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SULFATED OLIGOSACCHARIDES IN HUMAN LYSOSOMAL ENZYMES

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SUMMARY: Cathepsin D, arylsulfatase A and the α-chain of ß-hexosaminidase are synthesized in human fibroblasts as sulfated polypeptides. The sulfate is added posttranslationally. Its half-life is less than one-tenth of that of the respective polypeptide chains. The sulfate residues were found on asparagine-linked oligosaccharides sensitive to endoglycosidase F and peptide: N-glycosidase F and resistant to endoglycosidase H. Inhibition of formation of complex type oligosaccharides by 1-deoxy-manno-nojirimycin prevented sulfation, indicating that the sulfate residues were added to complex type oligosaccharides.

Lysosomal enzymes are synthesized on membrane bound ribosomes as glycosylated precursors of higher molecular weight. During the passage through the endoplasmic reticulum and Golgi the N-linked oligosaccharides are subject to processing which includes the formation of the mannose 6-phosphate recognition marker and complex type oligosaccharides. Following segregation from the secretory route lysosomal enzymes are subjected to proteolytic maturation. The small fraction of the lysosomal enzymes that escapes segregation is secreted (1,2). In the course of experiments, in which the sulfation of membrane and lysosomal proteins was studied (3), we noted the incorporation of [^{35}S]sulfate into lysosomal enzymes of human skin fibroblasts. In the present communication we report on the presence of sulfated oligosaccharides of the complex type in cathepsin D, arylsulfatase A and the α-chain of ß-hexosaminidase.

Abbreviations: PMSF, phenylmethane sulfonyl fluoride; dMM, 1-deoxy-manno-nojirimycin; endo F and H, endoglycosidases F and H; PNGase F, peptide: N-glycosidase F.

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MATERIALS AND METHODS

Materials. [^{35}S]Methionine (49.2 TBq/mmol) and [^{35}S]$NaSO_4$ (0.9-1.5 TBq/mg S) were from Amersham Buchler and ^{14}C-methylated protein standards from New England Nuclear. Immuno Precipitin, a 10 % Staphylococcus aureus cell wall preparation, was purchased from Bethesda Research Laboratories. Endo H was obtained from Seikagaku, Tokyo, endo F and PNGase F from Boehringer Mannheim. 1-Deoxy-manno-nojirimycin (dMM) and swainsonine was kindly provided by Dr. J. Legler, Cologne.

Cell culture. Human fibroblasts were grown in Eagle's minimal essential medium supplemented with 7.5 % fetal calf serum on 35 mm dishes. The cells were labeled with 0.5 ml medium containing either [^{35}S]methionine (1.5 MBq) or [^{35}S]sulfate (30 MBq) and chased in the presence of an excess of methionine (0.25 mg/ml) or Na_2SO_4 (1 mM), respectively. Where indicated cells were pretreated and labeled in the presence of swainsonine (10 µM) or dMM (5 mM). Extracts of cells and medium were prepared as described (4,5,6).

Immunoprecipitation of lysosomal enzymes. Sequential immunoprecipitation of cathepsin D, ß-hexosaminidase and arylsulfatase A from cells and medium extract and collection of the immune complexes with the aid of Immuno Precipitin were done as described (4,5,6). Solubilization of the immunoprecipitates in the presence of sodium dodecylsulfate and dithiothreitol and electrophoretic separation in 12.5 % polyacrylamide gels was carried out as described (7). Radioactivity in lysosomal enzymes was quantified by densitometry of the fluorograms.

Endo H treatment. The cell extracts were mixed with 5 vol of ice-cold acetone and kept for 5 h on ice. After centrifugation the pellets were dissolved in 0.04 ml 10 mM sodium phosphate, pH 6.0, 0.1 M NaCl and 1 mM PMSF. Endo H was added to a final concentration of 50 mU/ml. After incubation for 16 h at 37°C the same amount of the enzyme was added and incubation was continuied for another 14 h.

Endo F and PNGase F treatment. The immune complexes adsorbed to Immuno Precipitin were solubilized in 0.14 ml 0.1 M sodium phosphate pH 8.6, containing 1.2 % Nonidet P 40, 0.2 % SDS and 1 % mercaptoethanol by heating at 95°C for 6 min. After centrifugation the supernatants were adjusted to 1 mM PMSF, 5 mM iodoacetamide, 1 mM EDTA. Aliquots were incubated for 20 h at 37°C in the absence and presence of 50 mU Endo F or 10 mU PNGase F. The reaction was stopped by addition of an equal volume solubilizer (7), boiled for 5 min and subjected to gel electrophoresis. The reaction was stopped by addition of an equal volume solubilizer, boiled for 5 min and subjected to gel electrophoresis.

