

# Several cooperating binding sites mediate the interaction of a lysosomal enzyme with phosphotransferase

Ritva Tikkanen<sup>1</sup>, Minna Peltola,  
Carita Oinonen<sup>2</sup>, Juha Rouvinen<sup>2</sup> and  
Leena Peltonen<sup>3</sup>

Department of Molecular Genetics, Institute of Biomedicine, University of Helsinki and National Public Health Institute, Mannerheimintie 166, FIN-00300 Helsinki and <sup>2</sup>Department of Chemistry, University of Joensuu, PO Box 111, FIN-87101 Joensuu, Finland

<sup>1</sup>Present address: Abteilung Biochemie II, Georg-August University Goettingen, Gosslerstrasse 12d, D-37073 Goettingen, Germany

<sup>3</sup>Corresponding author

R.Tikkanen and M.Peltola contributed equally to this work

**Lysosomal targeting of soluble lysosomal hydrolases is mediated by mannose 6-phosphate receptors, which recognize and bind mannose 6-phosphate residues in the oligosaccharide chains of proteins destined for delivery to lysosomes. This recognition marker is generated by the sequential action of two enzymes, the first of which, UDP-*N*-acetylglucosamine phosphotransferase, recognizes lysosomal enzymes on the basis of a structural determinant in their polypeptide chains. This recognition event is a key step in lysosomal targeting of soluble proteins, but the exact nature of the recognition determinant is not well understood. In this study we have characterized the phosphotransferase recognition signals of human lysosomal aspartylglucosaminidase (AGA) using transient expression of polypeptides carrying targeted amino acid substitutions. We found that three lysine residues and a tyrosine residing in three spatially distinct regions of the AGA polypeptide are necessary for phosphorylation of the oligosaccharides. Two of the lysines are especially important for the lysosomal targeting efficiency of AGA, which seems to be mostly dictated by the degree of phosphorylation of the  $\alpha$  subunit oligosaccharide. On the basis of the results of this and previous studies we suggest a general model for recognition of lysosomal enzymes by the phosphotransferase.**

**Keywords:** aspartylglucosaminidase/lysosomal targeting/  
mannose 6-phosphate receptor/phosphotransferase

## Introduction

Lysosomal targeting of soluble lysosomal enzymes occurs mainly via the mannose 6-phosphate (Man 6-P) receptor pathway and is dependent on generation of a Man 6-P marker in the oligosaccharide chains of proteins destined for lysosomes. The initial step is accomplished by the Golgi-resident enzyme UDP-*N*-acetylglucosamine 1-phosphotransferase (phosphotransferase), which catalyzes the transfer of GlcNAc phosphate onto the  $\alpha$ -1,2-linked

mannose residues in the oligosaccharide chains of lysosomal enzymes. Another intra-Golgi enzyme, *N*-acetylglucosamine 1-phosphodiester  $\alpha$ -*N*-acetylglucosaminidase later exposes the Man 6-P marker by removing the covering GlcNAc. The Man 6-P is then recognized and bound by mannose 6-phosphate receptors and the lysosomal hydrolases are transported into the endosomal compartment. The phosphotransferase enzyme has only been preliminarily characterized and very little is known about its mode of function and of the interactions with its substrate molecules. Recently the bovine enzyme was purified to homogeneity and found to be a hexameric 540 kDa complex consisting of two disulfide-linked homodimers of 166 and 51 kDa subunits and two non-covalently associated 56 kDa subunits (Bao *et al.*, 1996a,b).

The phosphotransferase must specifically recognize and phosphorylate soluble lysosomal proteins, which represent only a minority of all soluble glycoproteins passing through the Golgi apparatus. Therefore, this recognition event is a key step in lysosomal targeting. Phosphotransferase recognizes its substrates on the basis of a three-dimensional polypeptide determinant that has been postulated to be common to soluble lysosomal enzymes (Reitman and Kornfeld, 1981; Waheed *et al.*, 1982), but the exact nature of this targeting determinant is not well understood. Soluble lysosomal enzymes do not exhibit any obvious sequence homologies beyond functional similarity and thus sequence comparisons have not revealed any common targeting motif.

Earlier studies based mainly on various mutagenesis strategies have suggested that lysine residues of soluble lysosomal enzymes are essential for interaction with the phosphotransferase, but no well-defined structural determinant has been identified that would exclusively mediate the initial interaction. So far, the lysosomal targeting determinant has been experimentally characterized in only two lysosomal enzymes, namely cathepsins D and L (Baranski *et al.*, 1990, 1991, 1992; Cantor and Kornfeld, 1992; Cuzzo and Sahagian, 1994; Cuzzo *et al.*, 1995; Dustin *et al.*, 1995), but the results have been somewhat puzzling. In cathepsin D the minimal phosphorylation determinant was found to consist of Lys203 and residues 265–292 in the C-terminal lobe of the molecule (Baranski *et al.*, 1990). However, subsequent studies have revealed that other elements in the N-terminal lobe also affect phosphorylation of the oligosaccharides (Baranski *et al.*, 1992). In procathepsin L, Cuzzo *et al.* (1995) have shown that Lys54 and Lys99 in the propeptide region are necessary for phosphorylation. On the basis of these studies, it is known that lysine residues on the surface of the protein molecules play a key role in recognition by the phosphotransferase.

