Four monoclonal antibodies inhibit the recognition of arylsulphatase A by the lysosomal enzyme phosphotransferase

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The critical step in the sorting of lysosomal enzymes is their recognition by a phosphotransferase in the Golgi apparatus. The topogenic sequences responsible for the recognition by this enzyme have so far only been defined for the lysosomal protease cathepsin D. We have generated four monoclonal antibodies directed against lysosomal arylsulphatase A (ASA). These antibodies inhibit the recognition of ASA by the phosphotransferase in vitro and thus define a region of topogenic sequences in the ASA polypeptide. The antibodies do not interfere with the enzymic activity nor with pH-dependent dimerization of ASA. The epitopes recognized by the antibodies have been located in the second quarter of the ASA polypeptide using chimeric mouse–human ASA molecules. Three of the monoclonal antibodies bind to identical or closely adjacent epitopes, which are formed by the interaction of amino acid residues 165–184 and 202–240. The fourth antibody recognizes a different epitope within amino acids 256–265.

INTRODUCTION

Lysosomal enzymes are synthesized on the rough endoplasmic reticulum and are co-translationally transported into the lumen of the endoplasmic reticulum. After cleavage of the signal peptide and the acquisition of N-linked oligosaccharide side chains lysosomal enzymes are transported to the Golgi apparatus. Whereas secretory proteins pass the Golgi apparatus, lysosomal enzymes specifically acquire mannose-6-phosphate residues, which serve as a lysosomal targeting signal (for a detailed review see [1]). In a late Golgi compartment these residues bind to mannose-6-phosphate receptors (M6PRs), which mediate further vesicular transport to the lysosomes. Upon acidification in a prelysosomal compartment, lysosomal enzymes dissociate from the receptors and are delivered to the lysosomes, whereas free receptors recycle. Synthesis of mannose 6-phosphate requires two enzymic reactions: a phosphotransferase (UDP-N-acetylglucosamine lysosomal enzyme N-acetylglucosamine-phosphotransferase, EC 2.7.8.17) transfers glucosamine 1-phosphate from UDP-N-acetylglucosamine to mannose residues of the high-mannose-type oligosaccharide side chains of lysosomal enzymes, creating N-acetylglucosamine-1-phospho-6-mannose residues. In a second reaction N-acetylglucosamine is removed by an α-N-acetylglucosaminidase, which generates the mannose-6-phosphate residues on lysosomal enzymes. Five different mannose residues within an oligosaccharide side chain can be phosphorylated but not more than two in a single oligosaccharide. A critical step in the sorting of lysosomal enzymes is the recognition by phosphotransferase. Insights into the mechanism of phosphorylation have come from two genetic diseases: mucolipidosis II and III. Both diseases are caused by the inability of the cells to synthesize the mannose-6-phosphate recognition marker. This leads to enhanced secretion of lysosomal enzymes, which results in a multiple intracellular deficiency. The disease is caused by deficiency of the phosphotransferase and two major complementation groups have been found [2]. Complementation studies, as well as biochemical studies, revealed that the phosphotransferase is composed of at least two subunits. One subunit may be involved in the recognition of lysosomal enzymes, whereas the other catalyses the transfer of N-acetylglucosamine 1-phosphate to mannose residues of the oligosaccharide side chains (see [2] for further references).

No obvious sequence similarities are detectable among lysosomal enzymes. The phosphotransferase recognition domain is therefore assumed to be a motif depending on the three-dimensional structure of lysosomal enzymes. It has been demonstrated that denatured lysosomal enzymes are not recognized by the phosphotransferase [3]. Recently, sequences involved in the recognition have been characterized in the lysosomal aspartyl protease cathepsin D [4]. This approach has used chimeric molecules constructed of different parts of cathepsin D and the homologous non-lysosomal zymogen pepsinogen. By introduction of different parts of cathepsin D into the pepsinogen background, the latter could be converted into a substrate of the phosphotransferase and was targeted to lysosomes via the M6PR pathway. To achieve this a sequence of 27 amino acid residues near the C-terminus of cathepsin D and a single lysine had to be introduced into the pepsinogen cDNA. The lysine was located in an area which, in the linear sequence, was not contiguous to the 27-amino-acid cathepsin D fragment. Crystallographic data from the pepsinogen, and recently from the cathepsin D molecule [5], allowed the conclusion that the sequences around the lysine residue and the sequences of the 27-amino-acid fragment are in proximity on the surface of the correctly folded enzyme. Although this represents the first characterization of a lysosomal topogenic signal, it is still unknown to what extent it is shared by other lysosomal enzymes.

Arylsulphatase A (ASA, EC 3.1.6.8) is a lysosomal enzyme, which is sorted via the M6PR pathway [6]. This enzyme initiates the degradation of cerebroside 3-sulphate. Cerebroside 3-sulphate is a sphingolipid mainly found in the myelin sheaths of the nervous system. ASA is synthesized as a 62 kDa protein, which carries three high-mannose-type oligosaccharide side chains ([7], H. J. Sommerlade, T. Selmer, K. von Figura, V. Gieselmann and

Abbreviations used: ASA, arylsulphatase A (EC 3.1.6.8); ASB, arylsulphatase B (EC 3.1.6.12); STS, steroid sulphatase (EC 3.1.6.2); M6PR, 46 kDa mannose-6-phosphate receptor; mAb, monoclonal antibody; BHK, baby hamster kidney.

