Recognition of Arylsulfatase A and B by the UDP-N-acetylglucosamine:lysosomal Enzyme N-Acetylglucosamine-phosphotransferase

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The critical step for sorting of lysosomal enzymes is the recognition by a Golgi-located phosphotransferase. The topogenic structure common to all lysosomal enzymes essential for this recognition is still not well defined, except that lysine residues seem to play a critical role. Here we have substituted surface-located lysine residues of lysosomal arylsulfatases A and B. In lysosomal arylsulfatase A only substitution of lysine residue 457 caused a reduction of phosphorylation to 33% and increased secretion of the mutant enzyme. In contrast to critical lysines in various other lysosomal enzymes, lysine 457 is not located in an unstructured loop region but in a helix. It is not strictly conserved among six homologous lysosomal sulfatases. Based on three-dimensional structure comparison, lysines 497 and 507 in arylsulfatase B are in a similar position as lysine 457 of arylsulfatase A. Also, the position of oligosaccharide side chains phosphorylated in arylsulfatase A is similar in arylsulfatase B. Despite the high degree of structural homology between these two sulfatases substitution of lysines 497 and 507 in arylsulfatase B has no effect on the sorting and phosphorylation of this sulfatase. Thus, highly homologous lysosomal arylsulfatases A and B did not develop a single conserved phosphotransferase recognition signal, demonstrating the high variability of this signal even in evolutionary closely related enzymes.

Soluble lysosomal enzymes are synthesized at the rough endoplasmic reticulum (ER). They are glycoproteins carrying N-linked oligosaccharide side chains. From the ER they are transported to the Golgi apparatus where they acquire mannose-6-phosphate (M6P) residues on their high mannose-type oligosaccharide side chains. In the trans-Golgi compartment the M6P-bearing enzymes bind to M6P receptors, which mediate the vesicular transport from the Golgi apparatus to the lysosomes (for review see Ref. 1).

The synthesis of M6P residues on the oligosaccharide side chains is a two-step process. The initial event is the recognition of soluble lysosomal enzymes by UDP-GlcNAc:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase (briefly phosphotransferase). This enzyme transfers N-acetylglucosaminyl phosphate to mannose residues of the α1,6 and α1,3 branches of the N-linked oligosaccharide side chains, creating N-acetylglucosamine-1-phospho-6-mannose residues. Subsequently, the terminal GlcNAc residue is removed by N-acetylglucosamine-1-phosphodiester α-N-acetylgalactosaminidase generating a M6P residue on the oligosaccharide side chains (1). Via these M6P residues the enzymes bind to M6P receptors, which mediate the further vesicular transport from the Golgi apparatus to the lysosomes.

Thus, the critical step in the sorting of soluble lysosomal enzymes is the recognition by the phosphotransferase. This enzyme is able to distinguish lysosomal enzymes from secretory proteins. Various efforts have been made to define the structure of the topogenic signal of lysosomal enzymes, which is recognized by the phosphotransferase. No apparent sequence similarities were found when different lysosomal enzymes were compared, instead it was shown the topogenic signal is defined within the three-dimensional structure of soluble lysosomal enzymes (2). Initial experiments with the lysosomal pro tease cathepsin D identified lysine 203 and amino acids 265–292 in the C-terminal lobe of the enzyme as minimal lysosomal targetting elements (3), but other amino acids also contribute to the phosphotransferase recognition of cathepsin D (4). Overall, the amino acids involved cover a rather large region of about 1630 Å² on the surface of cathepsin D. Further experiments demonstrated an additional independent phosphotransferase recognition domain in the N-terminal lobe of the enzyme (5). Other studies, however, could not confirm a phosphotransferase recognition domain in the C-terminal lobe of cathepsin D (6). Thus, despite numerous efforts the topogenic determinants of lysosomal enzymes are still not satisfyingly defined.

