Tyrosine Sulfation Is a trans-Golgi-specific Protein Modification

Patrick A. Baeuerle and Wieland B. Huttner

Cell Biology Program, European Molecular Biology Laboratory, 6900 Heidelberg, Federal Republic of Germany

Abstract. The trans-Golgi has been recognized as having a key role in terminal glycosylation and sorting of proteins. Here we show that tyrosine sulfation, a frequent modification of secretory proteins, occurs specifically in the trans-Golgi. The heavy chain of immunoglobulin M (IgM) produced by hybridoma cells was found to contain tyrosine sulfate. This finding allowed the comparison of the state of sulfation of the heavy chain with the state of processing of its N-linked oligosaccharides. First, the pre-trans-Golgi forms of the IgM heavy chain, which lacked galactose and sialic acid, were unsulfated, whereas the trans-Golgi form, identified by the presence of galactose and sialic acid, and the secreted form of the IgM heavy chain were sulfated. Second, the earliest form of the heavy chain detectable by sulfate labeling, as well as the heavy chain sulfated in a cell-free system in the absence of vesicle transport, already contained galactose and sialic acid. Third, sulfate-labeled IgM moved to the cell surface with kinetics identical to those of galactose-labeled IgM. Lastly, IgM labeled with sulfate at 20°C was not transported to the cell surface at 20°C but reached the cell surface at 37°C. The data suggest that within the trans-Golgi, tyrosine sulfation of IgM occurred at least in part after terminal glycosylation and therefore appeared to be the last modification of this constitutively secreted protein before its exit from this compartment. Furthermore, the results establish the covalent modification of amino acid side chains as a novel function of the trans-Golgi.

A major function of the Golgi complex is the covalent modification of proteins destined for lysosomes, secretory granules, and the plasma membrane. Golgi-specific processing of asparagine-linked oligosaccharides is particularly well characterized (for review see Kornfeld and Kornfeld, 1985; Farquhar, 1985). These reactions include (a) the specific removal of carbohydrate units, (b) the addition of new carbohydrates, and (c) the addition of residues other than carbohydrates, such as phosphate to N-linked oligosaccharides. The detailed characterization of these modification reactions has contributed significantly to the understanding of the structural and functional organization of the Golgi complex, which is currently viewed to comprise at least three distinct subcompartments, the cis-, medial, and trans-Golgi (for reviews see Farquhar and Palade, 1981; Tar, 1982; Dunphy and Rothman, 1985; Farquhar, 1985). Compared with the information about the localization of processing reactions involving N-linked oligosaccharides in the Golgi subcompartments, little is known about the localization of amino acid side chain modifications in the specific subcompartments of the Golgi.

Recent work has indicated that the sulfation of tyrosine residues is a widespread protein modification and that secretory proteins are the major physiological substrates for the sulfating enzyme(s) (Huttner, 1982; Hille et al., 1984; for review see Huttner and Baeuerle, 1987). One would therefore expect that the sulfation reaction should occur in one or more of those compartments that are part of the secretory pathway, i.e., the rough endoplasmic reticulum, the Golgi complex, and the various transport vesicles and storage granules. Indeed, a recent study indicated that after subcellular fractionation tyrosylprotein sulfotransferase activity is highest in Golgi-enriched membrane fractions and that the active site of this membrane-bound enzyme is oriented toward the Golgi lumen (Lee and Huttner, 1985).

Among the subcompartments of the Golgi, the trans-most structures, referred to as the trans-Golgi network (Griffiths and Simons, 1986), have recently received a great deal of attention. The trans-Golgi network has been proposed to have a key role in the sorting of lysosomal, plasma membrane, and secretory proteins into membrane vesicles that transport them to their specific destination. To understand the molecular mechanisms involved in these sorting processes, it would be helpful to identify protein components specific to the trans-Golgi network. Such protein components would provide new markers for the trans-Golgi network that could be used for a more refined description of this compartment and that could also be employed for its isolation. Furthermore, the characterization and comparison of several different trans-Golgi network-specific proteins may help to identify domains on these proteins that are involved in their specific localization and retention in the trans-Golgi network. Here, we report, using IgM as a model protein, that tyrosine sulfation specifically occurs in the trans-Golgi and

Dr. Baeuerle's present address is Whitehead Institute, Nine Cambridge Center, Cambridge, MA 02142.
apparently is the last modification of this secretory protein before its exit from the trans-Golgi.

Materials and Methods
Isotopes

L-[35S]methionine (carrier-free), L-[3,5-3H]lysolecine (1.5 TBq/mmol), L-[35S(U)]tyrosine (1.85 GBq/mmol), [35S]sulfite or sodium [35S]sulfate (carrier-free), L-[6-3H]fucose (0.93 TBq/mmol), and D-[4,5-3H(N)]galactose (1.7 TBq/mmol), and D-[2-3H(N)]mannose (0.89 TBq/mmol) were obtained from New England Nuclear (Dreieich, FRG). 3'-Phosphoadenosine 5'-phospho-[35S]sulfate (PAPS; 4.81 TBq/mmol) was kindly provided before its exit from the apparently is the last modification of this secretory protein.