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B. Schmidt, unpublished work). As is the case for other soluble lysosomal enzymes ASA is recognized in the Golgi apparatus by the phosphotransferase [6]. In vitro mutagenesis of potential glycosylation sites of ASA suggests that the mannose residues of the first and third oligosaccharide side chains are preferentially phosphorylated, whereas those of the second oligosaccharide side chain seem to be less efficient acceptors [6], H. J. Sommerlade, T. Selmer, K. von Figura, V. Gieselmann and B. Schmidt, unpublished work). The efficiency of the phosphorylation may be a result of the steric configuration between the phosphotransferase-recognition domain and the high-mannose oligosaccharide side chains.

In a search for topogenic sequences involved in the recognition of ASA by the phosphotransferase we have generated monoclonal antibodies (mAbs) and examined their ability to inhibit the phosphorylation of ASA in vitro. Here we present the characterization of four anti-ASA mAbs which interfere with the recognition of ASA in phosphorylation reaction in vitro, and describe the identification of the recognized epitopes.

MATERIALS AND METHODS

Materials

Cell-culture media, Eagle's minimal essential medium, RPMI 1690, medium 199, trypsin EDTA solution, and antibiotics were obtained from Seromed/Biochrom (Berlin). Fetal-calf serum, aminopterin stock solution and chemicals were obtained from Boehringer (Mannheim, Germany), Serva (Heidelberg, Germany), Merck (Darmstadt, Germany) or Sigma (Deisenhofen, Germany). Enzymes used for the synthesis of [β-32P]UDP-N-acetylgalosamine were purchased from Sigma. *Staphylococcus aureus* bacterial cell suspension (Pansorbin) was from Calbiochem.

[35S]Methionine (specific radioactivity > 600 Ci/mmol) and [γ-32P]ATP (specific radioactivity > 3000 Ci/mmol) was purchased from Amersham. The anti-ASA polyclonal antiserum was raised in goat and has been described previously [7]. Rhodamine-coupled anti-(mouse IgG) used for immunofluorescent staining were obtained from Sigma (Deisenhofen, Germany). Horseradish peroxidase-coupled goat anti-(mouse IgG) was from Medac (Hamburg, Germany). Immunoglobulin isotypes were determined with a kit from Paesel and Lorei (Frankfurt, Germany). Baby hamster kidney (BHK) cell lines stably transfected with cDNAs of ASA [9], arylsulphatase B (ASB; EC 3.1.6.12) [10] and steroidsulphotase (STS; EC 3.1.6.2) [11], as well as their culture conditions, have been described.

Purification of ASA and ASB

ASA was purified from human liver by a procedure which has been described previously [12]. Briefly, 1 kg of human liver was homogenized in 1 mM NaHCO₃/0.5 mM CaCl₂/100 μM phenylmethanesulphonyl fluoride using a Waring blender (three strokes, 10 s each). The homogenate was adjusted to pH 5 with acetic acid and centrifuged for 15 min at 10000 rev./min (11000 g) in a JA 10 Beckman rotor. Supernatant was precipitated with 70 % (w/v) ammonium sulphate. The precipitate was collected by centrifugation, dissolved in 0.5 litres of 10 mM Tris/HCl, pH 7.4, and any remaining particles were removed by filtration. The filtrate was passed over a 50-ml concanavalin A column, and after washing the column was eluted with 10 mM Tris/HCl (pH 7.0)/1 M NaCl/100 mM α-methylmannoside at 37°C. The eluate was concentrated in an Amicon chamber and applied to a Sephacryl S-300 gel-filtration column (2.6 cm × 100 cm) equilibrated with 20 mM Tris/HCl (pH 7.5)/0.9 % NaCl. Fractions with ASA activity were pooled, concentrated to 0.3 mg/ml protein and stored at 4°C. The final preparation had a specific activity of 19.9 units/mg of protein. The purification was 8600-fold with a yield of 15 %.

After mAbs were available the ASA used in the in vitro phosphorylation studies and epitope competition experiments was purified by an affinity affinity procedure from cells overexpressing ASA. These cells were cultured on Cytodex 3 beads (Pharmacia) in a Technne 1 litre cell-culture flask in which the beads are kept in suspension by gentle stirring of the medium. Cells used were BHK cells which overexpressed ASA as well as the 46 kDa M6PR. These cells have been described and secrete large amounts of ASA [13]. Medium was harvested several times, until due to confluence the cells started to detach from the beads. Medium was precipitated with 70 % (w/v) ammonium sulphate and dialysed against 10 mM Tris/HCl (pH 7.4)/150 mM NaCl and passed over a column to which the mAb 20B1 had been coupled. This antibody was purified by DEAE ion-exchange chromatography [14] and Sephacryl S-200 gel filtration from about 5 ml of mouse ascites. The purified antibody was coupled to Affigel 10 (Bio-Rad) according to the protocol supplied by the manufacturer. After binding of ASA, the column was washed with 10 mM Tris/HCl (pH 7.4)/150 mM NaCl and was eluted with 0.2 M glycine/HCl (pH 3.4). The eluate was immediately neutralized with saturated Tris. When the eluate was subjected to SDS/PAGE silver staining revealed that ASA was purified to apparent homogeneity in this one-step procedure. ASB was purified in a similar procedure. The cells overexpressing ASB were kindly provided by Dr. C. Peters (Göttingen, Germany) and the anti-ASB mAb by Dr. J. J. Hopwood (Adelaide, Australia).