Lysine residues, however, seem to play a critical role in the phosphotransferase recognition of various lysosomal enzymes, like cathepsin L (7), arylsulfatase A (ASA), arylsulfatase B (ASB) (9), cathepsin D (3, 4), cathepsin L (8, 10), and aspartylglucosaminidase (11). Comparisons of three-dimensional structures of non-homologous cathepsins A, B, and D in combination with peptide inhibition experiments of in vitro phosphorylation indicated that a β-hairpin loop structure containing the relevant lysines may be an important common recognition determinant in the cathepsins and in β-glucuronidase (12, 13). Comparable hairpin loops, however, where not found among those residues relevant for proper sorting of aspartylglucosaminidase (11). When the distance between critical lysine residues was determined, it was striking to find values of 34–35 Å in various non-homologous
Lysozymal Sorting of Arylsulfatase A and B

**In Vitro Mutagenesis of Lysine Residues**—Human ASA has a total of eight lysine residues. Five of these residues (Lys-367, Lys-393, Lys-457, Lys-457, and Lys-463) are located on the surface of the enzyme, two are partially accessible (Lys-302 and Lys-395), and one is not accessible from the solvent (Lys-123). Lys-123 and Lys-302 have been shown to be part of the active center (19, 24). In an initial *in vitro* mutagenesis experiment all lysines except for Lys-123 were replaced by alanine.

Expression plasmids containing the wild-type and lysine by alanine-substituted ASA cDNAs were transiently transfected into BHK cells in up to eight independent experiments. Forty-eight hours after transfection ASA activity was measured in the cells (see Fig. 1, *bottom line*). The K302A, K367A, K393A, and K433A substituted ASAs yielded 34–102% of the activity of wild-type ASA-expressing cells. Little or no ASA activity could be expressed from ASA cDNAs coding for K395A, K457A, and K463A-substituted enzymes (data not shown). Because the amount of enzyme activity expressed by a mutated ASA cDNA can be considered as an indicator for structural integrity, additional amino acid substitutions were introduced at positions 395, 457, and 463 to obtain expression of higher enzyme activities. Because of the close proximity of residues 393 and 395, we introduced various amino acid substitutions at position 395 on the background of the cDNA coding for the K393A-substituted enzyme to obtain an enzymatically active 393/395 double-substituted enzyme. An ASA cDNA coding for a double-substituted K393A/K395G or K393A/K395H enzyme expressed about 35 and 30% of enzyme activity compared with wild-type ASA, respectively. Four different amino acid substitutions (Arg, Ser, His, and Gly) were introduced at position 457, of which only the K457R- and K457S-substituted enzymes yielded satisfying en-
zyme activities of 45 and 25%, respectively. Thirteen different amino acid substitutions were introduced at position 463, of which only the K463R- and K463Q-substituted enzymes displayed residual enzyme activity of 39 and 14%, respectively.

To reveal whether the secretion of any of the enzymatically active lysine-substituted enzymes is enhanced, ASA activities in cells and in the media of transiently transfected BHK cells were compared (Fig. 1, A and B). Substitutions at positions 302, 367, 393, 395, and 433 had no effect on the distribution of enzyme activities; values were comparable to that of the wild-type enzyme. Numbers in the line at the bottom (Me) give the percentage of enzyme activity found in the media in n independent experiments. Numbers in the gray-shaded line at the bottom (Res) give the percentage of residual enzyme activity of each substituted enzyme relative to the wild-type enzyme. B, values of part A displayed as percentage of enzyme activity found in the media. The horizontal dashed line indicates secretion rates of wild-type ASA (WT).

**Fig. 1.** Enzyme activities in cells and media of BHK cells expressing lysine-substituted ASAs. A, BHK cells were transiently transfected with expression plasmids containing the ASA cDNAs with various lysine substitutions as indicated. ASA activity was measured in cells (Ce) and media (Me). N158Q/N350Q is an enzyme with only one non-phosphorylated oligosaccharide at Asn-184. We have shown previously that this enzyme is hypersecreted (23). Numbers in the line at the bottom (Me) give the average percentage of enzyme activity found in the media in n independent experiments. Numbers in the gray-shaded line at the bottom (Res) give the percentage of residual enzyme activity of each substituted enzyme relative to the wild-type enzyme. B, values of part A displayed as percentage of enzyme activity found in the media. The horizontal dashed line indicates secretion rates of wild-type ASA (WT).
ASA were consistently lower (25%) than the wild-type enzyme.

In contrast, all amino acid substitutions introduced at position 457 lead to an increase of ASA activity in the media to about 60% of total activity. Fig. 1A shows the absolute values for the amounts of ASA in cells and media, whereas Fig. 1B shows the same data expressed as percentage of total ASA activity found in the media. The latter presentation makes it more obvious that even mutant enzymes with low residual enzyme activity (K457G) show enhanced secretion.

