Interaction of Arylsulfatase A with UDP-N-Acetylglucosamine:Lysozanial Enzyme-N-Acetylglucosamine-1-phosphotransferase*

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The critical step in lysosomal targeting of soluble lysosomal enzymes is the recognition by an UDP-N-acetylglucosaminyllysosomal enzyme-N-acetylglucosamine-1-phosphotransferase. The structure of the determinant common to all lysosomal enzymes for proper recognition by the phosphotransferase is not completely understood. Our current knowledge is largely based on the introduction of targeted amino acid substitutions into lysosomal enzymes and analysis of their effects on phosphotransferase recognition. We have investigated the effect of eight anti-arylsulfatase A monoclonal antibodies on the interaction of arylsulfatase A with the lysosomal enzyme phosphotransferase in vitro. We also show that a lysine-rich surface area of arylsulfatases A and B is essential for proper recognition by the phosphotransferase. Monoclonal antibodies bind to at least six different epitopes at different locations on the surface of arylsulfatase A. All antibodies bind outside the lysine-rich recognition area, but nevertheless Fab fragments of these antibodies prevent interaction of arylsulfatase A with the phosphotransferase. Our data support a model in which binding of arylsulfatase A to the phosphotransferase is not restricted to a limited surface area but involves the simultaneous recognition of large parts of arylsulfatase A.

The correct lysosomal targeting of soluble lysosomal enzymes mainly depends on the synthesis of mannose 6-phosphate residues on their N-linked oligosaccharide side chains. These mannose 6-phosphate residues are lysosomal targeting signals. In the late Golgi compartments, lysosomal enzymes bind to mannose 6-phosphate receptors, which mediate the vesicular transport to the lysosomes (for review, see Refs. 1 and 2). The synthesis of mannose 6-phosphate residues is a two-step process: in the first reaction the UDP-N-acetylglucosaminyllysosomal enzyme N-acetylglucosamine-1-phosphotransferase (EC 2.7.8.17) (briefly called phosphotransferase)

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‡ The abbreviations used are: phosphotransferase, UDP-N-acetylglucosaminyllysosomal enzyme N-acetyl-glucosaminyl-1-phosphotransferase; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; SNA, sulfo-N-hydroxy-succinimidylacetate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride.

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lysosomal enzymes suggested that a loose consensus sequence in a β-hairpin structure may be common to several lysosomal enzymes (15). Whether this sequence is indeed part of the recognition determinant common to all lysosomal enzymes remains to be proven.

Arylsulfatases A (EC 3.1.6.8) and arylsulfatase B (EC 3.1.6.12) are lysosomal enzymes involved in the degradation of sulfated glycolipids and glycosaminoglycans, respectively. Arylsulfatase A is synthesized as a 62-kDa polypeptide, which bears three high mannose-type oligosaccharide side chains, two of which are phosphorylated by the phosphorytase (16, 17). The position of the recognition determinant in lysosomal arylsulfatase A and homologous sulfatases is unknown. Since our current knowledge on the requirements of the interaction of the lysosomal enzymes with the phosphorytase is based on the introduction of targeted amino acid substitutions we have chosen to characterize phosphorytase recognition determinant on arylsulfatase A with monoclonal antibodies. Recently, we reported the generation of four monoclonal antibodies specific for human arylsulfatase A (18). Fab fragments of these antibodies inhibited the synthesis of mannose 6-phosphate residues on arylsulfatase A in an in vitro phosphorylation system. Epitope mapping revealed that these antibodies recognized identical or adjacent epitopes on the enzyme, and it was assumed that the epitope was part of or close to the phosphorytase recognition determinant. In order to extend this study and to map the recognition determinant more precisely, we have generated eight additional antibodies and examined their ability to interfere with mannose phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture media RPMI 1640 for hybridomas and Dulbecco’s modified Eagle’s medium for all other cell types were from Life Technologies, Inc. Fetal calf serum was obtained from Biochrom. DNA digesting and DNA modifying enzymes were from New England Biolabs or Life Technologies, Inc. [γ-32P]ATP (3000 Ci/mmol) was from Amer sham. Enzymes used in the synthesis of [β-32P]UDP-N-acetylgalactosamine were purchased from Sigma. All other chemicals were of analytical or molecular biology grade and were from Merck, Serva, Sigma, or Fluka. MAb 5C6 (19) and NQ2/16-2 (20) were used as controls and were provided by Dr. Lemke, Kiel, Germany and Dr. Gordon, United Kingdom.