Production of mAbs

Female Balb/C mice were used for immunization. Mice were injected intraperitoneally with 20 μg of ASA in 100 μl of Freund's complete adjuvant. Animals were given boosters after 28 and 48 days with 10 μg of ASA and Freund's incomplete adjuvants. Production of antibodies was monitored by determination of anti-ASA titre in total serum of immunized mice. Mice having anti-ASA immunoglobulins were killed, splenocytes were prepared and were fused using poly(ethylene glycol) with AG8 myeloma cells at a ratio of 3:1 splenocytes to myeloma cells. Hybridomas were selected on feeder cells in 24-well plates. Peritoneal macrophages isolated from mice were used as feeders. Selection of hybridomas was started after 1 week by the addition of selection medium containing 100 μM hypoxanthine, 16 μM thymidine, and 0.4 μM aminopterine [14]. A total of 509 clones were obtained, 373 of which grew to an extent allowing screening for Ig with an e.l.i.s.a. To screen for hybridomas producing anti-ASA antibodies, 96-well plates were precoated with 125 ng/well of anti-ASA immunoglobulin, which had been isolated from goat antiserum by Protein A-Sepharose chromatography and incubated with 3 ng of ASA/well. Unbound ASA was removed and hybridoma supernatants were added. After incubation for 2 h hybridoma supernatants were removed, plates were washed and mouse immunoglobulins were detected by a peroxidase-coupled goat anti-(mouse IgG) antibody. Some 22 hybridomas screened positive for immunoglobulin production, four of which produced anti-ASA antibodies, as judged by their ability to immunoprecipitate ASA. Three hybridomas (11B5, 19C2, 20B1) were injected intraperitoneally into mice for the production of
as a. Quantities of 1.8–3 ml of ascites were obtained which had 90–40 mg of immunoglobulin/ml, as determined by a direct e.l.i.s.a.

**Immunoprecipitation and metabolic labelling**

BHK cells overexpressing the ASA cDNA were grown to confluency and harvested with trypsin. The cell pellet was washed with PBS/0.1 % fetal-calf serum to remove trypsin and homogenized by three 15 s bursts of sonication in 2 ml of H₂O. Hybridoma supernatants were dialysed against 50 mM Tris/HCId (pH 8.0)/0.15 M NaCl/0.05 % Triton X-100. A sample (14 ml) of BHK cell homogenate containing 5 m-units of ASA was mixed with 100 ml of dialysed hybridoma supernatant and 10 µg of goat anti-(mouse IgG) serum was added. After incubation for 30 min at room temperature and 16 h at 4 °C immunoglobulins were collected by incubation for 2 h with 60 µl of PanSorbin (Calbiochem). Bacteria were pelleted, washed once and ASA activity was determined in the pellets and in the supernatants.

The immunoprecipitation procedure for ASA from homogenates of metabolically labelled cells has been described in detail [6]. In the experiments described here the protocol was modified as follows: before preabsorption of cell homogenates with *Staphylococcus aureus* bacteria the DNA was removed by precipitation with protamine sulphate. The homogenate was adjusted to 0.03 % protamine sulphate and kept on ice for 10 min. DNA was precipitated by centrifugation for 5 min in an Eppendorf centrifuge. Subsequently homogenates were precleared with *Staph. aureus* bacteria. Samples (100 µl) of cell homogenates were mixed with 300 µl of 5-fold concentrated hybridoma supernatants and 132 µl of 0.4 M Tris/HCId (pH 7.5), 1.6 M KCl, 4 % (w/v) Triton X-100. Incubation was for 30 min at 20 °C and 4 °C overnight. Then 20 µg of goat anti-(mouse IgG) and 45 µl of *Staph. aureus* suspension was added. Immunocomplexes were allowed to bind for 2 h at 4 °C. Collection and washing of immunoprecipitates has been described [6].

**Indirect immunofluorescence**

Cells were grown at low density on glass coverslips (10⁴ cells/coverslip). Cells were washed with PBS, and fixed with 3 % (w/v) formaldehyde in PBS for 40 min at room temperature. Coverslips were then washed twice with 0.1 M glycine/Tris (pH 7.4) and incubated twice in the same buffer for 15 min. Glycine/Tris was removed, cells were washed with PBS and permeabilized with PBS/0.3 % Triton X-100 twice for 5 min. Triton X-100 was removed and coverslips were incubated three times for 5 min each with PBS/0.2 % gelatin to block unspecific binding. Cells were incubated with 100 µl of hybridoma supernatant or a 1:200 dilution of polyclonal anti-ASA antiserum raised in goat. After 3 h at room temperature antibodies were removed and coverslips were washed three times with PBS/0.2 % gelatin before a 1:200 dilution of the second antibody [anti-(mouse IgG) raised in rabbit and coupled with rhodamine] was added. The antibody was allowed to bind for 1 h at room temperature and after washing the coverslips were mounted on glass slides.