As a control we have used an ASA (N158Q/N350Q) in which the two attachment sites for phosphorylated N-linked oligosaccharide side chains at Asn-158 and Asn-350 were mutated. The remaining oligosaccharide side chain at Asn-184 is poorly phosphorylated (16), and the mutant is hypersecreted (23). In summary, the data demonstrate that only mutations at position 457 lead to enhanced accumulation of ASA activity in the medium.

**Immunoprecipitation of Lysine-substituted ASA in Cells and Media of Metabolically Labeled Cells**—To verify the results obtained by ASA activity determination, BHK cells which were transiently transfected with ASA cDNAs coding for the wild-type or the lysine-substituted ASAs were metabolically labeled with [35S]methionine for 1.5 h and chased for 6 h. ASA was immunoprecipitated from cells and media. Bars indicate the average of ASA cross-reacting material in the media. Incorporation of radioactivity was determined with a Fuji Bioimager. n indicates the number of independent experiments. The horizontal dashed line indicates average secretion rate of wild-type ASA. B, top shows a representative experiment. ASA cross-reacting material was quantified densitometrically. Ce, cells; Me, medium. Bottom, percentage of labeled ASA in the medium.

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experiments (Fig. 2A) only the K457S and K457R amino acid-substituted enzymes on average yielded an amount of ASA cross-reacting material in the media comparable to the non-phosphorylated, hypersecreted N158Q/N350Q ASA. Thus, these results are in accordance with enzyme activity determinations shown in Fig. 1.

Phosphorylation of Lysine-substituted ASA—The enhanced secretion of lysine 457-substituted ASA should correlate with a decreased amount of M6P residues on the N-linked oligosaccharide side chains. To determine the degree of mannose-phosphorylation, BHK cells transiently transfected with expression plasmids containing the wild-type and various lysine-substituted ASAs were metabolically labeled with [35S]methionine ([35S]) or [32P]orthophosphate ([32P]), and ASA or ASB were immunoprecipitated. The amount of [32P] and [35S] incorporated into ASA and ASB was quantified with a bioimager (Fuji). These arbitrary values for [32P] and [35S] incorporation were divided. The quotient for the wild-type was taken as 100%. The values for single experiments were obtained by comparison of the substituted enzymes to the respective wild-type controls and expressed as percent of wild-type incorporation for every experiment. The table at the bottom summarizes these values for a number of experiments performed with ASA and ASB, n.d., non-detectable. The N158Q/N350Q-substituted enzyme has been shown to bear no M6P (16) and served as a negative control. In contrast to ASA, ASB is synthesized as a 64-kDa precursor and processed intralysosomally to a mature 47-kDa form (20). Because it has been shown that in BHK cells mannose 6-phosphate residues are rapidly dephosphorylated upon arrival in the lysosome (30), the mature 47-kDa form is not visible in the panel showing the [32P] labeling. Phosphorylation rates for ASB were obtained by using the [35S] and [32P] incorporation of the 64-kDa ASB precursor only.

![Phosphorylation of ASA and ASB](image)

**Fig. 3. Phosphorylation of ASA and ASB.** BHK cells were transiently transfected with ASA or ASB cDNAs substituted as indicated at the top. Cells were either labeled with [35S]methionine ([35S]) or [32P]orthophosphate ([32P]), and ASA or ASB were immunoprecipitated. The amount of [32P] and [35S] incorporated into ASA and ASB was quantified with a bioimager (Fuji). These arbitrary values for [32P] and [35S] incorporation were divided. The quotient for the wild-type was taken as 100%. The values for single experiments were obtained by comparison of the substituted enzymes to the respective wild-type controls and expressed as percent of wild-type incorporation for every experiment. The table at the bottom summarizes these values for a number of experiments performed with ASA and ASB. ASB, n.d., non-detectable. The N158Q/N350Q-substituted enzyme has been shown to bear no M6P (16) and served as a negative control. In contrast to ASA, ASB is synthesized as a 64-kDa precursor and processed intralysosomally to a mature 47-kDa form (20). Because it has been shown that in BHK cells mannose 6-phosphate residues are rapidly dephosphorylated upon arrival in the lysosome (30), the mature 47-kDa form is not visible in the panel showing the [32P] labeling. Phosphorylation rates for ASB were obtained by using the [35S] and [32P] incorporation of the 64-kDa ASB precursor only.

