. In 12 oligosaccharides 6-O-sulfation occurred on the GlcNAc part of this core, and in 2 cases, 6-O-sulfation occurred on a GlcNAc residue β-3 linked to the Gal part of core 1 in 6 oligosaccharide-alditols, sulfation occurs on the C-3 of a terminal Gal residue contained in N-acetyllactosamine chains (25). Therefore, in a first step, we tested the activity of the GlcNAc-6-O-sulfotransferase on two oligosaccharide-alditols, OS1 and OS2 (see Table I). After incubation of these oligosaccharide-alditols with the microsomal preparation (which contains both activities Gal-3-O- and GlcNAc-6-O-sulfotransferase) and [35S]PAPS, two sulfated products were obtained: OS1 sulfated on the C-3 of the terminal galactose, corresponding to IVc-10 (see Table II and ref. 25), and OS2 sulfated on the C-6 of the terminal GlcNAc residue corresponding to IVc-19. No sulfation occurred on the C-6 of the internal GlcNAc of OS1, leading to oligosaccharide-alditol IVc-12 present in human respiratory mucins. No sulfation occurred either on OS3 corresponding to core 3 of mucins. It should be stressed that in carbohydrate chains of human respiratory mucins, no sulfation on GlcNAc residues β-3 linked to N-acetylgalactosaminol has been observed so far (25, 35).

Incubation of a pool of sialylated oligosaccharide-alditols III1 from human respiratory mucins (25) with the microsomal preparation led to the sulfation of two oligosaccharide-alditols on the C-3 of galactose. Prior degalactosylation of III1 with β-galactosidase is very limited due to sialylation of most Gal residues but allowed to obtain at least one oligosaccharide-alditol with a terminal nonreducing GlcNAc that could be 6-O-sulfated to generate IVc-19.

Four substrates having nonreducing terminal GlcNAc, 6-O-sulfated or not, could be galactosylated by the β1-4 galactosyltransferase from human bronchial mucosa. This enzyme probably recognizes terminal GlcNAc, 6-O-sulfated or not, and the influence of the sulfate group on the activity of the galactosyltransferase is only moderate (Table III). As a matter of fact, terminal N-acetyllactosamine units from human respiratory mucins may be nonsulfated on the GlcNAc residue (25). Altogether, these results suggest that in the biosynthesis of N-acetyllactosamine chains containing 6-O-sulfated GlcNAc, the sulfation of GlcNAc has to precede galactosylation and that the N-acetyllactosamine chains cannot be acceptors for this GlcNAc-6-O-sulfotransferase. These results are in good agreement with those observed for a 6-O-sulfotransferase from rat liver active on GlcNAc residues β-6 linked to mannose (40). It is likely that, in vivo, there is a competition between the GlcNAc-6-O-sulfotransferase and the β1-4 galactosyltransferase, acting both on the same GlcNAc residue, and that once the Gal residue is linked to the GlcNAc, the GlcNAc-6-O-sulfotransferase is inactive.

Only two human sulfotransferases active on mucins have been characterized so far: a Gal-3-O-sulfotransferase (26) and a GlcNAc-6-O-sulfotransferase, described here, both localized in respiratory mucosa. Other Gal-3-O- (39, 41) as well as GlcNAc-6-O-sulfotransferases (42–44) acting on animal mucin carbohydrate chains have also been described previously.

One of the best endothelial associated ligands for L-selectin is GlyCAM-1, a mucin-like glycoprotein (45) whose smallest O-glycans have a core 2 and a upper chain consisting of 6-O-sulfated derivatives of sLex either on the GlcNAc or on the Gal residues of the Galβ1-4GlcNAc chain (46). In the biosynthesis of GlyCAM-1, Crommie and Rosen (47) have observed that sialylation precedes both fucosylation and sulfation during biosynthesis. However, the temporal relationship between sulfation and fucosylation is still controversial (48, 49). In our case, no sulfation occurred using sLea-O-Met or α2-3-sialyl-N-acetyllactosamine as acceptor for the respiratory GlcNAc-6-O-sulfotransferase.

Because two oligosaccharide-alditols from human respiratory mucins were 6-O-sulfated on a GlcNAc β-1–3 linked to a Gal residue, we looked at the activity of this GlcNAc-6-O-sulfotransferase on GlcNAcβ1-3Gal-O-Met. The neosynthesized product coeluted exactly with the corresponding 6-O-sulfated standard.