**Production of Monoclonal Antibodies**—Female BALB/c mice 6 weeks of age were injected three times (day 1, 14, and 28) intraperitoneally with 100 µg of arylsulfatase A. Recombinant enzyme was purified by immunofinity purification as described earlier (18). Prior to injection the enzyme was reduced to aluminunhydroxide. Antibody titers were monitored 12 days after the last immunization. Animals scoring positive were boosted with 50 µg of arylsulfatase A and sacrificed 3 days later. Spleen cells of mice were fused with X63-Ag8.653 myeloma cells. Fusion was done as described (21). After fusion, cells were plated in 200 wells of 24-well hybridoma plates (Greiner) in RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin, 100 µM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine (Boehringer-Mannheim). The medium was further supplemented with 10% conditioned medium from the J774 cell line. Wells containing anti-aryl sulfatase A immunoglobulin secreting hybridomas were identified via a radioimmunounassay in which an iodinated secondary anti-mouse light chain antibody raised in rabbits was used (Dakopatts, Copenhagen). Clones in positive cells were subcloned and reanalyzed by radioimmunounassay. Immunoglobulin subclass determination was performed with a kit from Boehringer-Mannheim. MAb A3, C, E, and F are IgG2a, MAb A4, A2, and A4 are IgG1, and A5 is IgG2b. All antibodies have κ-light chains.

In part of the experiments three previously published mAbs have been used (18). The wide reading of this article these antibodies have been renamed, 19C2 is B1, 20D2 is B2, and 11B5 is D.

**Purification of Monoclonal Antibodies and Fab Fragments**—Hybridomas were cultured in the Hereaus Miniperm system, which in all cases yielded several milligrams of mAbs. IgG2a mAbs were purified via affinity chromatography on Protein A-Sepharose and IgG1 on Protein G-Sepharose (Pharmacia). Affinity chromatography was performed according to the manufacturers protocols. For production of Fab fragments, purified antibodies were adjusted to a concentration of 1–5 mg in phosphate-buffered saline, 5 mM cysteine. Papain was added at a molar ratio of 1:40 mol of IgG. Depending on the antibody, proteolysis was performed at 37 °C for 1 to 4 h. Reaction was terminated by the addition of iodoacetamide to a final concentration of 20 mM. Fab fragments were purified by gel filtration on a Bio-Gel P-15 column (Pharmacia) equilibrated with phosphate-buffered saline.

**Immunoprecipitation, SDS-PAGE, and Western Blots**—Immunoprecipitation of unlabeled arylsulfatase A under nondenaturing conditions was performed in 50 mM Tris/HCl, pH 7.4, 0.15 mM NaCl, 0.05% Triton X-100. Five to 10 milliliters of arylsulfatase A were incubated with 10 µg of antibodies for 12–16 h. To enhance precipitation, 10 µg of goat anti-mouse affinity purified antibodies (Dianova) were added for another 2 h before Ig were collected by the addition of 60 µl of Staphylococcus aureus suspension (Pansorbin, Calbiochem). After 30 min of continuous shaking, bacteria were pelleted for 4 min at 10,000 × g. Pellets were resuspended in immunoprecipitation buffer and washed once. Arylsulfatase A activity was determined in supernatants and pellets.

**Monoclonal Antibody Competition Assay**—96-well enzyme-linked immunosorbent assay plates were coated with 125 ng of mAbs and subsequently blocked with an excess of casein. Simultaneously, arylsulfatase A was incubated with a 10-fold molar excess of mAb in 20 mM Tris/HCl, pH 7.4, 0.15 mM NaCl. MAb-aryl sulfatase A complexes generated in liquid phase were then added to the mAb-coated wells and allowed to bind for 2 h at 37 °C. Afterward liquid phase was removed, the wells were washed twice, and arylsulfatase A bound to the wells was quantified by activity determination (18). If the antibody coupled to the well and complexed to the arylsulfatase A in the liquid phase compete and thus cannot bind arylsulfatase A simultaneously, the enzyme cannot be retained in the wells.