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Long-term Labeling

Cells were resuspended at a density of 1 x 10^7 cells/ml in labeling DME supplemented with 0.1% dialyzed FCS and allowed to equilibrate for 1 h. Cells were then labeled with a mixture of 85.5 MBq/ml [35S]sulfate and 12.3 MBq/ml [3H]galactose for 5 min in the incubator. Therefore the cell culture was immediately cooled on ice, and the cells were removed and collected by centrifugation at 4°C in a 50-ml tube (Falcon Labware). After a wash in ice-cold PBS, the cells were resuspended at a density of 1 x 10^7 cells/ml in complete DME supplemented with 0.1% FCS and allowed to equilibrate for 1 h. They were then labeled at 20°C for 10 min with 37 MBq/ml [35S]sulfate in the closed tube. Therefore the tube was placed on ice, the cells were collected by centrifugation at 4°C, washed in ice-cold PBS, split into several aliquots, and resuspended in 15-ml tubes in chase medium (complete DME supplemented with 0.1% FCS) of either 0, 20, or 37°C that had been pre-equilibrated in a 10% CO_2 atmosphere at 37°C. Immediately after resuspension, two aliquots were removed and cooled on ice (zero time values). The cell suspension remaining in the 50-ml Falcon tube was then returned to the incubator and placed on a slowly rotating platform during the chase period. Further aliquots of the well-equilibrated culture cell were removed at the indicated times and immediately placed on ice. Cells and media were further processed as described under Immunofluorometry of IgM.

20°C Block

Cells were resuspended in a 50-ml tube at a density of 1 x 10^7 cells/ml in labeling DME supplemented with 0.1% dialyzed FCS and allowed to equilibrate for 5 h on a slowly rotating platform in the incubator at 37°C. The tube was then closed to preserve the atmosphere, removed from the incubator, and placed in a 20°C waterbath for 5 min with occasional mixing. Cells were then labeled at 20°C for 10 min with 37 MBq/ml [35S]sulfate in the closed tube. Therefore the tube was placed on ice, the cells were collected by centrifugation at 4°C, washed in ice-cold PBS, split into several aliquots, and resuspended in 15-ml tubes in chase medium (complete DME supplemented with 0.1% FCS) of either 0, 20, or 37°C that had been pre-equilibrated in all cases in a 10% CO_2 atmosphere. The tubes were closed and placed on ice for (or the zero values) or in waterbaths of 20 or 37°C, respectively. Aliquots of the cell cultures in the waterbaths were collected after 0, 10, or 20 min and 35 as indicated, and placed on ice. Cells and media were further processed as described in Immunofluorometry of IgM.

Cell-free Sulfation

5 x 10^6 cells were pelleted at 150 g, washed once in ice-cold PBS, and resuspended in 0.5 ml of sulfation buffer (3 mM imidazole-HCl, pH 7.0, 0.25 mM sucrose, 25 mM NaF, 2.5 mM MgCl_2, 2.5 mM MnCl_2, 1 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF)). The cell suspension was passed five times through a steel ball homogenizer (Balch et al., 1984) with an 8-μm fitting. The homogenizer was rinsed with 0.5 ml sulfation buffer and the wash pooled with the homogenate. The lysate was incubated in the presence of 200 μg/ml hexokinase (Boehringer Mannheim) and 5 mM N(+)glucosamine for 10 min at 4°C (Schlossmann et al., 1984). 0.96 MBq/ml [35S]PAPS was added to the supernatant obtained after centrifugation at 10 min at 800 g and the reaction allowed for 2 h at 30°C. The reaction was stopped by the addition of 1% SDS following by boiling. An aliquot of the lysate was directly subjected to SDS-PAGE. IgM was purified from the remaining lysate as described in Immunofluorometry of IgM.

Immunoaffinity Purification of IgM

After labeling, cells were separated from the medium by centrifugation at 150 g for 5 min at 4°C. Cell culture supernatants were centrifuged at 10,000 g for 10 min to remove debris. Cells pellets were prepared for immunoaffinity purification by boiling for 5 min in 400 μl of an SDS-lysis buffer consisting of 2% SDS, 20 mM Tris-HCl, pH 7.5, 10 mM benzamidine,
10 mM e-caproic acid, 5 mM EDTA, aprotinin (300 kallikrein inhibitor units/ml), and 1 mM PMSF. The cleared culture media were used without this treatment. To both cell lysates and culture media 1.5 ml of 10% wt/vol NP-40 was added and the volume was brought to 15 ml in a 15 ml tube with buffer A (0.5 M NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, 5 mM EDTA, pH 7.5). Then 100 µl of a 50% gelled gel per volume suspension of anti-mouse IgG-agarose (A6531; Sigma Chemical Co.) equilibrated in buffer A containing 0.1% (wt/vol) sodium azide were added, followed by incubation for 2 h end-over-end at 4°C. The agarose beads were collected by centrifugation for 3 min at 500 g and washed four times with 15 ml of ice-cold buffer A. Bound IgM was eluted from the beads by boiling in 1 ml of the SDS-lysis buffer followed by centrifugation. The immunoaffinity-purified IgM in the supernatants was precipitated, after the addition of 50 µg bovine hemoglobin as carrier, in 80% acetone at -20°C. After centrifugation for 10 min at 10,000 g, the pellets were dissolved either in Laemmli sample buffer. Samples were subjected to SDS-PAGE.

Ricin-binding Assay
Acetone pellets containing immunoaffinity-purified IgM were dissolved in 20 µl 0.1 N NaOH and immediately brought to a pH of ~7.5 by the addition of 400 µl of a solution containing 100 mM sodium citrate, pH 5.5, 0.1% SDS, aprotinin (300 kallikrein inhibitor units/ml), and 1 mM PMSF. Samples were incubated overnight at 30°C in the presence or absence of 15 mM/µl endo H (Seikagaku, Tokyo, Japan). Proteins were precipitated using 80% acetone, and the resulting pellets were dissolved in Laemmli sample buffer for SDS-PAGE.