**Western-blot analysis**

Aliquots containing 500 ng of ASA were applied to an SDS/7.5 % (w/v) polyacrylamide gel. The gel was electrophoresed in 25 mM glycine/25 mM ethanolamine/20 % (v/v) methanol at 4 °C at 300 mA for 4 h. To control for efficient transfer the SDS/polyacrylamide gel was stained afterwards with Coomassie Blue. The nitrocellulose membrane was incubated at 4 °C overnight with 5 % (w/v) milk powder/0.05 % Triton X-100 in PBS to block unspecific binding. The membrane was cut into strips according to the lanes of the SDS/polyacrylamide gel and these strips were incubated with different antibodies in the buffer mentioned above. For controls a 1:100 dilution of polyclonal antiserum was used. Hybridoma supernatants were concentrated up to 5-fold and dialysed against PBS; ascites were diluted 1:100. Incubation with antibodies was for 5 h at room temperature. Strips were washed for 2 x 20 min and 2 x 5 min at room temperature with PBS to remove unbound antibody. For the detection of ASA-bound antibodies a horseradish peroxidase-coupled anti-(mouse IgG) antibody was added for 2 h. After washing the strips were developed for up to 1 h at room temperature.

**Phosphorylation of ASA in vitro**

Golgi membranes were prepared from male Sprague–Dawley rats obtained from the Tierzuchtanstalt Hannover. Animals were starved overnight and killed by decapitation the next morning. The liver was removed, cut into small pieces and homogenized three times for 30 s in a Braun Melsungen glass homogenizer (SS02) at low speed in 3 ml of 0.5 M sucrose/5 mM MgCl₂/50 mM Tris/HCId (pH 7.4) for each gram of liver tissue. Debris was removed by centrifugation at 6000 g for 10 min at 4 °C. Supernatant was recovered and adjusted to 0.17 unit/ml aprotinin. Supernatant (38 ml) was layered on to the top of a 20 ml 1.3 M sucrose cushion and centrifuged for 2 h at 25000 rev./min (90000 g) in an SW 28.2 rotor (Beckman). The membrane layer on top of the 1.3 M sucrose was removed and adjusted to 1.1 M sucrose by determination of the refractive index. A sample (27 ml) of this solution was overlayed with 7 ml of 1 M sucrose and 5 ml of 0.5 M sucrose and centrifuged for 30 min at 25000 rev./min (120000 g) in an SW 28.1 rotor (Beckman). The Golgi-membrane fraction appeared as a white band at the border of the 0.5 M and 1.0 M sucrose layer. This fraction was collected and adjusted to 0.25 M sucrose and 0.4 M NaCl. Membranes were pelleted at 45000 rev./min (200000 g) for 45 min in a Ti 60 rotor (Beckman). The pellet was resuspended in water and stored at −20 °C. Protein yield was about 1.4 mg. For details of the procedure see [15].

[^23]PUDP-N-acetylgalactosamine was synthesized according to a procedure described by Lang and Kornfeld [16]. The yield of radioactivity was 30–70 % and specific radioactivity 3000–4500 Ci/mmol. For the *in vitro* phosphorylation reaction the specific radioactivity was adjusted to 3–4.5 Ci/mmol. The phosphorylation assay contained various amounts of ASA or ASB, 35 mM sodium phosphate (pH 6.7), 22 µM [β-[^23]P]UDP-N-acetylgalactosamine, 1 % Triton X-100, 200 mM ADP, 10 mM CDP choline, 5 mM dimercaptosopropanol, 125 µM leupeptin and up to 7 µl of Golgi membranes in a final volume of 20 µl [17]. After incubation for various times 100 µg of BSA was added and the proteins were precipitated with 25 % (w/v) trichloroacetic acid. Pellets were washed with ethanol and subjected to SDS/PAGE. For quantification of phosphorylation the autoradiograph was quantified by densitometry or by determination of radioactivity in the eluted gel slices.

**Construction of chimeric ASA molecules**

For the mapping of the epitopes chimeric mouse–human ASA molecules were constructed. For this purpose we made use of a *PstI* restriction site conserved between mouse and human ASA.
Table 1 Immunoprecipitation of ASA with mAbs

ASA was immunoprecipitated from extracts of ASA-overexpressing BHK cells to determine reactivity with human ASA. Extracts of untransfected mouse Ltk- cells served as a source of mouse ASA to determine cross-reactivity with mouse ASA. After immunoprecipitation, enzyme activity was determined in the supernatant and in the pellet. The table summarizes the values for the four mAbs (11B5, 19C2, 20B1 and 20D2), a control anti-M6PR antibody and a polyclonal anti-(human ASA) antiserum (anti-ASA).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Human ASA (m-unit) Pellet</th>
<th>Human ASA (m-unit) Supernatant</th>
<th>% precipitated</th>
<th>Mouse ASA (m-unit) Pellet</th>
<th>Mouse ASA (m-unit) Supernatant</th>
<th>% precipitated</th>
</tr>
</thead>
<tbody>
<tr>
<td>11B5</td>
<td>3.8</td>
<td>0.0</td>
<td>100%</td>
<td>0.96</td>
<td>1.18</td>
<td>45</td>
</tr>
<tr>
<td>19C2</td>
<td>3.6</td>
<td>0.1</td>
<td>97%</td>
<td>0.13</td>
<td>1.69</td>
<td>7</td>
</tr>
<tr>
<td>20B1</td>
<td>3.5</td>
<td>0.2</td>
<td>95%</td>
<td>0.05</td>
<td>1.64</td>
<td>3</td>
</tr>
<tr>
<td>20D2</td>
<td>3.4</td>
<td>0.2</td>
<td>94%</td>
<td>0.05</td>
<td>1.73</td>
<td>3</td>
</tr>
<tr>
<td>Anti-M6PR</td>
<td>0.1</td>
<td>3.2</td>
<td>4%</td>
<td>0.07</td>
<td>1.77</td>
<td>4</td>
</tr>
<tr>
<td>Anti-ASA</td>
<td>1.9</td>
<td>0.0</td>
<td>100%</td>
<td>1.24</td>
<td>1.08</td>
<td>53</td>
</tr>
</tbody>
</table>