**Detection of Ocatmerization of Arylsulfatase A**—96-well enzyme-linked immunosorbent assay plates were coated with 100 ng of arylsulfatase A/well for 16 h at 4 °C. Purified arylsulfatase A can be labeled via incubation with [35S]Slycysteine.2 Labeled arylsulfatase A was incubated with a 10-fold molar excess of mAbs. The antibody complexed with [35S]aryl sulfatase A was added to the wells at neutral pH when octamerization of arylsulfatase A does not occur. Then pH of the wells was titrated to pH 4.5 and incubation continued for 1 h. Low pH favors octamerization of arylsulfatase A so that arylsulfatase A-antibody complexes should bind to solid phase arylsulfatase A if the antibody does not interfere with octamerization. Liquid phase was removed and wells were washed with 50 mM sodium acetate buffer, pH 4.5. Radioactivity bound to the plates was determined. As a control a duplicate plate was washed with neutral buffer, washes were reversed addition of [35S]aryl sulfatase A with arylsulfatase A bound to the wells.

**In Vitro Phosphorylation of Arylsulfatase A**—Preparation of Golgi membranes, [β-32P]UDP-GlcNAc and arylsulfatase A by immunofinity chromatography has been described in detail (18, 22). Purified arylsulfatase B was kindly provided by M. Evers and C. Peters, Freiburg, Germany. Quantification of radioactivity after SDS-PAGE was done with a Fuji Bioimager.

**Chemical Modification of Amino and Carboxyl Residues**—To modify carboxyl residues 50 µg of arylsulfatase A or arylsulfatase B were dialyzed against 20 mM sodium acetate, pH 5.2, and samples were divided into two parts of 50 µl each. One part received no addition and served as a control, the other part was supplemented with 20 µM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydroxycarbonyl (EDC) and 100 mM ethylenediamine. After incubation for 20 min at 30 °C, the reaction was stopped by increasing sodium acetate concentration to 0.66 M. Treated and untreated samples were subsequently dialyzed against Tris-buffered saline.

To modify amino groups of lysines, 50 µg of arylsulfatase A or arylsulfatase B were dialyzed against phosphate-buffered saline, pH 8.0, and samples were split into two aliquots. One aliquot received no additions and to the other 17.5 mM sulfono-hydroxy-succinimidyl acetyl (SNA) was added. Incubation was for 90 min on ice and reaction was terminated by dialysis against Tris-buffered saline. In each case, the success of the modification reaction was monitored by alterations in isoelectric focussing pattern.

**RESULTS**

**Characterization of Anti-aryl sulfatase A Monoclonal Antibodies**—Mice were immunized with recombinant human aryl sulfatase A and a total of 8 stable hybridomas secreting anti-
arylsulfatase A antibodies were obtained. These antibodies precipitated arylsulfatase A under nondenaturing conditions from extracts of Ltk− cells overexpressing the human arylsulfatase A cDNA. Since the sum of enzyme activity remaining in the supernatant (in all cases less than 10%) and bound to the mAbs in the pellet was in the same range as the activity in the supernatant when a irrelevant control antibody was used, it can be concluded that the antibodies do not interfere with arylsulfatase A activity (data not shown). All mAbs recognize human arylsulfatase A specifically, they do not precipitate rat or murine arylsulfatase A (less than 10% in all cases). They do not cross-react with arylsulfatase B an enzyme highly homologous to arylsulfatase A. None of the mAbs recognizes arylsulfatase A under denaturing conditions as revealed by Western blot analysis (data not shown).

Anti-arylsulfatase A Monoclonal Antibodies Inhibit in Vitro Phosphorylation of Arylsulfatase A—The synthesis of mammalian 6-phosphate residues, which represent the specific lysosomal targeting signal can be performed in vitro (18, 22). Purified lysosomal enzymes are incubated with a Golgi fraction as a source of lysosomal enzyme phosphotransferase and [β-32P]UDP-GlcNAc from which the radioactively labeled 32P is transferred to the oligosaccharide side chains of the substrate enzymes. Subsequently, phosphorylation is detected by SDS-PAGE analysis and autoradiography.

Fig. 1 shows an experiment in which recombinant arylsulfatase A purified to apparent homogeneity was phosphorylated in vitro in the absence or presence of control or anti-arylsulfatase A mAbs. Addition of an irrelevant control mAb has no effect on arylsulfatase A phosphorylation, whereas all mAbs (top panel) or their Fab fragments (bottom panel) inhibited phosphorylation almost completely. Addition of anti-arylsulfatase A mAbs or Fab fragments did not inhibit phosphorylation of arylsulfatase B, which was co-phosphorylated in the same sample, excluding nonspecific inhibition of phosphorylation by the addition of anti-arylsulfatase A mAbs.