To date three-dimensional structures of seven lysosomal enzymes have been reported, namely those of cathepsins

D, B and L (Jia *et al.*, 1995; Baldwin *et al.*, 1993; Metcalf and Fusek, 1993; Coulombe *et al.*, 1996), protective protein/cathepsin A (Rudenko *et al.*, 1995),  $\beta$ -glucuronidase (Jain *et al.*, 1996), arylsulfatase B (Bond *et al.*, 1997) and aspartylglucosaminidase (Oinonen *et al.*, 1995). Unfortunately, the coordinates are available in the databank only for cathepsins B and D and aspartylglucosaminidase, making direct structural comparisons difficult. On the basis of similarity in the three-dimensional structure of the previously defined targeting determinant of cathepsin D and the newly resolved structure of  $\beta$ -glucuronidase, it was suggested that there might be some sequence conservation in lysosomal targeting signals. A loose consensus sequence forming a  $\beta$ -hairpin structure was indicated as a putative lysosomal targeting determinant (Jain *et al.*, 1996). However, experimental evidence from mutagenesis studies supporting this hypothesis is lacking.

A reliable identification of the targeting determinant requires the use of site-directed mutagenesis and interpretation of the data on the basis of the three-dimensional structure of the protein. Here we present characterization of the phosphotransferase recognition determinant of human lysosomal aspartylglucosaminidase (AGA). AGA is a soluble lysosomal amidohydrolase that catalyzes the breakdown of glycoasparagines by removing the oligosaccharide chain attached to asparagine residues (Makino *et al.*, 1968). The AGA enzyme is a heterotetramer of two  $\alpha$  and two  $\beta$  subunits that are translated as a continuous precursor polypeptide which is rapidly cleaved after synthesis into the subunits in the endoplasmic reticulum. This proteolytic cleavage produces the 27 kDa pro- $\alpha$  and 17 kDa  $\beta$  subunits and results in establishment of the enzymatic activity of AGA (Ikonen *et al.*, 1993). Recent data suggests that the activating cleavage producing the (pro- $\alpha\beta$ )<sub>2</sub> heterotetrameric complex is autoproteolytic (Tikkanen *et al.*, 1996) and is triggered by the joining together of two precursor polypeptides (Riikonen *et al.*, 1996). We have recently determined the three-dimensional structure of the heterotetrameric human lysosomal AGA enzyme (Oinonen *et al.*, 1995). Each  $\alpha\beta$  dimer in the active tetramer forms a single domain, the core of which is formed by a four layer sandwich of  $\alpha$ -helices and  $\beta$ -sheets. The C-terminal end of the  $\alpha$  subunit extends outside this core and forms a loop structure on top of the molecule.

The AGA precursor contains two asparagine-linked glycosylation sites, Asn38 in the  $\alpha$  and Asn308 in the  $\beta$  subunit. Both sites are utilized, each tetramer thus bearing four oligosaccharides, and both oligosaccharides can also be mannose 6-phosphorylated (Tikkanen *et al.*, 1995). The enzymatically active tetrameric molecule is transported mainly via the Man 6-P receptor pathway into lysosomes, where the C-terminus of the pro- $\alpha$  subunit is further trimmed, resulting in the mature 24 kDa  $\alpha$  subunit (Ikonen *et al.*, 1993). This event can be utilized to monitor the entry of AGA into the endosomal/lysosomal compartment.

The well-defined structural features of the AGA molecule make it an excellent candidate for detailed analyses aimed at characterization of the phosphotransferase recognition site(s). We used *in vitro* mutagenesis and pulse-chase studies of the mutated polypeptides expressed in animal cells to study the effect of targeted substitutions on phosphorylation of the oligosaccharides of AGA and

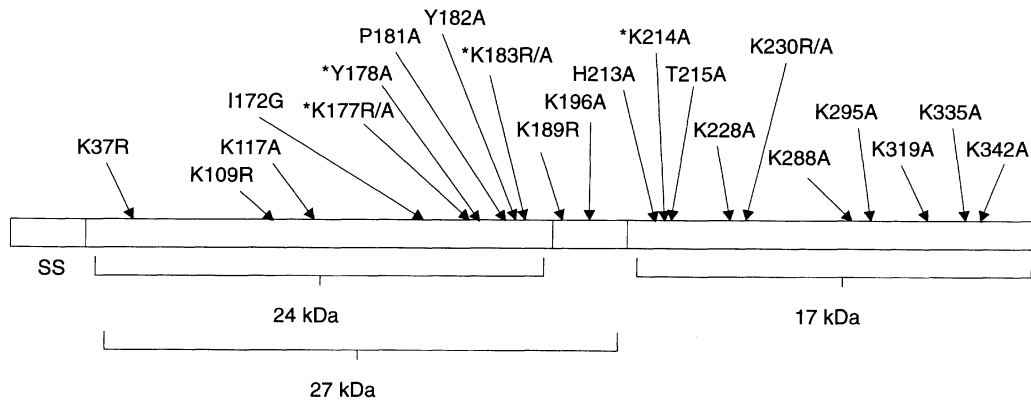
on lysosomal targeting. Three lysine residues and one tyrosine were found to be important for oligosaccharide phosphorylation. In the three-dimensional structure these residues are located in two distant regions and they form distinct but cooperating determinants that interact with the phosphotransferase. On the basis of the data from this and other studies we will also present some general rules for recognition of soluble lysosomal enzymes by the phosphotransferase.