(see Figure 5). In this case the C-terminal half of the human molecule was replaced with the respective mouse sequences (construct 1). In all other cases (constructs 2, 3 and 4) fragments of the mouse cDNA were amplified with oligonucleotides which introduced restriction sites that allowed direct cloning into the human cDNA. The constructs were cloned into the expression vector pBEH [6] and stably (construct 1 and 2) or transiently (construct 3 and 4) expressed in BHK cells.

Generation of Fab fragments

Fab fragments of mAbs were generated by digestion with papain following a protocol described in [14].

Other methods

ASA activity was measured with p-nitroatecholinsulphate. A sample (200 µl) of 10 mM p-nitroatecholinsulphate in 0.5 M sodium acetate, pH 5.0/10% (w/v) NaCl was incubated with up to 50 µl of ASA-containing solutions. After incubation for different times the reaction was stopped by the addition of 500 µl of 1 M NaOH and extinction was read at 515 nm. Activity towards the natural substrate cerobrosilicate was measured by using a fluorescently labelled analogue of this lipid [18]. The reaction conditions were identical to those of the natural substrate except that 1% (w/v) Triton X-100 was present. Lipids were extracted by chloroform/methanol and separated by t.l.c. and quantified as described previously [19]. Protein was determined either by the absorption at 280 nm or by the method of Lowry [20]. Silver staining of SDS/polyacrylamide gels was done by the protocol of Ansojne [21]. SDS/PAGE and fluorography have been described [22].

RESULTS

Characterization of mouse anti-ASA mAbs

In an initial experiment the four mAbs were used to precipitate ASA under non-denaturing conditions from extracts of BHK cells overexpressing the human ASA cDNA (BHK-ASA cells). Each of the four antibodies precipitated more than 90% of ASA activity. The amount of ASA present in pellets when ASA mAbs were used for immunoprecipitation was comparable with that in the supernatant of the control 46 kDa anti-M6PR antibody indicating that the anti-ASA mAbs did not inhibit the enzymatic activity of the enzyme (Table 1). In contrast, the polyclonal anti-ASA antiserum inhibited the enzyme by about 50%. In a similar assay mouse ASA was immunoprecipitated from lysates of mouse Ltk- cells (see Table 1). The mAb 11B5 precipitated about half of the mouse ASA activity, whereas the other mAbs showed no cross-reactivity.

To determine the specificity of the four mAbs, BHK cells overexpressing human ASA, ASB or STS were metabolically labelled overnight with [35S]methionine and cell extracts were subjected to immunoprecipitation with the mAbs and polyclonal antisera specific for ASA, ASB or STS (see Figure 1). The four mAbs precipitated only ASA but none of the homologous sulphatases.

To test whether the mAbs are suitable for immunofluorescence staining BHK-ASA cells were fixed with formaldehyde and permeabilized with Triton X-100. Fixed cells were incubated with hybridoma culture media and a second rhodamine-coupled antibody was used to visualize the mouse immunoglobulins. All mAbs gave the typical lysosomal granular perinuclear staining (results not shown).

To assay the reactivity of the mAbs towards denatured enzyme, ASA was subjected to SDS/PAGE and transferred on to a nitrocellulose membrane. The membranes were incubated with the polyclonal anti-ASA goat serum and hybridoma supernatants or ascites dilutions of the mAbs. Whereas the goat antiserum detected ASA polypeptides none of the mAbs showed a signal (results not shown). In an additional experiment [125I]-labelled ASA was carboxymethylated with iodoacetic acid and the reactivity of modified ASA with mAbs was tested. None of the mAbs immunoprecipitated the carboxymethylated ASA polypeptides under the conditions that allowed the precipitation of ASA activity (results not shown).

p-Nitroatecholinsulphate as well as the natural substrate of ASA, cerobrosilicate, was used to examine the influence of the mAbs on the enzymic activity of ASA. None of the antibodies inhibited the activity of the enzyme (results not shown).

mAbs inhibit the recognition of ASA by the phosphotransferase

In the Golgi apparatus lysosomal enzymes are specifically recognized by a phosphotransferase which transfers N-acetylglucosamine 1-phosphate on to mannose residues of oligosaccharide side chains. Phosphorylation of ASA can be followed in vitro using solubilized Golgi membranes from rat liver as a source of phosphotransferase and [α-32P]UDP-N-acetylglucosamine as a donor of the radioactive N-acetylglucosamine 1-phosphate. The anti-ASA mAbs and the Fab fragments were examined for interference with the in vitro phosphorylation of ASA. An affinity-purified antiserum
recognizing the 46 kDa M6PR [23] served as a negative control. Figure 2 shows that upon addition of a 10-fold molar excess of mAb or Fab fragment over ASA phosphorylation of ASA in vitro is completely inhibited. Fab fragments prepared from the anti-M6PR antibody had no effect.