Inhibition of arylsulfatase A phosphorylation may be explained in two ways. 1) The antibodies inhibit recognition of arylsulfatase A by the phosphotransferase, because they mask the determinant responsible for lysosomal enzyme recognition. In this case the arylsulfatase A-mAb complexes will not be able to interact with the phosphotransferase. 2) The antibodies may still allow arylsulfatase A to be recognized by the phosphotransferase as a lysosomal enzyme, but after binding, phosphorylation of oligosaccharides is sterically inhibited by the anti-arylsulfatase A mAbs. To differentiate between these possibilities, we developed an assay in which arylsulfatase A inhibits arylsulfatase B competitively. If arylsulfatase A-mAb complexes can still bind to the phosphotransferase, they should be able to compete arylsulfatase B phosphorylation. If the mAbs prevent arylsulfatase A from interacting with the phosphotransferase, arylsulfatase A-mAb complexes should not compete arylsulfatase B phosphorylation.

Fig. 2 shows results of three independent phosphorylation experiments. In these experiments arylsulfatase B and arylsulfatase A were co-phosphorylated in the same reaction. Arylsulfatase A was added in about 10-fold molar excess over arylsulfatase B. Addition of arylsulfatase A inhibits arylsulfatase B phosphorylation by about 40%. In all cases addition of anti-arylsulfatase A Fab fragments abolishes the inhibitory effect of arylsulfatase A on arylsulfatase B phosphorylation. Thus, Fab fragments of all mAbs prevent interaction of arylsulfatase A with the phosphotransferase.

Lysines Are Important for Recognition of Arylsulfatase A and Arylsulfatase B by the Phosphotransferase—The importance of lysines for lysosomal enzyme recognition has been documented
Effect of the Monoclonal Antibodies on pH-dependent Octamerization of Arylsulfatase A—The fact that all mAbs inhibited the interaction of arylsulfatase A with the lysosomal enzyme phosphotransferase raises the question of specificity. The mAbs may interfere unspecifically with any protein–protein interactions of arylsulfatase A simply by size. To get a measure of the specificity of mAb inhibition of phosphorylation we have investigated the influence of the mAbs on another protein–protein interaction of arylsulfatase A that can be performed in vitro. This is the pH-dependent octamerization of arylsulfatase A. At neutral pH, arylsulfatase A exists as a dimer, which upon acidification associates to form octamers (23). To examine whether the mAbs interfere with octamerization, 100 ng of purified arylsulfatase A was adsorbed to a 96-well microtiter plate. 35S-labeled arylsulfatase A was added at neutral pH to the wells. Afterward, pH in the wells was titrated to pH 4.5 under continuous shaking. At low pH, some of the fluid phase remains bound to the solid phase via interaction of the octamerization domain. If wells are washed at pH 4.5, labeled arylsulfatase A remains bound to the solid phase (bars Co pH 4.5 in Fig. 4) whereas washing at neutral pH reverses octamerization and removes most of the bound [35S]aryl sulfatase A (bars Co pH 7.4 in Fig. 4). To investigate whether mAbs interfere with this pH-dependent octamerization process, mAbs were added either to the wells to bind to solid phase arylsulfatase A before labeled fluid phase [35S]aryl sulfatase A was added (light bars in Fig. 4) or mAbs were preincubated with fluid phase [35S]aryl sulfatase A before addition to wells (dark bars, Fig. 4). After titration to pH 4.5 and washing for 1 h, wells were washed at pH 4.5 and counts remaining in the wells were determined. Compared with control mAb, none of the arylsulfatase A-specific mAbs inhibited binding of fluid phase [35S]aryl sulfatase A to solid phase enzyme, irrespective whether mAbs were incubated with the fluid phase [35S]aryl sulfatase A or unlabeled aryl sulfatase A bound to the wells. Radioactivity in all wells could be removed by subsequent washing at neutral pH, which reverses octamerization (data not shown). Control experiments showing that arylsulfatase A–mAb complexes are stable at pH 4.5 have been performed (data not shown).