## Results

### **Identification of residues necessary for the mannose 6-phosphorylation**

The role of individual amino acid residues in mannose 6-phosphorylation and lysosomal targeting of AGA was analyzed using site-directed mutagenesis and transient expression of the mutant polypeptides in COS-1 cells. Since lysine residues have been suggested to be essential recognition determinants in mannose 6-phosphorylation (Cuozzo and Sahagian, 1994), 15 of the 16 lysine residues of the AGA polypeptide (eight in the  $\alpha$  subunit and eight in the  $\beta$  subunit) were mutagenized either to arginines (most lysines in the  $\alpha$  subunit) or into alanines (those in the  $\beta$  subunit and Lys117 in the  $\alpha$  subunit) (Figure 1). Lys4 locates within the signal sequence and was not mutagenized. The effect of these substitutions on phosphorylation of the oligosaccharides and on lysosomal targeting efficiency of the AGA polypeptides was analyzed using pulse labeling with either [<sup>35</sup>S]Cys or [<sup>32</sup>P]P<sub>i</sub> in transiently transfected COS-1 cells. Activity measurements were performed to ensure that the mutant polypeptides exhibited normal enzyme activity to exclude the possibility that these substitutions resulted in misfolding of AGA which could be the cause of mistargeting. Of the lysine residues, Lys37, residing next to the *N*-glycosylation site of the  $\alpha$  subunit, and Lys117 could not be substituted for without disturbing the conformation of the polypeptide, whereas all other substitution mutants exhibited normal processing into subunits. We studied the effect of the produced substitutions on secretion, processing and phosphorylation of the mutant AGA polypeptides (Table I). Most of the substitutions resulted in wild-type phosphorylation ( $\pm 6\%$ ). For further studies we chose only those substitutions that resulted in a  $>10\%$  reduction in phosphorylation. Three lysine residues (Lys177, Lys183 and Lys214) were identified as putative parts of the GlcNAc phosphotransferase recognition determinant. Alanine substitutions were also produced for those  $\alpha$  subunit lysines whose substitution to arginine resulted in reduced phosphorylation of the oligosaccharides (Lys177 and Lys183) to test if the positive surface charge plays a role in phosphotransferase recognition. In both cases the alanine substitutions were found to result in a more severe phosphorylation defect than the arginine substitutions.

Lys177 and Lys183 are part of the C-terminal loop of the  $\alpha$  subunit, which forms an interesting extension into the tightly packed core structure of AGA. Furthermore, a sequence resembling the previously suggested short consensus sequence for GlcNAc phosphotransferase recognition (Jain *et al.*, 1996) could also be identified in this region, (Lys177 Tyr Cys Gly Pro Tyr Lys183), although in AGA it does not form a  $\beta$ -hairpin structure. Additional



**Fig. 1.** Schematic illustration of the mutagenized amino acids of the AGA polypeptide. The AGA polypeptide consists of a signal sequence (ss) and two subunits: the 27 kDa pro- $\alpha$ , which is further trimmed to the 24 kDa  $\alpha$  subunit in lysosomes, and the 17 kDa  $\beta$  subunit. The amino acids mediating interaction with the phosphotransferase are indicated with an asterisk.

alanine substitutions were produced for the neighboring residues Tyr178, Pro181 and Tyr182, whereas Ile172 was mutagenized to glycine. In pulse-chase experiments the Pro181Ala and Ile172Gly substitutions did not disturb either lysosomal targeting or phosphorylation, whereas both the Tyr178Ala and Tyr182Ala mutants exhibited reduced phosphorylation (Table I). Further analysis showed, however, that at least in part, the effect of Tyr182Ala substitution was due to reduced stability and degradation of the mutant polypeptides with this substitution.

The third lysine residue (Lys214) important for phosphorylation is located 40 Å distant from Lys183. To determine if this region also involves other residues, two nearby amino acids, Thr215 and His213, were mutagenized to alanine. The effect of these substitutions was analyzed as above. Pulse-chase studies with  $^{35}\text{S}$  and  $^{32}\text{P}$  labels showed that lysosomal targeting as well as phosphorylation was unaffected by the substitutions Thr215Ala and His213Ala.

### **The phosphotransferase recognition sites in the AGA polypeptide**

On the basis of the preliminary screening, those substitutions that seemed to produce an effect on phosphorylation and/or lysosomal targeting were analyzed in more detail. For further analysis we chose three different lysine substitutions (Lys177Ala, Lys183Ala and Lys214Ala) as well as mutant Tyr178Ala. In addition to single substitutions, we also produced mutants carrying various combinations of the alanine substitutions (Lys177Lys183Ala, Lys177Tyr178Lys183Ala, Lys183Lys214Ala and Lys177-Tyr178Lys183Lys214Ala). Pulse-chase analysis of  $^{35}\text{S}$ -labeled polypeptides with various chase times (1, 3 and 6 h) were performed to monitor lysosomal targeting efficiency of both the single and combination mutants. The targeting defect was already apparent at 3 h chase with mutants Lys183Ala, Lys214Ala and all the combination mutants, as evidenced by the slow appearance of the mature 24 kDa  $\alpha$  subunit and enhanced secretion of the polypeptides into the medium (data not shown). In particular, the two combination mutants in which the two lysines Lys183 and Lys214 were both substituted for were massively secreted into the medium, as shown in Figure