Neuraminidase Treatment
Acetone pellets containing immunoaffinity-purified IgM were dissolved in 20 µl 0.1 N NaOH and immediately brought to a pH of ~7.5 by the addition of 400 µl of a solution containing 100 mM sodium acetate, pH 5.0, 1 M NaCl, 1 mM CaCl₂, 1 mM MgCl₂. Then 80 µl of a 50% packed gel per volume suspension of ricin-agarose (L2757; Sigma Chemical Co.) equilibrated in buffer B was added. After 2 h of incubation at room temperature on a Viberax shaker, free and ricin-bound IgM were separated from each other by pelleting of the agarose beads for 5 min at 500 g. Protein in the supernatant was precipitated using 80% acetone and dissolved in Laemmli sample buffer. The pellets of the agarose beads were washed twice with buffer B, followed by elution of the bound IgM by boiling in Laemmli sample buffer. Samples were subjected to SDS-PAGE.

Polyacrylamide Gel Electrophoresis
SDS-PAGE was performed as described by Laemmli (1970). Processing of gels after electrophoresis was as described previously (Lee and Huttner, 1983).

Tyrosine Sulfate Analysis of the IgM Heavy Chain
Immunoaffinity-purified [³⁵S]sulfate- or [³⁵S] sulfate-[³⁴C]tyrosine double-labeled IgM heavy chain separated by SDS-PAGE under reducing conditions was analyzed by alkaline hydrolysis for the presence of tyrosine sulfate as described previously (Lee and Huttner, 1983; Huttner, 1984). Pronase digestion (Huttner, 1984) of IgM heavy chains in gel pieces was performed using 1 mg/ml pronase (Boehringer Mannheim) in 50 mM ammonium bicarbonate for 22 h at 37°C. The digest was lyophilized, redissolved in 200 µl H₂O, and excess pronase (as well as sulfated oligosaccharides) precipitated by 80% acetone. The resulting supernatant was dried in a speed-vac and subjected to two-dimensional thin-layer electrophoresis as described (Baeuerle and Huttner, 1984; Huttner, 1984).

Determination of the Stoichiometry of Tyrosine Sulfation
Immunoaffinity-purified [³⁴C]tyrosine- or [³⁴C]tyrosine-labeled IgM heavy chain separated by SDS-PAGE under reducing conditions was analyzed for the stoichiometry of tyrosine sulfonylation as described (Baeuerle and Huttner, 1985).

Quantitation of Radioactive Samples
Gel pieces were dissolved in H₂O. Samples were analyzed by liquid scintillation counting in Aquablu (Baker Co., Frankfurt, FRG). Double-labeled samples were counted using two separate windows. The spillover of the ³H radioactivity into the ³⁵S window was <1%, and the spillover of the ³⁵S radioactivity into the ³H window <4%.

Results

The Heavy Chain of IgM is Sulfated
Cell cultures of the hybridoma cell line 81-O4 were labeled with [³⁴C]tyrosine or [³⁵S]sulfate, and intracellular and secreted IgM was immunoaffinity-purified by anti-mouse IgG-agarose recognizing the light chains of IgM. After SDS-PAGE under nonreducing conditions (not shown), the secreted IgM had a slower mobility than the thyroglobulin dimer (M, 669,000), indicating that it was present in the pentameric 19S form. Under reducing conditions, two intracellular forms of the [³⁴C]tyrosine-labeled IgM heavy chain could be distinguished with apparent molecular masses of 71 and 76 kD (Fig. 1 A, fifth lane). (The proportion of the 71-kD form to the 76-kD form was found to vary with the age of the cultures.) In the medium, the heavy chain appeared as a diffuse band with a molecular mass ranging from 76-86 kD (Fig. 1 A, sixth lane). Heavy and light chains of IgM represented the major secretory proteins of the medium (Fig. 1 A, second lane). When IgM secreted by [³⁵S]sulfate-labeled cells was immunoaffinity-purified and analyzed on SDS gels under reducing conditions, the heavy chain was found to contain [³⁵S]sulfate (Fig. 1 A, eighth lane). SDS-PAGE of immunoaffinity-purified secreted IgM under nonreducing conditions indicated that the [³⁵S]sulfate label was contained in the 19S form of IgM (data not shown). Intracellularly, only the 76-kD form of the heavy chain of IgM incorporated [³⁵S]sulfate (Fig. 1 A, seventh lane). No incorporation of [³⁵S]sulfate was observed into the light chains (Fig. 1 A, eighth lane). The IgM heavy chain was a major sulfated protein in the medium (Fig. 1 A, fourth lane). In the cells, several sulfated bands in addition to the heavy chain were observed (Fig. 1 A, third lane) but most of them were apparently not secreted into the medium. The sulfation of IgM heavy chain in the hybridoma clone 81-O4 was not a singular observation. Two other IgM-secreting cell lines were tested, a rat hybridoma cell line (clone 13/305/9) and the mouse myeloma cell line WEHI 279.1. In both cases the IgM heavy chain was found to be sulfated (data not shown).