| ASA     | n | % of WT |
|---------|---|---------|
| WT      | 5 | 100     |
| K457R   | 5 | 33      |
| K457S   | 1 | 50      |
| K457G   | 1 | 31      |
| K433A   | 4 | 95      |
| K367A   | 3 | 106     |
| K393A   | 2 | 123     |
| N158Q   | 2 | n.d.    |
| N350Q   | 2 | n.d.    |
Lysosomal Sorting of Arylsulfatase A and B

ASAs, the K457R-substituted ASA showed only 33% of phosphorylation when compared with the wild-type enzyme. Because the replacement of lysine 457 by arginine conserves the positive charge, we also investigated the extent of phosphorylation of the K457S- and K457G-substituted ASAs. Compared with the wild-type enzyme these mutants showed residual phosphorylation of 50 and 31%, respectively.

Phosphorylation of Individual N-Linked Oligosaccharides—Wild-type ASA has three N-glycosylation sites. It was shown previously that only oligosaccharides at the first (Asn-158) and the third (Asn-350) glycosylation site are phosphorylated, whereas the second (Asn-184) is not (16). To examine how the K457R substitution affects the phosphorylation of the first and the third oligosaccharide side chain we analyzed the [32P]incorporation into individual oligosaccharide side chains. BHK cells were transiently transfected with the wild-type ASA and K457R-substituted cDNAs and labeled with [32P]orthophosphate for 3 h. After cell lysis 40 μg of unlabeled purified ASA was added, and ASA was immunopurified from the cell lysates. The purified enzyme was carboxymethylated and digested with trypsin. Tryptic peptides were separated by reversed-phase HPLC. The glycopeptides P9 and P18 were localized in the chromatographic fractions by MALDI-TOF mass spectrometry, detecting the hexose microheterogeneity characteristic for glycopeptides, and identified by N-terminal sequencing (FLIPPY and EPALAF for P9 and P18, respectively). The amounts of radioactivity (cpm) coeluting in fractions with P9 or P18 (oligosaccharides Asn-158 or Asn-350, respectively) are given and are corrected for background radioactivity (2-3 cpm per fraction, determined by liquid scintillation counting in the 156-1700 keV channel).

| Oligosaccharide | wt ASA | K457R ASA |
|----------------|--------|-----------|
| Asn-158        | 109 cpm| 155 cpm   |
| Asn-350        | 35 cpm | 0 cpm     |

The data also suggest that the K457S ASA is slightly better phosphorylated than the K457R- and K457G-substituted enzymes (Fig. 3, bottom). However, to prove that this difference is significant further experiments are needed, because we examined the K457S ASA only in one experiment to support the K457R results.

Although K457S- and K457G-substituted ASAs have only little residual enzyme activity, it is unlikely that reduction of phosphorylation is due to ER retention, because these mutants...

DISCUSSION

We have generated several enzymatically active lysine-substituted ASAs and ASBs to investigate the importance of lysine residues in the lysosomal sorting of these sulfatases. Only substitution of lysine 457 caused an increased secretion and reduced phosphorylation of ASA. Because lysine 302 has previously been identified as an element of the active center (19, 26) this residue could be expected not to contribute to the phosphotransferase recognition domain. Of the remaining lysines only residues 393, 433, and 457 are freely accessible to solvent. Lysine 395 is not fully surface-exposed, and lysine 463 is located within a groove, which is consistent with our result that they are not essential for recognition. Of the fully solvent-exposed residues lysine 433 of human ASA is not conserved in the murine ASA, which is in accordance with our result, that it is not essential for recognition of ASA by the phosphotransferase.

In all lysosomal enzymes investigated so far more than one lysine is involved in phosphotransferase recognition (4, 5, 8, 11). Simultaneous substitutions of these residues may (11) or may not (8) have additive effects on the extent of phosphorylation. In ASA substitution of a single lysine reduces phosphorylation by about 70%. This is consistent with the result that this ASA is secreted to an extent that is comparable to an ASA with increased lysine residues, although K457S and K457G show some increased activity in the media (Fig. 4). As for ASA, 32P incorporation into ASB was determined by labeling the cells with radioactive orthophosphate. This experiment yielded comparable phosphorylation rates for wild-type and K507A- and K507A/497A-substituted ASB, respectively (Fig. 3). Thus, substitution of these lysines has no effect on the sorting or phosphorylation of ASB.
are largely secreted and thus, not intracellularly retained. It should also be mentioned that our results for lysine 463 are not fully informative. Because most of the amino acid substitutions we introduced at Lys-463 did not yield enzymatically active enzyme and the K463R or K463Q ASA showed low secretion rates, we assume that this mutant is at least partially retained in the ER. Thus neither secretion rates nor extent of phosphorylation allow conclusion(s) about the importance of this residue in phosphotransferase recognition. However, in contrast to lysine 457 this residue is not fully surface exposed and
Lysosomal Sorting of Arylsulfatase A and B