RESULTS AND DISCUSSION

After labeling of fibroblasts for 1 h with [^{35}]sulfate, extracts of the cells and the culture medium were assayed for the presence of labeled lysosomal enzymes (Fig. 1). In cell extracts sulfate label was detected in the precursor (M_r 51 000), intermediate (M_r 47 000) and mature heavy chain (M_r 31 000) of cathepsin D, in the precursor (M_r 67 000) and mature (M_r 54 000) α-chain of ß-hexosaminidase and in arylsulfatase A (M_r 62 000). While this study was in progress, Waheed and van Etten (8)

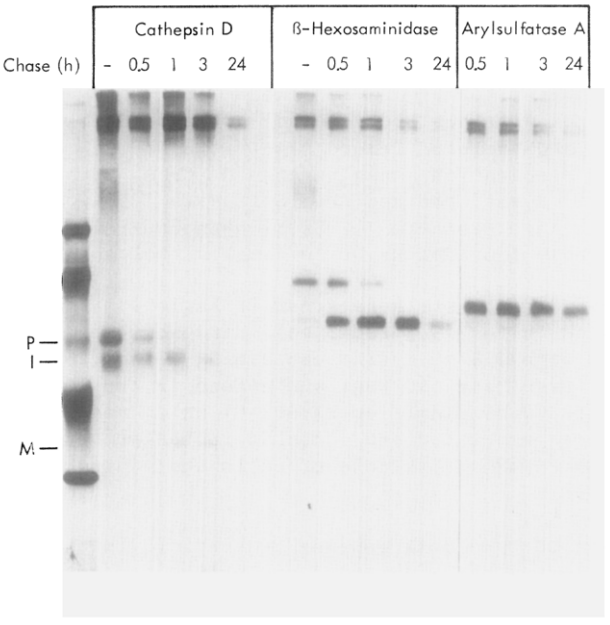

Fig. 1. Incorporation of sulfate into lysosomal enzymes. Fibroblasts were labeled for 1 h with [^{35}S]sulfate and either harvested or chased for the indicated time. Cathepsin D, ß-hexosaminidase and arylsulfatase A were immunoprecipitated electrophoretically separated and visualized by fluorography. The precursor (P), intermediate (I) and mature (M) forms of cathepsin D are indicated. The following ^{14}C-methylated standards were used: phosphorylase B (92.5kDa), bovine serum albumin, 69kDa; ovalbumin, 46kDa; carbonic anhydrase, 30kDa; cytochrome c, 12.3kDa.

reported on the sulfation of arylsulfatase A in human lung fibroblasts. During chase incubations for up to 24 h (Fig. 1) and 48 h (not shown) the sulfate label in cathepsin D, the α-chain of ß-hexosaminidase and arylsulfatase A was lost with a $t_{1/2}$ of <1h , 9 h and 26 h, respectively. These values are considerably shorter than the $t_{1/2}$ for [^{35}S]-methionine labeled cathepsin D (16.4 d, ref.9), ß-hexosaminidase α-chain (7 d, ref. 10) and arylsulfatase A (> 40 days, ref. 6,9). During the chase sulfated precursors of cathepsin D, ß-hexosaminidase α-chain and arylsulfatase A were secreted. The sulfate in the secreted precursors accounted for 7, 3 and 4 % of the sulfate incorporated into the respective lysosomal enzymes during a 3 h labeling period (not shown).

The experiment shown in Fig. 1 revealed that a high proportion of the sulfate was found after the 1 h labeling period into proteolytically processed forms of cathepsin D (M_r 47 000 intermediate and M_r 31 000 heavy

chain) and of ß-hexosaminidase α-chain (M_r 54 000 form). This is in contrast to the labeling with [^{35}S]methionine, which is found exclusively in the precursor forms of the two enzymes after a 1 h labeling period (see Fig. 3 for ß-hexosaminidase α-chain and ref. 4 for cathepsin D). One of the possible explanations is that sulfate is added to lysosomal enzymes shortly before (or even after) initiation of the proteolytic processing. That sulfate was added to preexisting precursors of ß-hexosaminidase α-chain and arylsulfatase A post-translationally was demonstrated by labeling fibroblasts in the presence of 0.5 mM cycloheximide. When cycloheximide was added 15 min prior to the labeling, the incorporation into both enzymes of [^{35}S]methionine was inhibited by more than 95 %, while that of sulfate was reduced only by 37 % (shown for arylsulfatase A in Fig. 2). If the lag phase of 10 min for incorporation of [^{35}S]sulfate into macromolecules after addition of [^{35}S]sulfate to the medium (11) and the 15 min pretreatment with cycloheximide is taken