Simultaneous Binding of Monoclonal Antibodies to Arylsulfatase A—To evaluate how many different epitopes are recognized by the mAbs, we investigated which of the mAbs can bind simultaneously to arylsulfatase A. Wells of a 96-well microtiter plate were precoated with the eight anti-arylsulfatase A mAbs and with a control antibody. Arylsulfatase A was preincubated with a 10-fold molar excess of antibodies for 16 h and then added to the wells containing the immobilized mAbs. In case
allow the conclusion that mAbs A1, A2, A3, A4, and A5 bind to acid sequence comparisons, and the criterion of surface location mined recently (23). Data from the chimeric enzymes, amino structure of the arylsulfatase A enzyme, which we have determined can be inferred from the three-dimensional map the regions to which the mAbs bind. Table II summarizes differences in the predicted amino acid sequences. Since the mAbs (B1 and B2) we have used in previously published experiments (18). (For alterations in mAb designations see “Experimental procedures.”) These antibodies compete with the first group of antibodies, but not with C, E, or F. This demonstrates that the mAbs recognize at least four different epitopes and that at least two mAbs can bind simultaneously to arylsulfatase A.

### Table I

| Immobilized mAbs | mAbs bound to ASA |
|------------------|-------------------|
| A1  | A2  | A3  | A4  | A5  | C   | E   | F   | B1  | B2  | CO  |
| A1  | 0   | 0   | 0   | 0   | 0   | 112 | 61  | 100 | 0   | 0   | 100 |
| A2  | 20  | 0   | 16  | 14  | 16  | 95  | 81  | 93  | 0   | 0   | 97  |
| A3  | 30  | 0   | 0   | 0   | 0   | 101 | 100 | 100 | 0   | 0   | 101 |
| A4  | 3   | 0   | 0   | 0   | 0   | 75  | 97  | 79  | 0   | 0   | 104 |
| A5  | 11  | 0   | 0   | 0   | 0   | 93  | 100 | 90  | 0   | 0   | 99  |
| C   | 106 | 91  | 82  | 91  | 92  | 0   | 57  | 101 | 91  | 91  | 99  |
| E   | 121 | 113 | 90  | 107 | 101 | 0   | 13  | 98  | 114 | 100 |
| F   | 106 | 71  | 74  | 94  | 108 | 66  | 44  | 0   | 71  | 81  | 100 |
| B1  | 0   | 0   | 0   | 0   | 0   | 129 | 113 | 103 | 0   | nd  | 100 |
| B2  | 0   | 0   | 3   | 8   | 0   | 101 | 104 | 98  | nd  | 0   | 100 |

Wells of a 96-well microtitr plate were coated with 125 ng of each of the mAbs listed in the left vertical lane. Arylsulfatase A was incubated with a 10-fold molar excess of the mAbs listed in the top horizontal lane. Arylsulfatase A-mAb complexes were then added to the wells and incubated for 2 h. Wells were washed twice and the arylsulfatase A remaining in the wells was quantified by activity determination. 100% is the value of wells to which only arylsulfatase A without prior incubation with antibodies has been added. The residual amount of arylsulfatase A bound when the antibody used for coating the wells and pretreatment with arylsulfatase A was identical was subtracted as background value. This value was less than 20% for all antibodies. Co designates control antibody.

aryl sulfatase A-bound mAbs compete binding of the immobilized mAbs arylsulfatase A should not bind to the immobilized mAbs in the wells. After 3 h incubation of arylsulfatase A-mAb complexes in the wells, wells were washed and arylsulfatase A remaining in the wells was quantified by activity determination. Results are shown in Table I.

Antibodies A1, A2, A3, A4, and A5 compete when binding to arylsulfatase A, which allows the conclusion that they recognize the same or adjacent epitopes. Antibody C partially competes binding of E, but not of F, and E also competes partially with F. It seems that these antibodies recognize different epitopes. We have performed similar experiments with two mAbs (B1 and B2) we have used in previously published experiments (18). (For alterations in mAb designations see “Experimental procedures.”) These antibodies compete with the first group of antibodies, but not with C, E, or F. This demonstrates that the mAbs recognize at least four different epitopes and that at least two mAbs can bind simultaneously to arylsulfatase A.

**Epitope mapping by immunoprecipitation of chimeric enzymes**

Four cDNAs expressing human/murine arylsulfatase A chimeras were transiently expressed and are shown schematically on the left of the table. Hatched parts indicate murine sequences, open parts human sequences. The amino acids at which the murine and human cDNA sequences were fused are indicated by position in the one-letter code. The right part lists the mAbs and a polyclonal arylsulfatase A rabbit antiserum (pAS) and their reactivity with the chimeric enzymes. + indicates that more than 90% of enzyme was precipitable, ~ that less than 10% of the enzyme was precipitable, and (−) indicates a 50% reduction in immunoprecipitation. Part of the data on antibodies D, B1, and B2 shown at the bottom have been published previously (18).