Sulfate Is Bound to Tyrosine and N-linked Oligosaccharides
Immunoaffinity-purified [³⁵S]sulfate-labeled IgM heavy chain separated by SDS-PAGE under reducing conditions was subjected to alkaline hydrolysis, a condition in which the tyrosine sulfate ester is stable (Huttner, 1984). Two-dimensional thin-layer electrophoresis of the hydrolysate revealed the
The IgM heavy chain of the hybridoma cell line 81-O4 is sulfated on tyrosine. (A) Hybridoma cell cultures were labeled for 18 h with \(^{[3}H\)tyrosine (\(^{3}H\)-Tyr) or \(^{35}S\)sulfate (\(^{35}S\)O\(^4\)). Aliquots of cell lysates (lanes C) and cell culture media (lanes M) were analyzed, either directly (total) or after immunoprecipitation of IgM, by reducing SDS-PAGE. Fluorograms are shown. The IgM heavy chains (HC) and light chains (LC) are indicated by brackets.

To determine the proportion of tyrosine sulfate relative to the total sulfate incorporated into the IgM heavy chain, cell cultures were double-labeled with \(^{[3}H\)tyrosine and \(^{35}S\)sulfate. After immunoaffinity purification and SDS-PAGE, radioactivity in the IgM heavy chain was eluted from the gel piece by pronase digestion and the \(^{35}S\)/\(^{3}H\) ratio was determined in an aliquot of the eluate. Another aliquot of the eluate was subjected to alkaline hydrolysis in barium hydroxide followed by neutralization with dilute sulfuric acid. In this method [\(^{35}S\)sulfate bound to carbohydrates is hydrolyzed and precipitated as barium \(^{35}S\)sulfate, whereas tyrosine \(^{35}S\)sulfate remains in the supernatant (Huttner, 1984). Fig. 2 (left) shows that, compared with the eluate (A), the \(^{35}S\)/\(^{3}H\) ratio in the neutralized supernatant (B) was decreased by 36%. Thin-layer electrophoresis indicated that all of the \(^{35}S\) in the neutralized supernatant was present as tyrosine sulfate (data not shown). Thus, tyrosine sulfate constituted 64% of the total sulfate present in the IgM heavy chain.

To investigate whether the 36% alkali-labile sulfate was bound to N-linked oligosaccharides, cell cultures were labeled with \(^{35}S\)sulfate in the presence of tunicamycin, an inhibitor of N-glycosylation (for review see Elbein, 1981). Compared with the 76-kD heavy chain of IgM secreted from control cells (Fig. 2, central panel, left lane), the non-glycosylated heavy chain of IgM secreted from tunicamycin-treated cells had a faster mobility in SDS-gels (Mr, 61,000) and was found in lesser amounts in the medium (Fig. 2, central panel, right lane). This 61-kD heavy chain was still sulfated. When the 61-kD IgM heavy chain, double-labeled with \(^{35}S\)sulfate and \(^{[3}H\)tyrosine, was analyzed by alkaline hydrolysis followed by neutralization, the \(^{35}S\)/\(^{3}H\) ratio determined in the pronase eluate (Fig. 2, right panel, A) was al-

Figure 1. The IgM heavy chain of the hybridoma cell line 81-O4 is sulfated on tyrosine. (A) Hybridoma cell cultures were labeled for 18 h with \(^{[3}H\)tyrosine (\(^{3}H\)-Tyr) or \(^{35}S\)sulfate (\(^{35}S\)O\(^4\)). Aliquots of cell lysates (lanes C) and cell culture media (lanes M) were analyzed, either directly (total) or after immunoprecipitation of IgM, by reducing SDS-PAGE. Fluorograms are shown. The IgM heavy chains (HC) and light chains (LC) are indicated by brackets.

Figure 2. The IgM heavy chain is also sulfated on N-linked carbohydrates. Hybridoma cell cultures were double-labeled for 18 h with \(^{[3}H\)tyrosine and \(^{35}S\)sulfate in the absence (control) and presence of 1 \(\mu\)g/ml tunicamycin. IgM was immunoaffinity purified from the media and separated by reducing SDS-PAGE. Gel pieces containing the IgM heavy chain were treated with pronase, and the ratio of \(^{35}S\) to \(^{3}H\) radioactivity in an aliquot of the eluates was determined (columns A). The value found for the heavy chain from control dishes was arbitrarily set to 1.0. The remainder of the eluates was subjected to alkaline hydrolysis followed by neutralization, and the ratio of \(^{35}S\) to \(^{3}H\) radioactivity in the neutralized supernatants was determined (columns B). For the control heavy chain the ratio was determined in triplicates (bar indicates SD). The recovery of \(^{35}S\) radioactivity of tyrosine \(^{35}S\)sulfate standard in the neutralized supernatant was equal to the recovery of \(^{3}H\) radioactivity of the \(^{[3}H\)tyrosine-labeled IgM heavy chain (both 90%). (Center) \(^{35}S\)sulfate-labeled IgM heavy chain of control (left lane) and tunicamycin-treated (right lane) cell cultures was immunoaffinity purified from the media and subjected to reducing SDS-PAGE. A fluorogram is shown. The arrowhead on the left indicates the position of the control heavy chain. The arrowhead with the asterisk on the right indicates the position of the IgM heavy chain secreted by tunicamycin-treated cell cultures.

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most identical to that in the neutralized supernatant (B). This indicated that in the absence of N-linked carbohydrates all sulfate incorporated into the heavy chain was present in an alkali-stable form. Thin-layer electrophoresis showed that this alkali-stable sulfate was indeed tyrosine sulfate (data not shown). The finding that tunicamycin treatment prevented the sulfation of IgM in alkali-labile linkage supported the assumption that the 36% of alkali-labile sulfate found in the control IgM heavy chain represented sulfate bound to N-linked carbohydrate. Consistent with the presence of sulfated N-linked oligosaccharides on the IgM heavy chain was the observation that treatment with endoglycosidase F removed part of the incorporated radioactive sulfate (data not shown). Inhibition of N-glycosylation by tunicamycin reduced the relative extent of tyrosine sulfation of the IgM heavy chain (see Fig. 1, fifth lane) to each other was investigated by pulse-chase labeling with [3H]tyrosine (Fig. 3). At the beginning of the chase period only the 71-kD form was present. After 40 min of chase ~50% of the 71-kD form had been converted into the 76-kD form. Thus, the 71-kD form was a precursor of the 76-kD form.