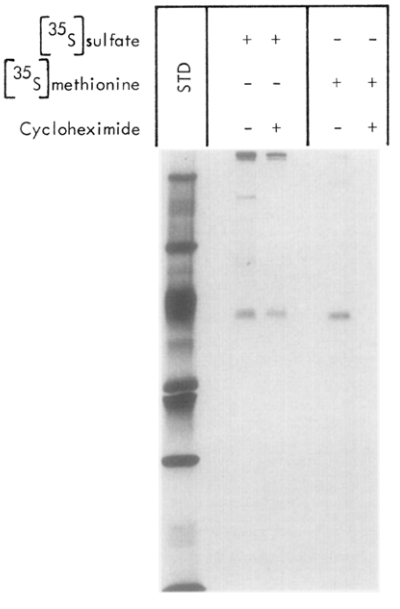

Fig. 2. Sulfation of arylsulfatase A in the presence of cycloheximide. Fibroblasts were incubated for 15 min in the absence or presence of 0.5 mM cycloheximide. The cells were then labeled with [^{35}S]sulfate or [^{35}S]methionine for 1 hour in the absence or presence of cycloheximide. Arylsulfatase A was immunoprecipitated, electrophoretically separated and visualized by fluorography.

into account, the inhibition of sulfation by about 40 % indicates that sulfation occurs about 1 h after synthesis of arylsulfatase A.

Sulfate residues in glycoproteins can be linked to either carbohydrate or tyrosine residues (12). When ß-hexosaminidase from cells labeled for 3 h with [^{35}S]sulfate was treated with endo F, complete loss of the label was observed . The M_r 67 000 α-chain precursor was converted F into a M_r 64 000 product and the (non sulfated) M_r 63 000 and M_r 61 000 precursor forms of the ß-chain into a M_r 53 000 product (Fig. 3). Treatment with PNGase F, resulted likewise in a complete desulfation of ß-hexosaminidase α-chain (not shown). These findings demonstrate that sulfate residues are bound to N-linked oligosaccharides.

To examine the type of N-linked oligosaccharides that carried the sulfate residues, the lysosomal enzymes were incubated with endo H, which cleaves the dichitobiosyl linkage of high mannose and hybrid type

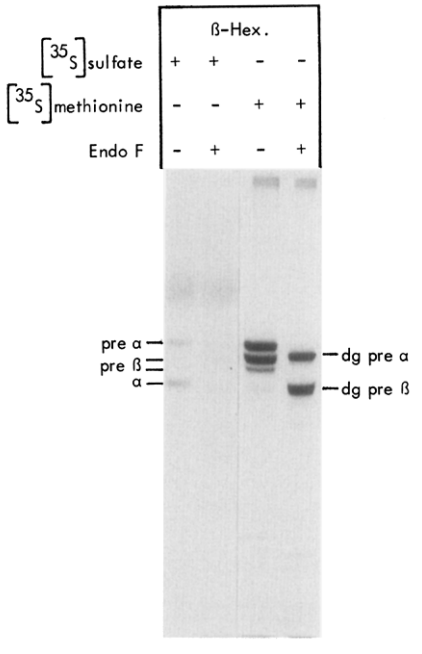

Fig. 3. Sensitivity of sulfate in ß-hexosaminidase to endo F. Fibroblasts were labeled with sulfate or [^{35}S]methionine for 3 h. ß-Hexosaminidase was immunoprecipitated from cell extracts and then incubated with or without endo F. The precursors of the α-chain and ß-chain of ß-hexosaminidase (pre-α, pre-ß) as well as the deglycosylated forms of the precursors (dg pre-α), (dg pre-ß) are indicated.