Conservation of lysines and potential N-glycosylation sites among sulfatases

Part A: six lysosomal sulfatases (arylsulfatase A/ASA, accession number AAB03341; N-acetylglactosamine-6-sulfatase/GAS6, P34058; arylsulfatase B/ASB, AAA51779; iduronate-2-sulfatase/IDS, P22304; glucosamine-6-sulfatase/GL6S, P15586; sulfoglucosamine sulfaminidase/SPHM, NP 000190), and four non-lysosomal sulfatases (steroidsulfatase/STS, P08842; arylsulfatase D/ASD, I37166; arylsulfatase E/ASE, P51690; and sea urchin arylsulfatase/ASU, SO1793) were individually aligned with the ASA amino acid sequence. The top line shows lysine residues of ASA, and the other lines show the residues, which are found in equivalent positions in the other sulfatases. When a lysine is conserved, this is indicated by **bold letters**. When a lysine is found within three positions towards the N or C terminus of the equivalent residue, this is indicated by *italic letters*. The first letter/number combination gives the residue found in the equivalent position and the second combination the next adjacent lysine. Part B summarizes the conservation of potential N-glycosylation sites corresponding to Asn-158 and Asn-350 in ASA. If potentially glycosylated asparagines were found within 15 amino acids toward the N or C terminus of the amino acid equivalent to Asn-158 or Asn-350 of ASA, they were considered as conserved. *Numbers in parentheses* give the number of amino acid residues between the residue aligned to Asn-158 or Asn-350 and the actual asparagine of the nearest potential N-glycosylation site in amino acid residues.

| Lysosomal sulfatases       | ASA (K123) | K302 | K307 | K309 | K315 | K433 | K437 | K439 | K457 | K463 |
|---------------------------|------------|------|------|------|------|------|------|------|------|------|
| GAS6                     | K140       |      | R276 |      | Q397 | K399 | R448 | S466 |      |      |
| ASB                      | K145       |      |      | R385 | S417/K414 | L419 | R479 | S500/K497 | H506/K507 |      |
| IDS                      | K135       |      |      |      | G415 | K436 | L438 |      | K483 |      |
| GL6S                     | K149       |      |      |      | N405 | S430 | S432 | A483 |      | N504/K502 |      |
| SPHM                     | K123       | R262 |      |      | Q365 | N391 | K393 | R443 | R465 | K470 |

| Non-lysosomal sulfatases  | STS (K134) | K368 |      |      |      |      |      |      |      |      |
|---------------------------|------------|------|------|------|------|------|------|------|------|------|
| ASB                      | K145       |      |      |      |      |      |      |      |      |      |
| ASE                      | K145       |      |      |      |      |      |      |      |      |      |
| ASU                      | K156       |      |      |      |      |      |      |      |      |      |

| Lyosomal sulfatases       | Oligosaccharide Asn-158 | Oligosaccharide Asn-350 |
|---------------------------|-------------------------|-------------------------|
| GAS6                      | –                       | –                       |
| ASB                       | –                       | –                       |
| IDS                       | –                       | –                       |
| GL6S                      | –                       | –                       |
| SPHM                      | –                       | –                       |

| Non-lysosomal sulfatases  | Oligosaccharide Asn-158 | Oligosaccharide Asn-350 |
|---------------------------|-------------------------|-------------------------|
| STS                       | –                       | –                       |
| ASD                       | –                       | –                       |
| ASE                       | –                       | –                       |
| ASU                       | –                       | –                       |

is therefore unlikely to play a role in phosphotransferase recognition.

For ASA it has been shown that the oligosaccharide side chains at Asn-158 and Asn-350 are phosphorylated to a similar extent, whereas the oligosaccharide at Asn-184 bears no M6P (161). In the K457R ASA only oligosaccharide at Asn-158 is phosphorylated, whereas no radioactivity was detected in fractions containing oligosaccharide at Asn-350 (Table I). Thus, substitution of lysine 457 abolishes phosphorylation of oligosaccharide Asn-350 completely but still allows for residual phosphorylation of mannose residues residing at oligosaccharide A-158 only.