The two intracellular forms of the IgM heavy chain were characterized with regard to the state of processing of their N-linked carbohydrates (Fig. 4). The 71-kD form incorporated [3H]mannose and trace amounts of [3H]fucose but no [3H]galactose (Fig. 4, top). Treatment with endo-β-N-acetylglucosaminidase H (endo H), an enzyme known to remove N-linked oligosaccharides that have not been modified by medial Golgi-specific enzymes (Tarentino et al., 1974), markedly increased the electrophoretic mobility of the 71-kD heavy chain (Fig. 4, lower left). The mobility of the 71-kD form was unaffected by treatment with neuraminidase (Fig. 4, lower right). The intracellular 71-kD form of the IgM heavy chain is a precursor of the 76-kD form. A hybridoma cell culture was pulse-labeled for 3 min with [35S]methionine followed by a 40-min chase. At the indicated chase times, aliquots of the cell culture (cells plus medium) were lysed in SDS, and IgM was immunopurified from the lysates and analyzed by reducing SDS-PAGE and fluorography. The radioactivity in the total IgM heavy chain (71- plus 76-kD form) present at a given chase time.
5, lower right). These results indicated that IgM containing the 71-kD form of the heavy chain was localized in the rER and the cis- and medial Golgi but had not yet acquired the trans-Golgi-specific modifications galactose and sialic acid (Griffiths et al., 1982; Roth and Berger, 1982; Roth et al., 1985; Geuze et al., 1985). (In view of the absence of galactose from the 71-kD heavy chain, we suggest that the small portion of fucose-labeled, 71-kD form [see Fig. 4] was contained in IgM that had been processed by a fucosyltransferase localized in the medial Golgi, which acted on the innermost N-acetylglucosamine of the oligosaccharides [Kornfeld and Kornfeld, 1985]. It is unclear whether this fucose-labeled 71-kD heavy chain was still present in medial Golgi cisternae or whether it had already reached the trans-Golgi but not yet acquired galactose.) No detectable amounts of [35S]sulfate were incorporated into the 71-kD form (Fig. 4, top), indicating that neither tyrosine nor carbohydrate sulfation of the IgM heavy chain occurred in the rER, the cis-Golgi, and the medial Golgi before fucosylation.

The state of oligosaccharide processing of the intracellular 76-kD form of the IgM heavy chain was very similar to that of the 76–86 kD forms found in the medium (collectively referred to as 76-kD form). In both forms the ratio of [3H]mannose to [3H]tyrosine was less compared with that of the 71-kD form, indicating the loss of mannose residues (Fig. 4, top). Both 76-kD forms incorporated [3H]fucose and [3H]galactose. Treatment of the 76-kD forms of cells and medium with neuraminidase increased their mobility in SDS gels (Fig. 4, lower right), indicating the presence of sialic acid residues on the heavy chain. Thus the intracellular 76-kD form of the IgM heavy chain was present in the trans-Golgi compartment and/or post-Golgi vesicles. (The observation that the 76-kD form showed a small increase in electrophoretic mobility after endo H digestion [Fig. 4, lower left] does not contradict this conclusion, since it has been reported that two of the five high mannose type oligosaccharides added to the heavy chain of IgM may remain endo H sensitive [Hickman et al., 1972].) In contrast to the intracellular 71-kD form, the intracellular 76-kD form did incorporate [35S]sulfate (Fig. 4, top). This indicated that, intracellularly, the sulfated form of IgM was localized largely, if not exclusively, in the trans-Golgi compartment and/or post-Golgi vesicles. However, the results obtained so far did not exclude the possibility that sulfation occurred late in the medial Golgi on the 71-kD form of the heavy chain followed by rapid transport of IgM to the trans-Golgi and conversion of the heavy chain to the 76-kD form, which then appeared as the predominant band upon long-term labeling.

**Tyrosine Sulfation of the Heavy Chain Takes Place after Exit of IgM from the Medial Golgi**

Hybridoma cells were labeled with [35S]sulfate for short periods of time and the IgM heavy chain was analyzed for the presence of trans-Golgi-specific modifications. The proportion of tyrosine sulfate to total sulfate incorporated into the heavy chain after 5 min of labeling was similar to that observed after long-term labeling. After immunoaffinity purification of IgM and SDS-PAGE, [35S]sulfate incorporation was detectable by fluorography after 4 min of labeling, but not after 1 and 2 min of labeling, and was confined to the 76-kD form (Fig. 5, top). The 76-kD heavy chain that came sulfate-labeled during a 4-min pulse bound to ricin-agarose, indicating the presence of galactose (Baenziger and Fiete, 1979), and was sensitive to neuraminidase treatment, indicating the presence of sialic acid. When the kinetics of [35S]sulfate incorporation into the IgM heavy chain was investigated (Fig. 5, bottom) a lag phase of ~2 min was observed before the incorporation started to increase linearly. Most likely this lag phase reflected the time requirements for radioactive sulfate uptake, PAPS synthesis, and PAPS translocation. From the kinetics in Fig. 5 it is apparent that ~80% of the [35S]sulfate found in the IgM heavy chain after labeling for 4 min actually became incorporated during the third and fourth minute after addition of the label. Thus, during incubation of cells with radioactive sulfate for 4 min, IgM was effectively labeled only for ~2 min. Since, after this short labeling period, essentially all of the labeled IgM heavy chain was in the terminally glycosylated 76-kD form, it is very unlikely that tyrosine sulfation occurs late in the medial Golgi.