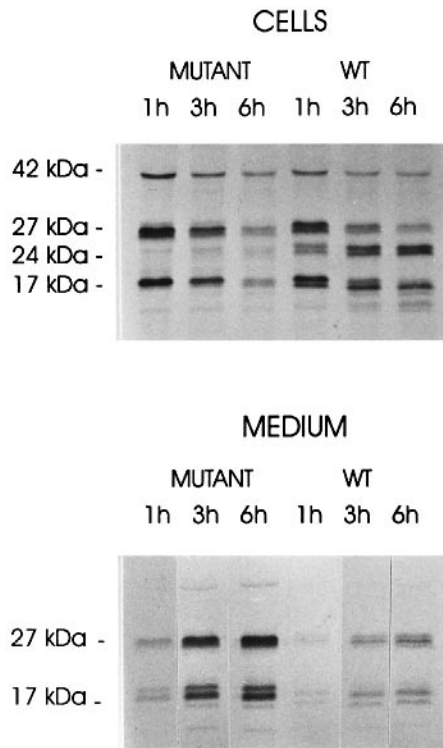
**Table I.** Preliminary screening of the lysine and other substitutions in the AGA polypeptide

Substitution	Activity	Precursor processing	Secretion	Phosphorylation
Lys37Arg	BG	misfolding	n.d.	n.d.
Lys109Arg	as WT	as WT	as WT	as WT
Lys117Ala	BG	misfolding	n.d.	n.d.
Lys177Arg	as WT	as WT	as WT	reduced
Lys177Ala	as WT	as WT	as WT	reduced
Lys183Arg	as WT	as WT	Increased	reduced
Lys183Ala	as WT	as WT	Increased	reduced
Lys189Arg	as WT	as WT	as WT	as WT
Lys196Ala	as WT	as WT	as WT	as WT
Lys214Ala	as WT	as WT	increased	reduced
Lys228Ala	as WT	as WT	as WT	as WT
Lys230Ala	as WT	as WT	as WT	as WT
Lys288Ala	as WT	as WT	as WT	as WT
Lys295Ala	as WT	as WT	as WT	as WT
Lys319Ala	as WT	as WT	as WT	as WT
Lys335Ala	as WT	as WT	as WT	as WT
Lys342Ala	as WT	as WT	as WT	as WT
Ile172Gly	as WT	as WT	as WT	as WT
Tyr178Ala	as WT	as WT, unstable	as WT	reduced
Pro181Ala	as WT	as WT	as WT	as WT
Tyr182Ala	reduced	degradation	reduced	reduced
His213Ala	as WT	as WT	as WT	as WT
Thr215Ala	as WT	as WT	as WT	as WT

The effect of targeted amino acid substitutions on phosphorylation and secretion of the mutant AGA polypeptides transiently expressed in COS cells was determined in pulse-chase experiments with  $^{35}\text{S}$  or  $^{32}\text{P}$  label. Each determination was performed at least twice. As WT, the numeric values deviate from those obtained for the wild-type AGA by not more than 6%; BG, background; n.d., not determined.

2 for the combination mutant containing all four substitutions.

Since pulse-chase analysis can be used to monitor the fate of polypeptides synthesized during a limited period of time, immunofluorescence staining was performed with cells transfected with the wild-type AGA or with the combination mutant carrying all four substitutions. These analyses show that even at steady-state these mutant polypeptides are not detected in significant amounts in lysosomes, but rather reveal an endoplasmic reticulum/Golgi-like staining pattern, as expected for a protein that is present in the secretory pathway. In contrast, the wild-

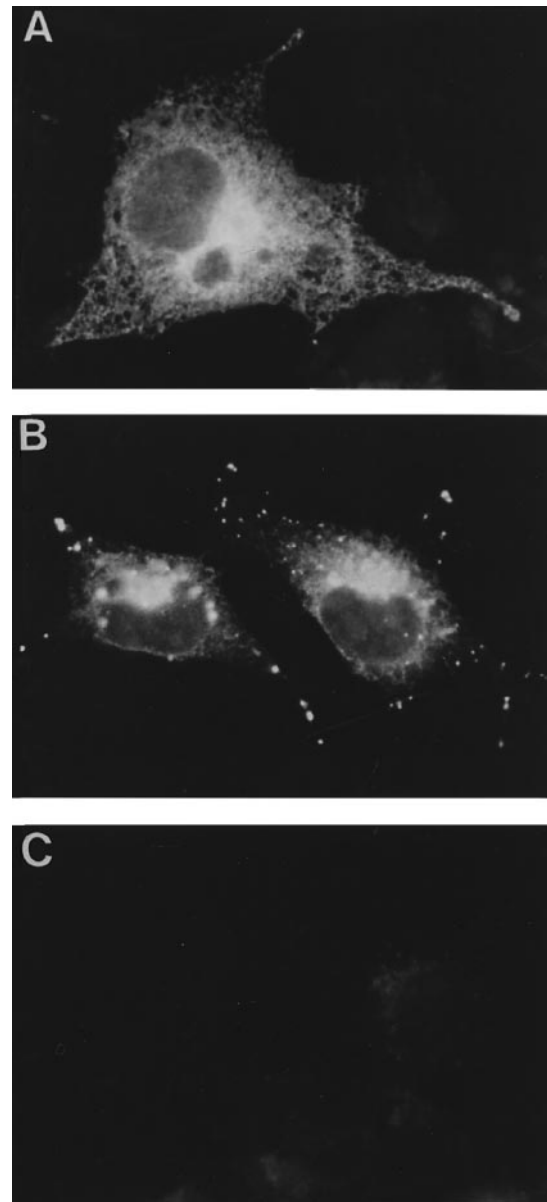


**Fig. 2.** Pulse-chase analysis of the Lys177Tyr178Lys183Lys214Ala mutant and wild-type (WT) AGA polypeptides expressed in COS-1 cells. The cells were transfected with the mutant or wild-type AGA cDNA constructs and pulse-labeled with [ $^{35}$ S]Cys for 1 h and chased for 1, 3 or 6 h as indicated. The proteins were immunoprecipitated from the cells and medium with an AGA-specific antiserum and separated by 14% SDS-PAGE. The molecular weights of the polypeptides are indicated.

type AGA displays a clear Golgi and lysosomal staining (Figure 3).