In conclusion, we have characterized a GlcNAc-sulfotransferase in human respiratory mucosa which transfers sulfate to the C-6 position of terminal GlcNAc residues of carbohydrate chains from human respiratory mucins and which can be substituted further by a galactose residue to produce Galβ1-4(HO3S-6)GlcNAc. This enzyme is inactive on N-acetyllactosamine chains, suggesting that 6-O-sulfation of GlcNAc has to precede galactosylation during mucin-type oligosaccharide biosynthesis. The same results have been observed for a GlcNAc-6-O-sulfotransferase from rat liver acting on GlcNAcβ1–6Man sequences (40). Structural determinations of oligosaccharide-alditols supported these conclusions.
charide-alditols isolated from CF and non-CF mucins have shown that CF mucins were predominantly 6-O-sulfated on GlcNAc, whereas non-CF mucins may be more 3-O-sulfated on Gal (25, 35). Enzymatic properties and $K_m$ of the two sulfotransferases responsible for sulfation of respiratory mucins are rather similar except for their optimum pH. The GlcNAc-6-O-sulfotransferase described here has an optimal pH (6.7) higher than that of the Gal-3-O-sulfotransferase described previously (6.1) (26). This observation is interesting within the context of CF since some reports have suggested that mutations of CFTR (especially ΔF508) could be responsible for defective acidification of the trans-Golgi/trans-Golgi network pH (whose normal pH is 6.0) leading to modifications in the sulfation and glycosylation processes and notably to hyperactivity of some sulfotransferases (13, 14). Altogether these results suggest that the GlcNAc-6-O-sulfotransferase described here might be responsible for the predominance of 6-O-sulfation in CF respiratory mucins (25) and for oversulfation of CF mucins, which has been recently shown to be a primary defect (21).

Acknowledgments—We are indebted to Prof. J. J. Lafitte for kindly providing human respiratory mucosa and to Dr. K. L. Matta for the sulfated substrate HO3S-6GlcNAcβ1-3Gal-O-Met. We thank Dr. Yves Plancke for running the NMR spectra.

REFERENCES
1. Lambin, G., Lhermitte, M., Klein, A., Houdret, N., Scharfman, A., Ramphal, R., and Roussel, P. (1991) Am. Rev. Respir. Dis. 144, S19–S24
2. Gendler, S. J., and Spicer, A. P. (1995) Annu. Rev. Physiol. 57, 607–634
3. Puchert, N., Pigot, F., Busine, M. P., Debailleul, V., Degand, P., Laine, A., and Aubert, J. P. (1995) Biochem. Soc. Trans. 23, 800–805
4. Roussel, P., and Lambin, G. (1996) in Glycoproteins and Disease (Montreuil, J., Vliegenthart, J. F. G., and Schachter, H., eds) pp. 351–393, Elsevier, Amsterdam
5. Roussel, P., Lambin, G., and Ramphal, R. (1996) in Environmental Impact on the Airways, from Injury to Repair (Chrétién J., and Dussier, D., eds) Vol. 93, pp. 427–469, Marcel Dekker, New York
6. Habiy, N. (1988) Chest 94, 975–1008
7. Riordan, J. R., Rommens, J. M., Kerem, B. S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plasvik, N., Chou, J. L., Drumm, M. C., and Roussel, P. (1995) in Pronephron, M. D., Fulimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 150, 76–85
8. Whittemore, R. M., Pearce, L. B., and Roth, J. A. (1986) Arch. Biochem. Biophys. 249, 464–471
9. Lo-Guidice, J. M., Herz, H., Lambin, G., Plancne, Y., Roussel, P., and Lhermitte, M. (1997) Glycoconjugates 14, 113–125
10. Suzuki, S., and Strominger, J. L. (1969) J. Biol. Chem. 245, 257–266
11. DeLuca, S., and Silber, J. B. (1968) J. Biol. Chem. 243, 2725–2729
12. Miller, R. R., and Waechter, C. J. (1979) Arch. Biochem. Biophys. 198, 31–41
13. Kuhns, W., Jain, R. K., Matta, K. L., Myrsnocnough, C., Borefield, A., Parasekva, C., and Brockhausen, I. (1995) Glyobiology 5, 689–697
14. Spiro, R. G., Yasumoto, Y., and Bhoyroo, V. (1996) Biochem. J. 319, 209–216
15. Varas, S. F., Dole, K., Yang, J., Matta, K. L., Myrsnocnough, C., Borefield, A., Parasekva, C., and Brockhausen, I. (1994) Eur. J. Biochem. 222, 415–424
16. Slomiany, A., Murt, V. L. N., Liu, Y. H., Carter, S. R., and Slomiany, B. L. (1988) Arch. Oral Biol. 33, 669–676
17. Carter, S. R., Slomiany, A., Gwozdzinski, K., Liu, Y. H., and Slomiany, B. L. (1988) J. Biol. Chem. 263, 11977–11984
18. Goo, Y., and Hotta, K. (1993) Glycoconjugate J. 10, 326 (abstr.)
19. Laski, I. A., Singer, M. S., Dowbenko, D., Imali, Y., Hennel, E. J., Fennie, C., Gillett, N., Watson, S. R., and Rosen, S. D. (1992) Cell 69, 927–938
20. Hemmerich, H., Leffler, H., and Rosen, S. D. (1995) J. Biol. Chem. 270, 12065–12074
21. Cramm, D., and Rosen, S. D. (1995) J. Biol. Chem. 270, 22614–22624
22. Scudde, P. R., Shalilah, K., Dufflin, K. L., Streeter, P. R., and Jacob, G. S. (1994) Glyobiology 4, 929–933
23. Chandrasekaran, E. V., Jia, R. K., Larsen, R. D., Wlasichuk, K., and Matta, K. L. (1995) Biochemistry 34, 2925–2936