In line with this conclusion were observations made after
Figure 6. The secretion kinetics of [3H]galactose- and [35S]sulfate-labeled IgM are very similar. A hybridoma cell culture was pulse-labeled for 5 min with a mixture of [3H]galactose and [35S]sulfate followed by a 60-min chase. At the indicated chase times, aliquots were removed in triplicate, and cells and medium separated and analyzed directly by reducing SDS-PAGE. The [H] and 35S radioactivity in the band corresponding to the IgM heavy chain was determined by liquid scintillation counting. The labeled IgM heavy chain found in the medium at the various chase times is expressed as percent of the total (intracellular plus secreted) labeled IgM heavy chain ([3H]galactose, solid circles; [35S]sulfate, open circles) and is plotted on a logarithmic time scale. Bars indicate SD. In a different experiment, hybridoma cell cultures were pulse-labeled for 10 min with [3H]tyrosine followed by a 4-h chase. At the indicated chase times (solid triangles), cells and media were separated, and IgM was immunoaffinity-purified from cell lysates and cell culture media and analyzed by reducing SDS-PAGE. The radioactivity in the [3H]tyrosine-labeled IgM heavy chain was determined and the secretion kinetics is expressed as above. The times at which the linear secretion kinetics intercepts the 50% line (half-solid arrows) and, after extrapolation (dashed lines), the 0% line (open arrows), are indicated on the abscissa.

Treatment of cell cultures with the drug monensin (data not shown). Monensin has been reported to perturb Golgi processing reactions primarily at the level of the trans-cisternae and to lead to an accumulation of newly synthesized proteins in the medial Golgi (Griffiths et al., 1983; Tartakoff, 1983). After labeling cell cultures with [3H]tyrosine in the presence of 10^{-6} M monensin, only the 71-kD form but not the 76-kD form of the IgM heavy chain was found. The 71-kD form accumulated intracellularly and did not incorporate any [35S]sulfate.

Tyrosine Sulfation of IgM Occurs in the Same Compartment as Galactosylation

To investigate whether sulfation of IgM occurs in the trans-Golgi or later during the intracellular transport in post-Golgi transport vesicles, the kinetics of secretion of IgM pulse-labeled with radioactive sulfate was compared with that of IgM pulse-labeled with radioactive galactose. The amount of [3H]galactose and [35S]sulfate incorporated into the IgM heavy chain after a 5-min pulse labeling increased slightly during the first 15 min of the chase, with identical rates for both isotopes, and plateaued thereafter (data not shown). This indicated that the specific activity of the cosubstrate for sulfation (PAPS) and that of the cosubstrate for galactosylation (UDP-galactose) decreased rapidly and with similar relative rates during the chase. The observation that the amount of [3H]galactose and [35S]sulfate incorporated into the total heavy chain (cells plus medium) did not decrease during the chase showed that both modifications were irreversibly attached to the IgM heavy chain. These findings provided the necessary basis for the quantitative comparison of secretion kinetics of galactose- and sulfate-labeled IgM (presented as a semi-logarithmic plot in Fig. 6). No significant difference in the secretion kinetics of [3H]galactose- and [35S]sulfate-labeled IgM heavy chains was observed. These secretion kinetics, however, clearly differed from that of [3H]tyrosine-labeled heavy chain, which appeared much later in the medium (Fig. 6). These results strongly suggest that sulfate addition to tyrosine (as well as to N-linked carbohydrate) occurs at a time point during intracellular transport of IgM very similar to that of galactose addition, i.e., in the trans-Golgi, and not much later, e.g., in transport vesicles shortly before exocytosis.

Plotting the secretion of IgM pulse-labeled with [35S]sulfate, [3H]galactose, or [3H]tyrosine on a logarithmic time scale yielded straight lines (linear regression coefficients \(\geq 0.9966\)) (Fig. 6). Intercepts of the linear secretion kinetics with the 50% line gave the \(t_0\) values (\(t_0 = 20\) min for [3H]galactose and [35S]sulfate; \(t_0 = 94\) min for [3H]tyrosine). Intercepts of the extrapolated linearized secretion kinetics with the 0% line gave the \(t_a\) values, which are the times of first appearance of the labels at the cell surface (\(t_a = 2\) min for [3H]galactose and [35S]sulfate; \(t_a = 26\) min for [3H]tyrosine). The secretion of pulse-labeled IgM during the course of our measurements can be described as: percent of total IgM secreted = \((50)/(\log(t_a/t_0)) \cdot \log(t/t_0)\). The first order kinetics observed for the transport of pulse-labeled IgM to the cell surface indicates the existence of a rate-limiting transport step between the sites of sulfation and galactosylation (i.e., the trans-Golgi) and the cell surface.