Phosphorylation of the oligosaccharides of the mutant AGA enzymes was studied using pulse-chase labeling. The wild-type and mutant polypeptides were labeled with [ $^{32}$ P] $P_i$ , immunoprecipitated from the cells and the medium and analyzed by SDS-PAGE and autoradiography. Each of the single substitutions resulted in a noticeable decrease in phosphorylation, but different substitutions exhibited phosphorylation defects of varying degrees (Figure 4). Endoglycosidase H digestion was performed with the labeled, immunoprecipitated wild-type polypeptides to demonstrate that labeling with  $^{32}$ P was oligosaccharide-specific and thus could be removed upon digestion. We observed no remaining label after digestion with endoglycosidase H, suggesting that there is no phosphorylation of AGA in the polypeptide backbone that would interfere with our phosphorylation assay (data not shown). We also studied the half-life of phosphorylation of the over-expressed AGA. Even after 6 h chase we observed no reduction in phosphorylation of the wild-type AGA, indicating that the Man 6-P groups of AGA are stable under the conditions used in this study (2.5 h pulse, 3 h chase) (data not shown).

To measure quantitatively the effect of these substitutions on secretion and lysosomal targeting of the polypeptides as well as on phosphorylation of the oligosaccharides, pulse-chase experiments were performed in parallel with both [ $^{35}$ S]Cys (1 h pulse, 4.5 h chase) and [ $^{32}$ P] $P_i$  (2.5 h

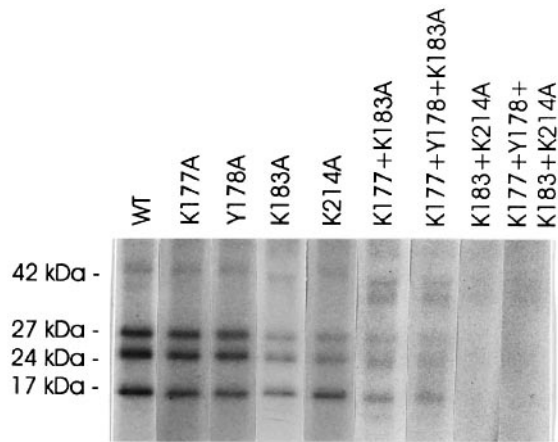


**Fig. 3.** Immunofluorescence staining of AGA polypeptides in transfected COS cells. (A) Lys177Tyr178Lys183Lys214Ala; (B) wild-type AGA; (C) untransfected COS-1 cells.

pulse, 3 h chase). Secretion of the mutant polypeptides was measured as the fraction (% secreted) of the [ $^{35}$ S]Cys-labeled AGA polypeptides immunoprecipitated from the medium and the cells after 1 h label and 4.5 h chase, as determined by scanning of the autoradiographs. Lysosomal targeting efficiency was determined as the percentage of processed  $\alpha$  subunit from the total intracellular and secreted  $\alpha$  subunit pool (pro- $\alpha$  +  $\alpha$ ) of the  $^{35}$ S-labeled AGA polypeptides. Phosphorylation was determined as the percentage of wild-type phosphorylation in the parallel experiments, by determining the relative total amount of immunoprecipitated phosphorylated polypeptides (intracellular + secreted) correlated with transfection efficiency/expression level (relative amount of total immunoprecipitated  $^{35}$ S-labeled polypeptides) (Table II). Substitutions of Lys177 or Tyr178 resulted in a noticeable but relatively minor reduction in phosphorylation of the oligosaccharides and neither secretion nor lysosomal targeting efficiency

of the polypeptides was altered. Other single substitutions as well as their combinations result in a more severe defect in phosphorylation (exhibiting 4.2–40% of wild-type phosphorylation) associated with increased secretion and decreased lysosomal targeting of the polypeptides (5.8–29%) (Table II).

Our assay allows for quantification of the effect of the produced substitutions on phosphorylation of the oligosaccharides in each subunit separately, since both the  $\alpha$  and the  $\beta$  subunit carry only one oligosaccharide chain. All single substitutions and their combinations that resulted in reduced phosphorylation of the oligosaccharides affect phosphorylation of both subunits (Table II). However, the subunit phosphorylation ratio ( $\alpha/\beta$ ) is similar to that of the wild-type AGA only in the case of the Lys177Ala and Tyr178Ala mutants, whereas all other mutants exhibit considerably lower ratios (Table II). This indicates that although overall phosphorylation of AGA is reduced by all these substitutions, some of them display a stronger



**Fig. 4.** Phosphorylation of the mutant and wild-type AGA polypeptides. COS cells were transfected with one of the mutant or wild-type AGA cDNA constructs and pulse-labeled with [ $^{32}$ P]orthophosphate for 2.5 h and chased for 3 h. The substitutions are indicated above the lanes with the standard one letter abbreviations due to space restrictions.

effect on phosphorylation of the  $\alpha$  subunit sugar chain. Interestingly, the reduced phosphorylation ratio also correlates with enhanced secretion and reduced lysosomal targeting efficiency. In the case of the mutant polypeptides exhibiting reduced phosphorylation ratios, lysosomal targeting efficiency is not improved even after prolonged chasing (18 h), whereas the fraction of secreted polypeptides is increased as compared with shorter chase times (data not shown). This further indicates that these polypeptides are incapable of being internalized by endocytosis.