### Table II

| Structure of chimeric ASA | I | II | III | IV | mAb |
|--------------------------|---|----|-----|----|-----|
| A280                     | + | -  | -   | +  | A1  |
| R143                     | + | -  | -   | +  | A2  |
| R244                     | + | -  | -   | nd | A3  |
| L462                     | + | +  | -   | +  | A4  |
|                           | - | +  | +   |    | C   |
|                           | - | +  | +   | (+)| E   |
|                           | + | +  | +   | nd| B1  |
|                           | + | +  | -   | nd| B2  |
|                           | + | +  | (−) | + | D   |
|                           | + | +  | +   | + | pAS |
Lysosomal Enzyme Phosphotransferase

| Mab  | Epitopes                                                                |
|------|-------------------------------------------------------------------------|
| A1   | Arg^{19}                                                                |
| A2   | Met^{56}-Gly^{26}                                                       |
| A3   | Val^{19}                                                                |
| A4   | Arg^{27}                                                                |
| A5   |                                                                        |
| B1   | Ala^{105}-Asp^{160}                                                     |
| B2   | Met^{206}-His^{206}                                                     |
|      | Ala^{299}-Glu^{240}                                                     |
| C    | Ala^{165}-Asp^{160}                                                     |
|      | Met^{206}-His^{206}                                                     |
|      | Asp^{166}                                                               |
|      | Ala^{299}-Glu^{240}                                                     |
| D    | Ala^{266}                                                               |
|      | Thr^{250}                                                               |
| E    | Ala^{257}-Leu^{159}                                                     |
| F    | Leu^{262}-Ala^{307}                                                     |

**DISCUSSION**

We have generated eight anti-human arylsulfatase A mAbs to study the interaction of this lysosomal enzyme with the lysosomal enzyme phosphotransferase.

Results are based on an *in vitro* system in which mannose 6-phosphate residues as specific lysosomal targeting signals are synthesized *in vitro* on oligosaccharide side chains of recombinant arylsulfatase A. All 8 anti-aryl sulfatase A mAbs or their Fab fragments, respectively, were able to inhibit *in vitro* phosphorylation of arylsulfatase A completely. This inhibition may have three different explanations. If all mAbs bind close to the lysosomal enzyme recognition determinant, it can be expected that they prevent interaction of arylsulfatase A with the phosphotransferase. Alternatively, the interacting surface of arylsulfatase A with phosphotransferase may be so large that any antibody will interfere with recognition irrespective of the distance of its epitope to the recognition determinant. Third, Fab fragments may bind to areas outside the recognition site, such that arylsulfatase A-Fab fragment complexes may still be recognized by the phosphotransferase, but due to steric hindrance phosphate cannot be transferred to the oligosaccharide side chains.

The latter possibility has been excluded by competition experiments. Arylsulfatase A-Fab fragment complexes do not inhibit arylsulfatase B phosphorylation. This allows the conclusion that the Fab fragment complexed arylsulfatase A does not interact with the phosphotransferase. If the effect of the Fab fragments would be limited to steric hindrance of oligosaccharide phosphorylation, binding to the phosphotransferase via the recognition determinant should have been possible and arylsulfatase A-Fab fragment complexes should inhibit arylsulfatase B phosphorylation.

To distinguish between the remaining two possibilities, we attempted to locate the epitopes more precisely. Data from immunoprecipitation experiments of human/murine chimeric arylsulfatase A and mAb competition experiments suggest that the mAbs, described here and previously (18), recognize at least 6 different epitopes, all of which depend on the native status of the enzyme (see Tables II and III). Table III summarizes the location of the epitopes of anti-aryl sulfatase A mAbs and lists possible amino acids being part of these epitopes. Data from these experiments are consistent with those from competition experiments, in which different mAbs were tested for their capability to bind arylsulfatase A simultaneously. MAbs mapping to the same region by chimera immunoprecipitation also compete when binding to arylsulfatase A. Vice versa, mAbs C, E, and F, which map to different parts than A1 to A5 do not compete binding of these mAbs. Similarly, the different epitope mapping pattern of mAbs C, E, and F as revealed by immunoprecipitation of chimeric enzyme is also reflected in competition experiments. C and F do not compete, but at least partial competition can be seen between C/E and E/F. On the other hand, competition experiments also show that antibodies mapping to the same regions according to chimeric arylsulfatase A immunoprecipitation must recognize different epitopes. MAbs B1, B2, and C show the same reactivity pattern toward chimeric arylsulfatase A enzymes (Table II and 3), but B1 and B2 do not compete binding of C proving recognition of different epitopes.