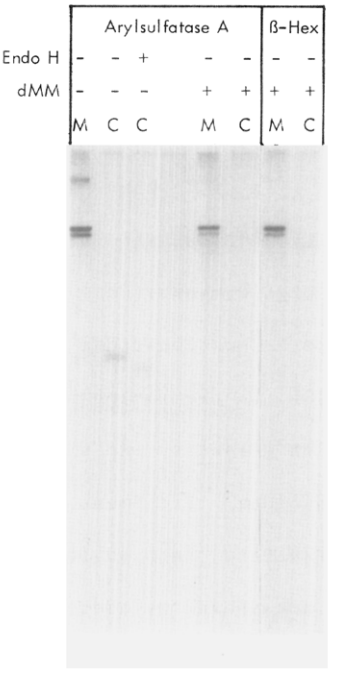

Fig. 4. Sensitivtiy of sulfate in ß-hexosaminidase and arylsulfatase A to endo H and sulfation in the presence of dMM. Fibroblasts were incubated in the absence or presence of 5 mM dMM for 4 h and then labeled for further 4 h with [^{35}S]sulfate in the presence or absence of dMM. ß-Hexosaminidase and arylsulfatase A were immunoprecipitated from the media (M) and cell extracts (C). The latter were incubated with or without endo H.

N-linked oligosaccharides (13). Endo H treatment did not release significant amounts of sulfate from ß-hexosaminidase or arylsulfatase A, while it decreased the size of the latter by 1.5 to 2kDa due to removal of one endo H sensitive oligosaccharide chain (Fig. 4, the lower amount of sulfate in the endo H-treated sample is due to technical failure). These results indicate that the sulfate resides in endo H-resistant oligosaccharides. Since sulfation may render high mannose and hybrid type oligosaccharides resistant to endo H (14,15), additional experiments were performed to determine out which type of the N-linked oligosaccharides carried the sulfate residues. Fibroblasts were metabolically labeled with [^{35}S]sulfate in the presence of 1-deoxy-*manno*-nojirimycin (dMM), which inhibits several α-mannosidases including the Golgi α-mannosidase I (16). As a result the processing of oligosaccharides in lysosomal enzymes, such as ß-hexosaminidase, arylsulfatase A and B, to

complex (or hybrid) type oligosaccharides is prevented, while formation of mannose 6-phosphate recognition markers is not impaired (17). Treatment with dMM abolished the sulfation of ß-hexosaminidase α-chain and arylsulfatase A (Fig. 4). In a further experiment, fibroblasts were metabolically labeled with [^{35}S]sulfate in the presence of swainsonine, which inhibits the Golgi α-mannosidase II. Swainsonine inhibits the formation of complex type oligosaccharides and promotes that of hybrid type oligosaccharides (18). In the latter one of the branches may terminally be glycosylated with galactose and sialic acid residues similar to complex type oligosaccharides. Treatment with swainsonine reduced the incorporation of [^{35}S]sulfate into arylsulfatase A (Fig. 5) and ß-hexosaminidase α-chain (not shown) by about 65 %, while synthesis of the corresponding [^{35}S]methionine-labeled polypeptides was unaffected. The resistance of sulfate to endo H, the sensitivity of sulfation to dMM and swainsonine indicate that the sulfate groups are linked to the sugar

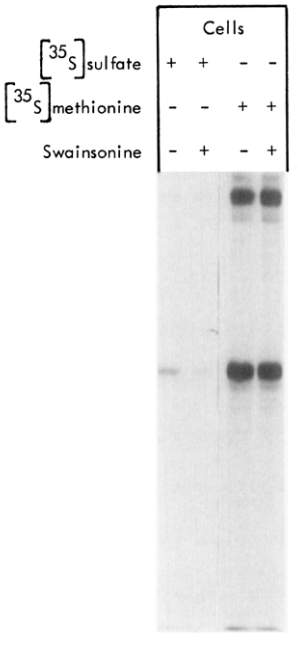

Fig. 5. Sulfation of arylsulfatase A in the presence of swainsonine. Fibroblasts were preincubated with and without 10 µM swainsonine for 1 h and then metabolically labeled with [^{35}S]sulfate and [^{35}S]methionine for 16 h in the absence or presence of swainsonine. Arylsulfatase A was immunoprecipitated from the extracts.

moieties which are added to lysosomal enzymes during terminal glycosylation of complex oligosaccharides.

Our studies provide no information on the stoichiometry of the sulfation. The role of sulfated oligosaccharides in lysosomal enzymes remains a matter of speculation. If the sulfate residues are of functional significance at all, they should be of importance for processes occuring during transport from the trans-Golgi, the presumed site of sulfation (19), to the lysosomes or shortly after entry into lysosomes. Their rapid loss after proteolytic processing renders a role of sulfate for stability or activity of lysosomal enzymes rather unlikely.

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