The phosphorylation of lysosomal enzymes requires the recognition of two sites on the acceptor lysosomal enzymes by the phosphotransferase. One site is represented by the proteinaceous determinant of the protein backbone, which distinguishes lysosomal enzymes from non-lysosomal glycoproteins. The other sites are the mannose residues on the oligosaccharide side chains which become phosphorylated. Inhibition of phosphorylation of ASA can therefore be due to two different effects. Either the antibodies cover the proteinaceous phosphotransferase-recognition domain of ASA or they cover the phosphate-accepting mannose residues. In the latter case ASA–mAb complexes can still bind to the phosphotransferase but phosphorylation is impossible. Binding of the phosphotransferase with the lysosomal enzyme would not be inhibited by the antibodies. To distinguish between these two possibilities a competition experiment was designed. ASA, a closely related lysosomal sulphatase, has a similar high affinity for the phosphotransferase. For both ASA and ASB a $K_m$ value of about 2 $\mu$M was determined. ASA was phosphorylated in vitro in the presence of ASA. The amounts of both enzymes were adjusted such that ASA inhibited the phosphorylation of ASB by about 50% (Figure 3). We reasoned that, if the mAbs interfere with the recognition by the phosphotransferase of the proteinaceous determinant of ASA, addition of anti-ASA mAbs should abolish the inhibition of ASB phosphorylation by ASA. On the contrary, if anti-ASA mAbs inhibit the phosphorylation of ASA by interfering with the
Table 2 Influence of mAbs on the pH-dependent dimerization of ASA

ASA was covalently coupled to Sepharose 4B beads. Beads were incubated with soluble ASA at neutral pH before the pH was lowered to pH 4.5. A control reaction was maintained at pH 7.0. Beads were pelleted and ASA activity was measured in the supernatant. The dimerization took place in the presence of a 10-fold molar excess of the Fab fragments of the antibodies indicated, which had been allowed to bind to the soluble ASA before beads were added.

<table>
<thead>
<tr>
<th>pH</th>
<th>Fab fragments of</th>
<th>ASA complexed with</th>
<th>Activity in supernatant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-M6PR</td>
<td>11B5</td>
<td>19C2</td>
</tr>
<tr>
<td>4.5</td>
<td>16</td>
<td>28</td>
<td>26</td>
</tr>
<tr>
<td>7.0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

The pH of the mixtures was lowered to 4.5 such that the soluble ASA could dimerize with immobilized ASA. The beads were pelleted and the remaining ASA activity in the supernatant was measured. This experiment was done either in the presence of a 10-fold molar excess of Fab fragments of two anti-ASA mAbs or with the control anti-M6PR antibody. In all cases the soluble ASA was found to dimerize with immobilized ASA (see Table 2). When beads were subsequently washed at neutral pH ASA activity reappeared in the supernatant (results not shown). To exclude the possibility that due to low pH the Fab fragments dissociate from the ASA, antigen immunoprecipitation experiments were performed. ASA–Fab fragment complexes were incubated with an anti-(ε light chain) antibody at pH values of 4.5 and 7.4. Upon addition of protein A–Sepharose ASA activity was quantitatively immunoprecipitated at both pH values, demonstrating that the ASA–Fab fragment complex is resistant to pH 4.5.

MAPPING OF THE EPITOPE RECOGNIZED BY THE FOUR mABS

To identify the epitopes recognized by the four mAbs we have made use of the observation that these mAbs did not (19C2, 20B1 and 20D2) cross-react, or only weakly (11B5), with mouse ASA. We constructed chimeras of the mouse and human ASA cDNA and overexpressed those hybrid ASA molecules in BHK cells.

Cells were harvested 48 h after transfection and the mAbs were used to precipitate ASA activity from cell homogenates. The constructs made are summarized in Figure 4. The reactivity with mAbs is indicated. Constructs 1 and 2 reacted with all mAbs, indicating that all epitopes are located between amino acid residues 143 and 280, which represents the second quarter of the molecule. Chimeras 3 and 4 were constructed to narrow down epitopes further. mAbs 19C2, 20B1 and 20D2 still precipitated about 50% of construct 3 but failed to recognize construct 4. 11B5 showed full reactivity with all constructs, locating its epitope between amino acid residues 244 and 280.

In order to examine whether the mAbs recognize the same epitope we performed competition experiments. mAbs were precoated on to 96-well plates. Human ASA, preincubated with a 10-fold (see Table 3) molar excess of mAbs, was then allowed to react with the mAbs (see Table 4) and the amount of residual activity was determined. This allowed the relative importance of each epitope to be judged.