In COS-1 cells, human AGA protein was overexpressed ~20- to 30-fold as compared with endogenous AGA. Therefore, it is possible that this high overexpression might cause mistargeting and exaggerate the effect of the produced substitutions on targeting and phosphorylation of AGA. To test this, we also expressed wild-type AGA and the mutants containing the substitutions Lys183Ala or Lys214Ala or their combinations in NIH 3T3 cells (indicated in Table II), resulting in 5- to 7-fold lower expression levels. The results concerning secretion, lysosomal targeting and phosphorylation of the wild-type and mutant AGA proteins were found to be in good agreement with the results obtained in COS-1 cells (Table II).

## Discussion

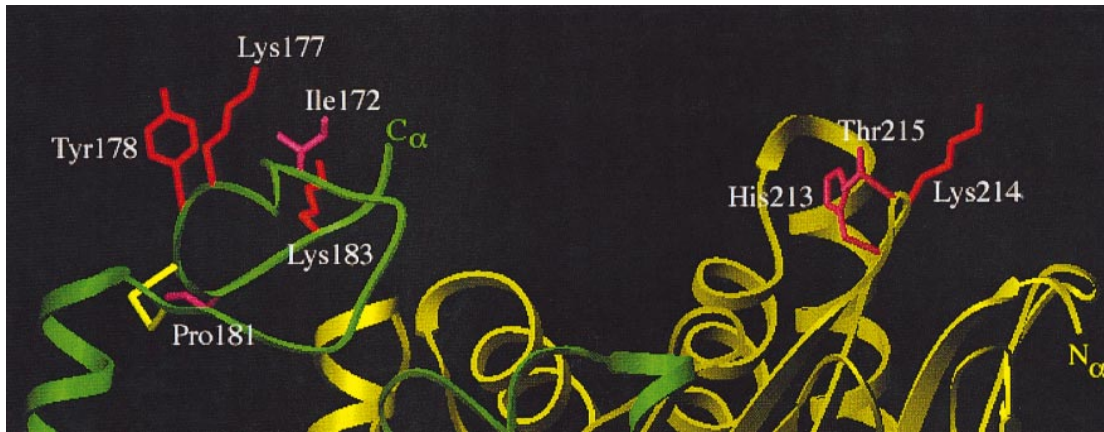
A systematic mutagenesis screening of the lysine residues in the AGA polypeptide revealed three lysine residues important for proper phosphorylation of the oligosaccharides, namely Lys177 and Lys183 in the  $\alpha$  and Lys214 in the  $\beta$  subunit. On the basis of the three-dimensional structure of AGA determined earlier by us (Oinonen *et al.*, 1995), these three lysines clearly protrude from the surface of the AGA molecule. We selected several candidate residues in the close vicinity of the lysines in the three-dimensional structure and analyzed their role in phosphorylation, but of these only Tyr178 was clearly demonstrated to be a part of the phosphorylation signal of AGA (Figure 5). However, Lys183 and Lys214 seem to be the major parts of the determinant, Lys177 and Tyr178 playing

**Table II.** Effect of targeted substitutions on phosphorylation and lysosomal targeting of AGA

	Number of experiments	Phosphorylation (% wild-type) (cells + medium)	Phosphorylated $\alpha/\beta$ ratio		Lysosomal targeting efficiency	Secretion (% total $^{35}$ S-labeled polypeptides)
			Cells	Medium		
WT <sup>a</sup>	5	100	2.5 ± 0.25	3.1 ± 0.22	52 ± 3.6 (43)	32 ± 3.1 (24)
Lys177Ala	3	86 ± 5.0	2.9 ± 0.085	2.7 ± 0.55	58 ± 6.2	26 ± 4.1
Tyr178Ala	3	58 ± 4.9	2.9 ± 0.58	3.6 ± 0.88	62 ± 2.2	27 ± 1.9
Lys183Arg	2	54 ± 4.8	2.7 ± 0.35	3.3 ± 0.50	49 ± 1.4	43 ± 5.6
Lys183Ala <sup>a</sup>	4	28 ± 7.2 (35)	1.0 ± 0.087	1.2 ± 0.11	27 ± 1.4 (25)	62 ± 7.1 (50)
Lys214Ala <sup>a</sup>	4	39 ± 2.9 (30)	1.2 ± 0.38	1.3 ± 0.13	29 ± 1.5 (27)	56 ± 12 (49)
Lys177Lys183Ala	4	29 ± 4.5	1.4 ± 0.25	1.9 ± 0.33	23 ± 4.7	59 ± 5.5
Lys183Lys214Ala <sup>a</sup>	3	4.2 ± 0.8 (2.3)	nd	nd	10 ± 4.8 (16)	83 ± 6.4 (59)
Lys177Tyr178Lys183Ala	4	22 ± 1.0	1.7 ± 0.049	2.2 ± 0.14	23 ± 5.8	62 ± 3.2
Lys177Tyr178Lys183Lys214Ala <sup>a</sup>	4	7.7 ± 1.3 (9.5)	nd	nd	5.8 ± 1.3 (13)	74 ± 7.7 (59)
Pro181Ala	3	106 ± 5.0	2.4 ± 0.10	2.5 ± 0.050	53 ± 2.8	30 ± 2.6