Transport of Tyrosine-sulfated IgM to the Cell Surface is Blocked at 20°C

Matlin and Simons (1983) and Saraste and Kuismanen (1984) reported that the intracellular transport of viral membrane glycoproteins from the Golgi to the cell surface can be arrested in a reversible manner by reducing the temperature to 20°C. This led to an accumulation of terminally glycosylated viral proteins in the trans-most structure of the Golgi, designated the trans-Golgi network (see Griffiths and Simons, 1986). This compartment is further characterized by the presence of galactosyl- and sialyltransferases (Geuze et al., 1985; Roth et al., 1985). We investigated whether sulfate-labeled secretory IgM is also reversibly arrested in its intracellular transport at 20°C. When labeling was performed at 20°C, only the 76-kD form of the heavy chain incorporated sulfate (Fig. 7, left). The band of cellular heavy chain was broader (76–86 kD) after sulfate-labeling at 20°C than after labeling at 37°C (cf. Fig. 7, left, with Fig. 1 A, and Fig. 4), which probably reflected a longer residence time in the trans-Golgi resulting in a higher degree of sialylation and thus slower electrophoretic mobility. The proportion of tyrosine sulfate to the total sulfate incorporated into the heavy chain was similar (59%) to that observed after long-term labeling at 37°C (64%, see Fig. 2). The incorporation of radioactive sulfate into IgM at 20°C was reduced by a factor of 5.15 as compared with 37°C (\(Q_{10} = 2.6\)). The terminally glycosylated 76-kD IgM heavy chain pulse-labeled for 10 min with [35S]sulfate at 20°C was not detectable in the medium after 15 min (Fig. 7, left and right) and 30 min (Fig.
Figure 7. [35S]Sulfate-labeled IgM is not transported to the cell surface at 20°C. A hybridoma cell culture was pulse-labeled for 10 min at 20°C with [35S]Sulfate followed by a 30-min chase at the indicated temperatures. The percentages of sulfate-labeled IgM heavy chain secreted to 37°C (Fig. 7, right) were very similar to those of galactose-chase at 37°C. Also, sulfate-labeled macromolecules of high molecular mass (presumably proteoglycans) were not secreted at 20°C (Fig. 7, left). The IgM arrested intracellularly at 20°C appeared in the medium when the temperature was raised to 37°C (Fig. 7). The percentages of sulfate-labeled IgM heavy chain secreted within 15 and 30 min after the temperature was shifted back to 37°C (Fig. 7, right) were very similar to those of galactose-labeled IgM secreted in pulse-chase experiments at 37°C (cf. Fig. 6). Even after continuous sulfate-labeling at 20°C for 80 min (at which time ~90% of the total sulfate-labeled IgM was still found intracellularly; data not shown), the proportion of the intracellularly arrested sulfate-labeled IgM that was secreted during a subsequent 20-min chase at 37°C was similar (57%) to that of galactose-labeled IgM secreted in pulse-chase experiments at 37°C (cf. Fig. 6). This suggested (a) that the reduced temperature inhibited a step in the intracellular transport of IgM that occurred shortly after galactosylation, and (b) that tyrosine and carbohydrate sulfation of the terminally glycosylated 76-kD IgM heavy chain also took place before this temperature-sensitive transport step. In the case of viral membrane proteins in epithelial cells, the 20°C block affects the exit from the trans-Golgi (Matlin and Simons, 1983; Saraste and Kuismans, 1984). Constitutively secreted proteins and viral membrane proteins apparently reach the cell surface in the same transport vesicles (Stroos et al., 1983). In view of these observations it is likely that at 20°C the transport of IgM in hybridoma cells is also blocked at the exit from the trans-Golgi, and that tyrosine and carbohydrate sulfation of IgM occur before exit from this compartment.

Tyrosine Sulfation is the Last Modification of IgM in the trans-Golgi

A postnuclear supernatant of an 81-O4 cell homogenate was depleted of endogenous ATP in order to prevent vesicle transport between compartments in vitro (Balch et al., 1984; Davey et al., 1985). The cell-free sulfation of IgM was then investigated using [35S]PAPS under conditions known to allow translocation of this nucleotide into Golgi membrane vesicles (Schwarz et al., 1984). The Golgi vesicles appeared to be largely intact, since the pattern of cell-free protein sulfation was similar to that observed after sulfate labeling of intact cells and was strikingly different from that seen with detergent-lysed membrane vesicles where tubulin was the major sulfated protein (Lee and Huttner, 1985; Huttner and Baeuerle, 1987). Only the 76-kD form of the IgM heavy chain incorporated sulfate in the cell-free system (Fig. 8), and ~50% of the incorporated sulfate was bound to tyrosine (as compared with 64% after in vivo labeling, see Fig. 2). The cell-free sulfated IgM heavy chain quantitatively bound to ricin-agarose, indicating the presence of galactose (Fig. 8, left). After treatment with neuraminidase, the cell-free sulfated IgM heavy chain shifted in its mobility in SDS-gels to faster-migrating forms, indicating the presence of sialic acid (Fig. 8, right). These results show that both tyrosylprotein sulfotransferase and carbohydrate sulfotransferase are active in vesicles that contain the heavy chain of IgM in an already terminally glycosylated form and imply that tyrosine and carbohydrate sulfation of IgM occur after galactosylation and sialylation.

If sulfation of the IgM heavy chain occurs after galactosylation and sialylation and thus is the last modification before exit from the trans-Golgi, one may expect that the average degree of sulfation of the terminally glycosylated 76-kD heavy chain from cells is less than that from the medium. This was indeed found to be the case, since the ratio of sulfate/tyrosine incorporation into the cellular 76-kD form was ~0.75 of that observed for the heavy chain from the medium (Table 1).