Figure 4 Mapping of the epitopes recognized by the mAbs

Using the human and mouse ASA cDNA chimeras were constructed. The constructs were expressed in BHK cells, ASA was immunoprecipitated from cell extracts and enzyme activity was determined in the supernatants, as well as in the immunoprecipitated pellets. The bar represents the coding sequence, while parts are human sequences, hatched parts are mouse sequences. Potential N-glycosylation sites are indicated by q. The restriction sites used for fusion in the human ASA cDNA are indicated as well as the amino acid in the human ASA at which the fusion occurred. The Table on the right-hand side shows the reactivity of the mAbs with the respective construct on the left-hand side. + indicates that more than 90% of ASA activity was immunoprecipitable, (+) indicates that 50% was immunoprecipitable and — shows that the mAb precipitated less than 10% of enzyme activity. The amount of antibodies added was sufficient to ensure a complete precipitation of the equivalent amount of human ASA.
Table 3  Competition of antibody binding to ASA

96-well plates were precoated with the mAbs indicated in the left-hand column. Purified ASA was incubated with a 10-fold excess of each mAb. Free ASA or ASA–mAb complexes were allowed to bind to the antibody-coated wells. After 2 h unbound material was removed and the amount of bound ASA was determined by an e.i.s.a. using the polyclonal anti-ASA goat antiserum. The amount of bound ASA when free ASA was used as antigen was taken as 100%. The residual amount of ASA bound when the antibody used for precoating the plates and preincubation of ASA were identical was 22%, 8%, 43% and 34% for antibodies 11B5, 19C2, 20B1, and 20D2, respectively. These values were subtracted as background levels.

<table>
<thead>
<tr>
<th>mAb incubated</th>
<th>Amount of ASA bound (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11B5</td>
</tr>
<tr>
<td>11B5</td>
<td>0</td>
</tr>
<tr>
<td>19C2</td>
<td>16</td>
</tr>
<tr>
<td>20B1</td>
<td>28</td>
</tr>
<tr>
<td>20D2</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 4  Summary of the characteristics of the mAbs

The table summarizes the properties of the four mAbs. It shows the IgG subclass, the suitability of antibodies for immunoprecipitation (IP), immunofluorescence (IF) and Western-blot analysis (WB). The position of the epitope recognized is given in the last column.

<table>
<thead>
<tr>
<th>mAb</th>
<th>IgG subclass</th>
<th>IP</th>
<th>IF</th>
<th>WB</th>
<th>Epitope (ASA amino acid residues)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11B5</td>
<td>IgG(_a) (\kappa)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>256–265</td>
</tr>
<tr>
<td>19C2</td>
<td>IgG(_k) (\kappa)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>202–240</td>
</tr>
<tr>
<td>20B1</td>
<td>IgG(_k) (\kappa)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>202–240</td>
</tr>
<tr>
<td>20D2</td>
<td>IgG(_k) (\kappa)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>202–240</td>
</tr>
</tbody>
</table>

to bind to the mAb-precoated plates. After incubation for 2 h unbound complexes were removed and the amount of ASA bound to the plates was determined by an indirect e.i.s.a. using a polyclonal anti-ASA antiserum. Results of the competition experiments are shown in Table 3. Comparably high binding was observed for ASA–mAb complexes with 19C2, 20B1 and 20D2 to plates precoated with 11B5 (37% to 46%), while binding to plates precoated with 19C2, 20B1 and 20D2 was low. Conversely complexes of ASA with 11B5 still bound to plates precoated with 19C2, 20B1 and 20D2 (13% to 28%). These data suggest that antibodies 19C2, 20B1 and 20D2 recognize the same or closely adjacent epitopes, whereas 11B5 bind to a different epitope.

**DISCUSSION**

The four mAbs described (Table 4) here were raised against human ASA. The antibodies precipitated ASA under native conditions as well as in the presence of low concentrations of SDS (0.1%), as used for immunoprecipitation from extracts of metabolically labelled cells. However, carboxymethylation or SDS/PAGE followed by transfer to nitrocellulose abolished recognition by the mAbs. This indicates that the epitopes recognized are dependent on the correct folding of the enzyme.

No cross-reactivity with the homologous sulphatases ASB and STS could be detected. When tested with mouse ASA three mAbs (19C2, 20B1, 20D2) showed no, and one (11B5) only weak, reactivity with the mouse enzyme.

Using chimeric molecules constructed from mouse and human ASA cDNAs the epitopes recognized by the four mAbs could be located in the second quarter of the molecule. When the amino acid residues 143–194 were replaced by mouse sequences antibodies 19C2, 20B1 and 20D2 lost their reactivity partially and precipitated only half of the enzyme activity. When in addition amino residues 194–244 were replaced by mouse sequences reactivity was lost completely. This gradual loss of reactivity indicated that sequences present between residues 143 and 194 and 194 and 244 interact to form the epitopes and thus have to be in close spatial proximity on the surface of the molecule. Antibody 11B5 cross-reacts weakly with the mouse ASA but retains full reactivity with all of the constructs, which demonstrates that the epitope locates to the region in between the SstII and PstI sites which correspond to amino acids 244–280. That antibody 11B5 recognizes an epitope that is different from that of the other antibodies is also supported by the data from the competition experiments. If data in Table 3 are read columnwise from left to right it shows that 11B5–ASA complexes bind more efficiently to precoated 19C2, 20B1 and 20D2 than they do to 11B5. In all other cases mAb–ASA complexes bind better to 11B5 than to the other precoated antibodies. This is in accordance with the results obtained from the chimeric molecules.