The effect of selected amino acid substitutions on phosphorylation of the oligosaccharides and on lysosomal targeting of the mutant AGA polypeptides was determined quantitatively. Phosphorylation was correlated with the expression level as determined from parallel experiments using  $^{35}$ S label. Expression levels of the mutants were within the range 80–120% of the expression level of the overexpressed wild-type AGA. Values are given with the standard deviations. nd, not determined due to the very low level of phosphorylation.

<sup>a</sup>Constructs also expressed in NIH 3T3 cells, the results for which are given in parentheses and represent a mean of two parallel samples.



**Fig. 5.** The mutated amino acid residues of AGA. The  $\alpha$  polypeptide chain is in green and the  $\beta$  polypeptide chain in yellow. The residues affecting phosphorylation and lysosomal targeting (Lys177, Tyr178, Lys183 and Lys214) are colored red. The neighboring residues that were mutated but which did not affect phosphorylation are colored pink (Ile172, Pro181, His213 and Thr215). Tyr182 has been omitted from the picture for clarity. The figure was drawn with the program SETOR (Evans, 1993).

only a less important, yet clearly detectable role in the recognition event.

#### **The lysosomal targeting signals in the $\alpha$ and $\beta$ subunits**

Two parts of the phosphotransferase recognition signal of AGA are located at the C-terminal end of the  $\alpha$  subunit, the first comprising Lys177 and Tyr178, the second Lys183. Interestingly, the loose lysosomal targeting consensus sequence (Lys Tyr Pro Lys Gly Tyr in  $\beta$ -glucuronidase) suggested by Jain *et al.* (1996) could also be identified in this region. In AGA the same residues are present, but the actual sequence is different and interrupted by a Cys that is part of a disulfide bridge (Lys Tyr Cys Gly Pro Tyr Lys). Furthermore, in AGA these residues do not form a  $\beta$ -hairpin. The C-terminal end of the mature, lysosomal  $\alpha$  subunit of AGA is a 24 residue polypeptide that forms a loop structure held together by a disulfide bridge followed by a short C-terminal extension, but the functional significance of this region has previously been unknown (Oinonen *et al.*, 1995). Interestingly, the most common mutation causing aspartylglucosaminuria, a lysosomal accumulation disease, results in substitution of Cys163 (Ikonen *et al.*, 1991) and disruption of the disulfide bond to Cys179 holding the C-terminal loop of the  $\alpha$  subunit together. The mutant polypeptides are neither processed into subunits nor phosphorylated (Peltola *et al.*, 1996), indicating the significance of folding of this region in both processing of the AGA precursor into subunits and in oligosaccharide phosphorylation.

Lys177 and Lys183 are accessible on the surface of the molecule and are likely to be in direct contact with the phosphotransferase. The flat face of the Tyr178 phenol ring is also exposed to the solvent. Tyr182, whose substitution resulted in lower phosphorylation but also in reduced stability of the AGA molecule, is also an important part of this structure, affecting the conformation of the C-terminal loop structure. However, alanine substitution of Tyr182 cannot result in severe misfolding, since this region is located close to the subunit processing site and large structural alterations would result in prevention of processing of the precursor molecule into subunits. In the

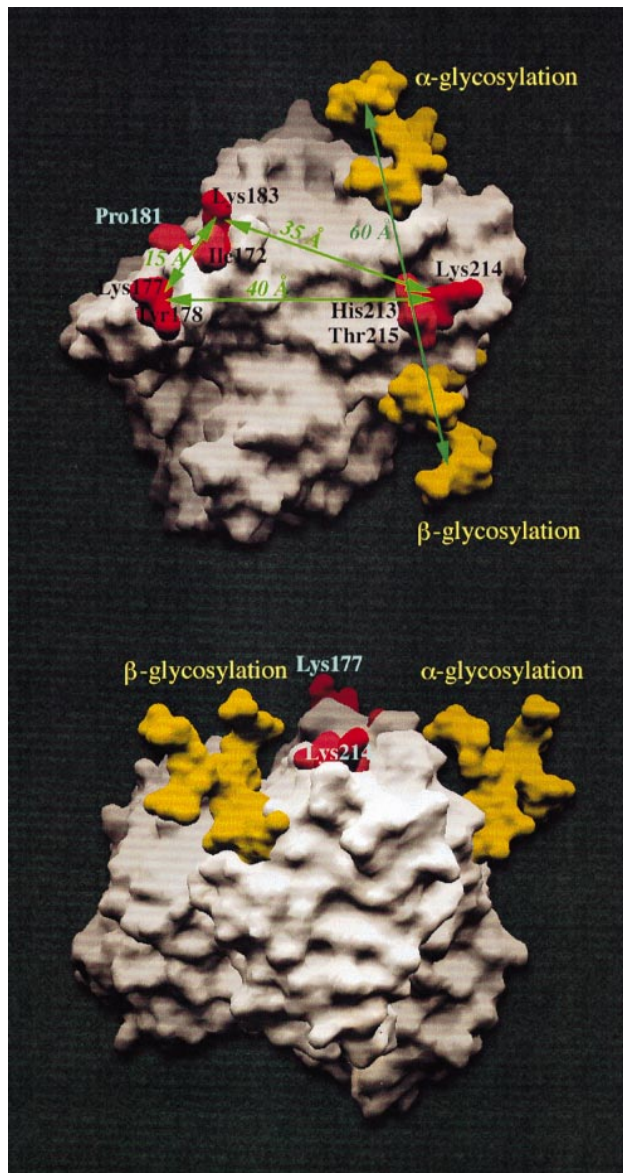
case of the Tyr182Ala mutant normal processing of the precursor into subunits was found to occur without delay.