Discussion

We have shown that protein tyrosine sulfation specifically oc-
curs in the trans-Golgi. The enzyme catalyzing this post-translational modification (a) was localized via its activity towards a physiological substrate protein, IgM, rather than via translational modification (a) was localized via its activity to-the 3H and 35S radioactivity in the 76-kD forms of the heavy chain. Values are given after subtraction of background, which was 25 cpm for 35S and 19 cpm for 3H. Numerals in parenthesis give the 35S/3H ratios with the value of the secreted 76-kD form arbitrarily set to 1.0.

| 76-kD form | Dish | [3S]Sulfate cpm | [3H]Tyrosine cpm | 35S/3H ratio |
|------------|------|----------------|----------------|-------------|
| Cellular   | 1    | 90             | 598            | 0.1505 (0.79)|
|            | 2    | 100            | 677            | 0.1477 (0.75)|
| Secreted   | 1    | 4,423          | 23,249         | 0.1902 (1.0)|
|            | 2    | 4,145          | 20,994         | 0.1974 (1.0)|

The intracellular 76-kD form of the IgM heavy chain contains less sulfate than the secreted form. Hybridoma cell cultures were double-labeled with [3H]tyrosine and [35S]sulfate for 18 h, and IgM was immunofluorinity-purified from the cells and medium followed by reducing SDS-PAGE and determination of the 3H and 35S radioactivity in the 76-kD forms of the heavy chain. Values are resolved by pulse-chase experiments. It is therefore possible that tyrosine sulfation occurs in both the trans-Golgi cisternae and the trans-Golgi network.

Under all experimental conditions, the sulfotransferase activity catalyzing the sulfation of N-linked oligosaccharides on IgM was found to colocalize with tyrosylprotein sulfotransferase in the trans-Golgi. Moreover, the cell-free sulfation of terminally glycosylated IgM in membrane vesicles after addition of [35S]PAPS implies that the translocation system for PAPS resides, at least in part, in trans-Golgi membranes. Thus, the trans-Golgi appears to be the major intracellular site where the various types of protein sulfation occur.

Tyrosine-sulfated proteins leave the cell by constitutive as well as regulated pathways (see Huttner and Baeuerle, 1987). These various secretory pathways are thought to diverge at the level of the trans-Golgi (Kelly, 1985; Griffiths and Simons, 1986). The localization of tyrosylprotein sulfotransferase activity in the trans-Golgi is not only consistent with these observations, but is also economical, since enzymes with post-trans-Golgi locations would require distinct localization signals and retrieval mechanisms.

It is unclear why an amino acid modification like protein tyrosine sulfation is confined to the trans-Golgi. In the case of N-glycosylation, the localization of specific processing enzymes to the endoplasmic reticulum and specific parts of the Golgi can be explained by the sequential formation of the final oligosaccharide structures. However, in the case of protein tyrosine sulfation, the presence of carbohydrates is not a requirement for this modification to occur, since unglycosylated proteins also become tyrosine-sulfated (see Huttner and Baeuerle, 1987). Hence, there is no a priori reason why protein tyrosine sulfation should not occur in a compartment other than the trans-Golgi. One possible explanation is that, for reasons of economy, the PAPS-translocating system is confined to a single subcompartment of the Golgi. This may have evolved to be the trans-Golgi, since PAPS is also the cosubstrate for carbohydrate sulfotransferases, the specific trans-Golgi localization of which may be necessary for the sequential formation of the final oligosaccharide structures. It is also conceivable that the trans-Golgi localization of protein tyrosine sulfation is related to a specific property of this compartment. It is worth noting that, within the secretory pathway, most modifications adding negatively charged groups to proteins have been shown (sialylation, Roth et al., 1985; N-linked oligosaccharide sulfation, tyrosine sulfation, see this study) or are likely (serine and threonine phosphorylation of secretory proteins, Rosa, P., and W. B. Huttner, unpublished data) to reside in the trans-Golgi.

IgM is the first member of the immunoglobulin supergene family shown to be tyrosine sulfated under physiological conditions. Tyrosylprotein sulfotransferase apparently recognizes tyrosine residues in the vicinity of acidic amino acids (Lee and Huttner, 1983; 1985) and consensus features of tyrosine sulfation sites have been delineated (Huttner et al., 1986; Hortin et al., 1986; Huttner and Baeuerle, 1987). In the constant part of the mouse IgM heavy chain, the sequence surrounding tyrosine 210 (Cys1 domain) conforms best with the consensus features, the only violation being the presence of a cysteine residue in position 213, which is disulfide bonded to cysteine 153 (Kehry et al., 1979). Disulfide bonds near tyrosine sulfation sites may be the

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**Table 1. Relative Sulfate Content of the Cellular and Secreted 76-kD Forms of the IgM Heavy Chain**

| 76-kD form | Dish | [3S]Sulfate cpm | [3H]Tyrosine cpm | 35S/3H ratio |
|------------|------|----------------|----------------|-------------|
| Cellular   | 1    | 90             | 598            | 0.1505 (0.79)|
|            | 2    | 100            | 677            | 0.1477 (0.75)|
| Secreted   | 1    | 4,423          | 23,249         | 0.1902 (1.0)|
|            | 2    | 4,145          | 20,994         | 0.1974 (1.0)|
cause of substoichiometric tyrosine sulfation (Huttner and Baeuerle, 1987), as observed here for the IgM in which every heavy chain and 90% containing only unsulfated heavy chains, whether they are clustered (10% of the pentamers containing only sulfated heavy chains and 90% containing only unsulfated heavy chains), or whether an intermediate situation exists. The functional significance of tyrosine sulfate in the C1 domain of IgM remains to be elucidated and may be related to the functional significance of tyrosine sulfate in the Clxl domain of IgM.

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