The combination of all data allows location of likely positions of epitopes in the three-dimensional model of arylsulfatase A as presented in Fig. 5. Amino acids determining the epitope of mAbs A1 to A5 (see Table III) are located on one side of the enzyme. Amino acids of B1/B2 and C are found on top and on the opposite side of arylsulfatase A. Antibodies B1 and B2 do not compete with C, although chimeric immunoprecipitation locates them to the same amino acid residues. This is understandable in view of the three-dimensional model: the residues determining the epitopes of these mAbs are found in three distant locations so that simultaneous binding of these antibodies is conceivable. Epitope of D has been determined previously (18) and is found on the same side as those of B1, B2, and C. Epitopes of mAbs E and F can be less precisely defined. However, their epitopes must be located in some distance to those of the other antibodies, because they do not, or in the case of mAb E only partially, compete binding of these antibodies to arylsulfatase A. Thus, as illustrated in Fig. 5 epitopes of mAbs are found on different parts of the surface of arylsulfatase A. Therefore, the inhibition of phosphorylation cannot be explained by binding of all antibodies to a restricted area on the surface of arylsulfatase A.

Modification of lysines specifically inhibits the recognition of arylsulfatase A and arylsulfatase B by the phosphotransferase. In arylsulfatase A only five lysines are clustered on the surface of the enzyme (23). These five lysines are not evenly distributed on the enzyme surface, but are located in a single cluster (see Fig. 5). Interestingly, this lysine cluster is in close proximity and partly overlaps the octamerization determinant of arylsulfatase A. The mAbs (except possibly for E and F) have epitopes that are distant from this region, which is functionally supported by the fact that none of the mAbs interferes with octamerization. Thus, there is no evidence that any of the mAbs or Fab fragments bind close to the lysine containing lysosomal enzyme recognition determinant in arylsulfatase A, yet all are able to inhibit binding of arylsulfatase A to the phosphotransferase. The fact that the mAbs do not inhibit octamerization, but phosphorylation suggests that the area interacting with the phosphotransferase is larger than the octamerization area, which covers about 900 Å (23) and thus larger than the lysine cluster necessary for proper recognition.

One may argue that the size relation of arylsulfatase A to Fab fragments (62 versus 50 kDa) is unfavorable, so that an
unspecific inhibition of protein-protein interactions can be expected. However, this seems to be unlikely, since Fab fragments have been shown to specifically inhibit functions of protein domains, which are limited to a few amino acids. Microinjections of Fab fragments generated against pentapeptides contained in the cytoplasmic tail of the 46-kDa mannose 6-phosphate receptor have shown that Fab fragments binding to amino acids 43–48 interfere with proper sorting of the receptor, whereas Fab fragments directed against amino acids 38–43 do not (26). This demonstrates that Fab fragments despite of their size can be used to discern protein-protein interactions even of adjacent small amino acid sequences. Co-crystallization of lysozyme and lysozyme-binding Fab fragments revealed that the binding site of the Fab fragment is a flat structure and the fragment extends from the binding site into the surrounding solution in a rather stretched conformation (27). The Fab fragment leaves most of the surface of the antigen accessible. The size of the interacting area of lysozyme and lysozyme Fab-fragments is 600 Å². This is only a fraction of the total arylsulfatase A surface area. Thus, the ability of the mAbs to inhibit interaction with the phosphotransferase cannot be explained by the assumption that single Fab fragments cover large parts of the arylsulfatase A surface making them inaccessible to other molecules. This is supported by our demonstration that none of the mAbs interferes with enzyme activity or octamerization and that at least two mAbs can bind simultaneously to the enzyme.