Lysine 457 may represents only one residue of the recognition domain and its loss may lead to an overall reduction of affinity for the phosphotransferase particularly affecting oligosaccharide Asn-350. Alternatively, ASA may have two independent recognition domains. The one responsible for phosphorylation of oligosaccharide Asn-158 could be lysine independent and located within another surface area of ASA. The latter possibility is supported by the fact that in the three-dimensional structure of ASA, oligosaccharide Asn-350 is closest to lysine 457, whereas oligosaccharide Asn-158 is located on the opposite site of the molecule (see Fig. 5). Introduction of novel N-glycosylation sites at various positions into cathepsin D has demonstrated that an increased distance to the phosphotransferase recognition domain correlates with a decreased phosphorylation of oligosaccharides (27). If lysine 457 is part of an independent recognition domain, which mainly directs the phosphorylation of oligosaccharide Asn-350, its substitution may affect phosphorylation of oligosaccharide Asn-350 more severely than that of oligosaccharide Asn-158.

Given the structural diversity of lysosomal enzymes, it seems unlikely that all of them have developed a similar distinct phosphotransferase recognition domain. This has led to the hypothesis that the phosphotransferase recognizes lysosomal enzymes through the contact to various surface located key residues preferably lysines (8, 11). These residues must have a particular position relative to each other that would allow for considerable structural variation in the different lysosomal enzymes. Thus, in cathepsin D and cathepsin L critical lysine residues in both non-homologous enzymes are 34 Å apart (8). Similar values of 34.31 and 35 Å were described for desoxyribonuclease I (14) and aspartylglucosaminidase (11), respectively. These similar distances suggest that the spacing of lysine residues on the surface is important for the recognition by the phosphotransferase.

However, at least in ASA a single lysine is critical. Thus, the suggested interlysine spacing of about 34 Å is also not a conserved feature of a topogenic structure common to all lysosomal enzymes. Even if one assumes that lysines other than 457 may also weakly contribute to phosphorylation, the distance between Lys-457 and the other lysines in no case reaches 34 Å. The largest distance of 25 Å is found between Lys-457 and Lys-367.

With the exception of lysine 54 of cathepsin L, all lysines that have been identified to be essential for phosphotransferase recognition of lysosomal enzymes are located in non-structured loop regions. Based on three-dimensional comparisons it has been suggested that at least in cathepsins A, B, and D and
\(\beta\)-glucuronidase a \(\beta\)-hairpin loop may be the common structural determinant of the phosphotransferase recognition domain (12, 13). The existence of a critical lysine residue in a helix in ASA demonstrates that this cannot be the case for all lysosomal enzymes. Lysines need not be located in unstructured loop regions to be important for mannose phosphorylation.

The diversity of lysosomal enzymes makes the existence of a discrete structurally conserved topogenic domain unlikely. However, the lysosomal sulfatases are homologous (15), and thus one may expect that during evolution a conserved phosphotransferase recognition domain has developed at least among the members of such an enzyme family. Sulfatases show a high degree of conservation in the N-terminal part, which decreases toward the C terminus (28) Thus, it is surprising to find a lysine (Lys-457) relevant for lysosomal sorting in a region showing the least conservation among sulfatases. Lysine 457 of ASA is strictly conserved in iduronate-2-sulfatase region showing the least conservation among sulfatases. Lysine 457 of ASA is strictly conserved in iduronate-2-sulfatase, among the members of such an enzyme family. Sulfatases show a high degree of conservation in the N-terminal part, which decreases toward the C terminus (28) Thus, it is surprising to find a lysine (Lys-457) relevant for lysosomal sorting in a region showing the least conservation among sulfatases. Lysine 457 of ASA is strictly conserved in iduronate-2-sulfatase region showing the least conservation among sulfatases. Lysine 457 of ASA is strictly conserved in iduronate-2-sulfatase.

The process by which the phosphotransferase recognizes lysosomal enzymes is still enigmatic. The only common feature of the recognition domain that has been identified so far is the necessity of lysine residues, but the position of these lysines important within the three-dimensional structure of lysosomal enzymes does not show striking similarities present in all enzymes investigated so far. Finally, our results show that even evolutionary related enzymes such as the sulfatases did not develop a single common recognition domain.