Since human and mouse ASA sequences are highly homologous, epitopes can be defined more precisely by a comparison of both sequences (see Figure 5). For antibodies 19C2, 20B1 and 20D2 sequences between residues 143 and 194 and 194 and 244 contribute to the formation of the epitope. In fragment 143–194 one or more out of three non-conservative substitutions within a pentapeptide are likely to be part of the epitope (Ala\(^{165}\) → Asp; Thr\(^{166}\) → Ile; Asp\(^{169}\) → Lys). Ser\(^{198}\) → Thr is a conservative substitution and due to the presence of an oligosaccharide side chain at Asn\(^{194}\) this residue might not be accessible to the antibody. In fragment 194–244, the substitution of which causes a complete loss of reactivity of mAbs 19C2, 20B1 and 20D2, seven amino acids differ between the mouse and human sequence. Except the His\(^{206}\) → Arg substitution none of these exchanges is conservative.

![Figure 5  Amino acid sequence of the region 143–280 recognized by the mAbs](image-url)

The amino acid sequence of human ASA between residues 143 and 280 is shown. To facilitate the alignment with the constructs shown in Figure 4 the positions of the restriction sites used for fusion of mouse and human sequences are indicated. Letters in the lower line show amino acid substitutions found in the mouse ASA sequence. Two potential N-glycosylation sites are boxed. Amino acids underlined are conserved among all lysosomal sulphatases cloned so far [26].
Two (Asp216 → Gly and Glu238 → Lys) involve changes of polarity. Since the amino acid substitutions are not clustered, the epitope can only be localized between residues 202 and 240. The fragment 244–280 recognized by the 11B5 antibody shows four adjacent substitutions so the epitope may be located to residues 256–265.

ASA can be phosphorylated in vitro by the lysosomal enzyme phosphotransferase and we examined whether Fab fragments of the mAbs interfere with the phosphorylation of ASA in vitro. Addition of a 10-fold molar excess of Fab fragments caused complete inhibition of phosphorylation. This inhibition may be due to two different effects. The binding of the Fab fragment could cover the phosphate-accepting manno residues. As the epitopes map close to two potential N-glycosylation sites this may be a plausible explanation. On the other hand binding of the Fab fragment may interfere with the recognition of ASA by the phosphotransferase. To differentiate between the two possibilities we have performed competition experiments in which the phosphorylation of the homologous lysosomal sulphatase ASB was partially inhibited by ASA. The addition of Fab fragments led to a full restoration of ASB phosphorylation, indicating that in contrast with ASA the ASA-mAb complexes did not function as competitors. This strongly indicates that those complexes are not recognized by the phosphotransferase. Since all epitopes map to the same region of the molecule it is not surprising that each of the antibodies inhibited the phosphorylation. Since the gradual loss of reactivity of antibodies 19C2, 20B1, 20D2 in constructs 3 and 4 demonstrated a spatial interaction of non-adjacent amino acid residues the epitopes recognized by 11B5 and the other antibodies might be in even closer proximity than it appears from the linear amino acid sequence. Since none of the antibodies failed to inhibit phosphorylation we cannot exclude the possibility that the inhibition of phosphorylation is a rather unspecific effect due to sterical hindrance exerted by Fab fragments bound to a site distant from the phosphotransferase-recognition domain. However, at least two other functions of the ASA polypeptides were not affected by binding of the mAbs. One is the enzymic activity towards natural and artificial substrates and the other is the pH-dependent dimerization of ASA. The enzyme is a monomer at pH 7, but dimerizes at pH 4.5. This demonstrates that binding of the mAbs neither interfered with the accessibility of the active site nor with the domains mediating dimerization.

Studies in other systems have shown that binding of Fab fragments to pentapeptide epitopes can functionally dissect closely adjacent epitopes. An example is the characterization of peptide sequences of the cytoplasmic tail of the 46 kDa M6PR by microinjection of Fab fragments from polyclonal antibodies directed against pentapeptide fragments of the 52-amino-acid tail. In this example Fab fragments directed against the amino acid residues 38–42 did not interfere with signal-mediated trafficking of the receptor, whereas those directed against the pentapeptide 43–48 did [25]. Thus Fab fragments, which are large in comparison to the peptides they recognize, can functionally distinguish neighbouring sequences as short as pentapeptides.

The lysosomal protease cathepsin D is so far the only example in which sequences involved in the recognition by the phosphotransferase have been located [4]. The topogenic sequences of cathepsin D (amino acid residues 203 and 265–292) are located in the C-terminal quarter of the molecule. As already mentioned there are no obvious sequence similarities among lysosomal enzymes, nor do they show a common pattern of glycosylation. Comparison of the cathepsin D and ASA data also reveals that the location of the topogenic domains is not conserved. Topogenic cathepsin D sequences are located in the C-terminal part, whereas those of ASA seem to reside in the N-terminal part of the molecule. This conclusion is supported by the comparison of amino acid sequences of lysosomal sulphatases having different functions [26]. It is reasonable to assume that the topogenic motif is conserved among homologous sulphatases. The degree of sequence similarity is higher in the N-terminal sequences of sulphatases, whereas towards the C-terminus sequences become more divergent. Thus it is likely that a common topogenic motif is encoded in the N-terminal part of the molecule.

It will be necessary to generate a broader panel of anti-ASA mAbs to define more precisely the signals involved in the recognition of ASA by the phosphotransferase. The results of this study have revealed the feasibility of this approach.

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REFERENCES

8. Reference deleted.