Our data support a model in which lysosomal enzyme phosphotransferase interacts with large surface areas of arylsulfatase A, a view that can be brought into line with results of targeted amino acid substitutions in cathepsin D (10, 12) and aspartylglucosaminidase (11). In cathepsin D two lysine residues 34 Å apart (203 and 293) cooperate to form part of a phosphotransferase recognition determinant. When these residues are mutated 30% of normal phosphorylation still remains (12), demonstrating the involvement of more residues and thus, most likely a larger surface area in the formation of a fully efficient recognition determinant. In aspartylglucosaminidase three distantly located lysine residues contribute to form a recognition signal. In addition, the minimal regions establishing the cathepsin D recognition determinants were identified as lysine 203 and amino acid residues 265–292. When these residues were introduced into the non-lysosomal homologous protease glycopepsinogen, the chimeric molecule was recognized by the phosphotransferase, but to a much lesser extent than cathepsin D (6). Obviously, residues beyond lysine 203 and amino acids 265–292 contribute to efficient expression of the lysosomal enzyme recognition determinant, and it has been shown that such sequences can be found in the amino-terminal lobe of cathepsin D (8, 10). A minimal recognition sequence may be necessary for recognition, but its efficiency can be enhanced by additional even distant areas on the same molecule. The cooperativity of various surface areas in the formation of a fully expressed lysosomal enzyme recognition determinant can be explained by different models in which the distant parts of a lysosomal enzyme bind subsequently or simultaneously to phosphotransferase (10). Phosphotransferase may initially bind lysosomal enzymes through a lysine-dependent recognition determinant and starts to phosphorylate accessible oligosaccharide side chains. After completion of this phosphorylation step, the enzyme dissociates and rotates to get access to another oligosaccharide side chain. However, the lysosomal enzyme remains in the vicinity of the phosphotransferase ensuring a high probability of rebinding even at weaker lysine-dependent or independent sites, present at different locations on the lysosomal enzyme surface. This process may continue until several oligosaccharide side chains have been phosphorylated. If this model is correct, it is surprising that none of the arylsulfatase A-Fab fragment complexes inhibits the phosphorylation of arylsulfatase B. Since the lysine region on arylsulfatase A is not covered by any of the Fab fragments, an initial contact through this region should not be disturbed by Fab fragments and arylsulfatase A-Fab fragment complexes should be able to inhibit phosphorylation of arylsulfatase B competitively. Thus, a model which depends on an initial high affinity binding of restricted area and with subsequent rotation and rebinding at weaker sites seems not likely.

Alternatively, phosphotransferase may contact the surface of

**FIG. 5. Location of epitopes in the three-dimensional model of arylsulfatase A.** The figure shows different views of a three-dimensional model of arylsulfatase A. Coordinates according to Brookhaven Protein Data Bank accession number PDB Id: 1AUK. A and B, surface-located lysines are shown in red, residues involved in octamerization in green. Possible epitopes of mAbs A1 to A5 are depicted in blue, those of B1, B2 and C mAbs are yellow, that of D, gray. Asparagines bearing N-linked oligosaccharide side chains are shown in black or pink. They are labeled Ol1, Ol2, and Ol3 indicating oligosaccharide side chains linked to asparagines 158, 184, and 350, respectively. Ol2 (pink) is not phosphorylated by the lysosomal enzyme phosphotransferase (17). B shows a view of the enzyme after vertical rotation by 180° compared with A. C and D, lysine/octetamerization area and oligosaccharide labeling as in A and B. Possible epitopes of mAb E appear dark purple, those of F, tan. Epitopes can be less clearly defined because numerous amino acid residues are different among mouse/rat and human arylsulfatase A in the respective regions. D shows a view of the enzyme after vertical rotation by 180° compared with C.
lysosomal enzyme at multiple sites simultaneously. The initial contact may depend on a lysine containing recognition determinant and subsequent binding is stabilized via additional contacts. Since phosphotransferase is a large multimeric enzyme of 9 times the molecular weight of an arylsulfatase A monomer, such an explanation seems applicable. Since all Fab fragments irrespective of their binding sites prevent interaction of arylsulfatase A with the phosphotransferase, it may be assumed that lysosomal enzymes enter a large active site pocket covering most of lysosomal enzymes surface. However, since lysosomal enzymes have different shapes it seems unlikely that they all fit tightly into a large inflexible active site of phosphotransferase. Thus, after initial contacts are made perhaps by charge-dependent interactions with lysines a conformational change in the phosphotransferase may be induced which causes embracement of the lysosomal enzyme. This embracement is only stabilized when additional contact points are established, which may then occur at distant points on the surface of a lysosomal enzyme. In this case the arylsulfatase A-Fab fragment complexes may still interact with phosphotransferase but Fab fragments may interfere sterically with embracement and establishment of secondary stabilizing contacts. Since arylsulfatase B phosphorylation cannot be inhibited by arylsulfatase A-Fab fragment complexes, in the embracement model the initial lysine dependent contact must be of low affinity. Our data does not allow distinction between the latter models, but in any case support a model in which large areas of the surface of lysosomal enzymes simultaneously interact with the phosphotransferase.

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