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REFERENCES

1. Kornfeld, S., and Melman, I. (1989) Annu. Rev. Cell Biol. 5, 483–525
2. Lang, L., Reitman, M. L., Tang, J., Roberts, R. M., and Kornfeld, S. (1984) J. Biol. Chem. 259, 14663–14671
3. Baranski, T. J., Faust, P. L., and Kornfeld, S. (1990) Cell 63, 281–291
4. Baranski, T. J., Koenig, A., and Kornfeld, S. (1991) J. Biol. Chem. 266, 23365–23372
5. Baranski, T. J., Cantor, A. B., and Kornfeld, S. (1992) J. Biol. Chem. 267, 23342–23348
6. Sedlacek, B., Fortenberry, S. C., and Chirgwin, J. M. (1995) J. Cell Sci. 108, 2067–2075
7. Cuozzo, J. W., and Sahagian, G. G. (1994) J. Biol. Chem. 269, 14490–14496
8. Cuozzo, J. W., and Sahagian, G. G. (1995) J. Biol. Chem. 270, 15611–15619
9. Gieselmann, V., Schmidt, B., and von Figura, K. (1992) EMBO J. 11, 2664–2669
10. Lukseng, K. E., Elsiger, M. A., Mort, J. S., Potier, M., and Peshehetsky, A. V. (1999) Biochemistry 38, 73–80
11. Jain, S., Drendel, W. B., Chen, Z., Mathews, F. S., Sly, W. S., and Grupp, J. H. (1996) Nat. Struct. Biol. 3, 375–381
12. Nishikawa, A., Gregory, W., Frenz, J., Caia, J., and Kornfeld, S. (1997) J. Biol. Chem. 272, 14008–14012
13. Parenti, G., Meroni, G., and Ballabio, A. (1997) Curr. Opin. Genet. Dev. 7, 386–391
14. Sommerlade, H., J., Selmer, T., Ingendoh, A., Giesselmann, V., von Figura, K., Neifer, K., and Schmidt, B. (1994) J. Biol. Chem. 269, 20977–20981
15. Peters, C., Schmidt, B., Rommerskirch, W., Rupp, K., Zuhldorf, M., Vingron, M., Meyer, H. E., Pohlmann, R., and von Figura, K. (1990) J. Biol. Chem. 265, 3374–3381
16. Sommerlade, H. J., Hille-Rehfeld, A., von Figura, K., and Giesselmann, V. (1994) Biochem. J. 297, 123–130
17. Lukatela, G., Kraus, N., Theis, K., Giesselmann, V., von Figura, K., and Saenger, W. (1998) Biochemistry 37, 3654–3664
18. Bond, C. S., Clements, P. R., Ashby, S. J., Collyer, C. A., Harrop, S. J., Hopkins, M. J., and Guss, J. M. (1997) Structure 5, 277–289
19. Stein, C., Giesselmann, V., Kreysing, J., Schmidt, B., Pohlmann, R., Waheed, A., Meyer, H. E., O'Brien, J. S., and von Figura, K. (1989) J. Biol. Chem. 264, 1525–1530
20. Artelt, P., Morrell, C., Ausmeier, M., Fitzeck, M., and Hauser, H. (1988) Gene (Amst.) 68, 213–219
21. Giesselmann, V., Schmidt, B., andvon Figura, K. (1992) J. Biol. Chem. 267, 12362–12366
22. Schmidt, B., Selmer, T., Ingendoh, A., and von Figura, K. (1995) Cell 82, 271–278
23. Dierks, T., Schmidt, B., and von Figura, K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11963–11968
24. Waldow, A., Schmidt, B., Dierks, T., Bulow, R., and Figura, K. (1999) J. Biol. Chem. 274, 12284–12288
25. Cantor, A. B., and Kornfeld, S. (1992) J. Biol. Chem. 267, 23357–23363
26. Tomatsu, S., Fukuda, S., Masane, M., Suwaga, K., Fukao, T., Yamaguchi, A., Hori, T., Ikawa, H., Ogawa, T., Nakashima, Y., Hanyo, Y., Hashimoto, T., Iwata, H., Ogawa, T., and Orii, T. (1991) Biochem. Biophys. Res. Comm. 181, 677–683
27. Cantor, A., and Kornfeld, S. (1990) J. Biol. Chem. 265, 23349–23356
28. Bresciani, R., and von Figura, K. (1996) Eur. J. Biochem. 238, 669–674