The third targeting determinant, located in the  $\beta$  subunit of AGA, seems to consist of Lys214, which also clearly extends outwards from the surface of the molecule. Lys214 is located at a  $\beta$ -hairpin between the first two  $\beta$ -strands of the  $\beta$  subunit. Recently Jain *et al.* (1996) have suggested that this kind of  $\beta$ -hairpin structure could be part of the lysosomal targeting motif. However, this  $\beta$ -hairpin of AGA is embedded in the structure, with only His213, Lys214, Thr215 and His217 exposed to the solvent, and this region does not match with the consensus recognition sequence that has been suggested by Jain *et al.* Moreover, phosphorylation of AGA, which would suggest that the interaction area on the  $\beta$  subunit is restricted.

On the basis of the three-dimensional structure of the AGA heterotetramer the three signals are located far from each other, on opposite faces of the molecule, when the residues in each  $\alpha\beta$  heterodimer are considered. However, determinants provided by different halves of the tetramer, i.e. the  $\alpha$  subunit loop with Lys177, Tyr178 and Lys183 from one dimer and the  $\beta$  subunit site with Lys214 from the other dimer, place the residues on the same face of the tetrameric AGA molecule (Figure 6). Therefore, each AGA molecule contains two recognition sites, both of which involve residues from both  $\alpha\beta$  dimers of the tetramer. The *N*-glycosylation sites of AGA are located 60 Å from each other and are not in the immediate vicinity of the phosphotransferase-binding sites. However, molecular modeling of the *N*-glycans in both subunits suggests that the mannose residues that can become phosphorylated are at approximately the same spatial level in the molecule as the targeting signal residues.

#### **Interaction with the phosphotransferase is cooperative.**

Our mutagenesis data suggest that the recognition sites of AGA function in a cooperative manner. Since all three sites of AGA are necessary for full phosphorylation of the oligosaccharide on either subunit, binding of the phosphotransferase must take place simultaneously at all



**Fig. 6.** The solvent-accessible surface of AGA in two orientations. The protein surface is shown in white and the modeled glycan surface in yellow. The mutated amino acid residues are colored red (reduced phosphorylation) or pink (phosphorylation level unchanged). The figure was drawn with the program GRASP (Nicholls *et al.*, 1991).

sites. Although Lys177 and Tyr178 clearly play a role in binding of the phosphotransferase, as evidenced by the reduced phosphorylation resulting from their single substitution, the main part of the determinant seems to be made up of Lys183 and Lys214, whose combined substitution results in a similar defect in targeting and phosphorylation as combined substitution of all four residues. However, we cannot exclude the possibility that there are other residues in the vicinity of the targeting determinants or in the C-terminal propeptide of the  $\alpha$  subunit that participate in phosphotransferase binding, since both the above-mentioned combination mutants still have some residual phosphorylation (4.2 and 7.7%). However, these residues would be only minor contributors to binding.

The productive interaction must be able to occur with the AGA molecule consisting of two dimerized precursor

polypeptides, prior to processing into  $\alpha$  and  $\beta$  subunits (Riikonen *et al.*, 1996). This is supported by our earlier findings on phosphorylation of active site mutants of AGA whose processing into subunits is prevented (Tikkanen *et al.*, 1996). These molecules are transported into the lysosomes as precursor dimer complexes and their oligosaccharides are apparently phosphorylated (Peltola *et al.*, 1996). Normal processing of the AGA precursor takes place very rapidly after synthesis, in the ER (Ikonen *et al.*, 1993), whereas the phosphotransferase seems to be located within the Golgi apparatus (Goldberg and Kornfeld, 1983). Thus, interaction of AGA with the phosphotransferase normally occurs only after processing of AGA into its subunits has taken place. This would suggest that the conformations of the precursor and the processed forms of AGA are not grossly different, since both forms are compatible with recognition by the phosphotransferase and oligosaccharide phosphorylation, as evidenced by phosphorylation of the above-mentioned mutations preventing processing of AGA.

### **The $\alpha$ and $\beta$ subunits are unequally phosphorylated**

We were also able to measure directly the phosphorylation ratio of oligosaccharides located in different *N*-glycosylation sites on a lysosomal enzyme and the effect of targeted substitutions not only on overall phosphorylation but also on the phosphorylation ratio. The degree of phosphorylation of the  $\alpha$  and  $\beta$  subunits was not equal even in wild-type AGA, where the  $\alpha$  subunit is phosphorylated more efficiently than the  $\beta$  subunit. Substitution of individual residues at any of the three identified sites decreased phosphorylation of the oligosaccharides on both subunits and none of the produced substitutions affected phosphorylation of only one subunit.

Phosphorylation of the  $\alpha$  subunit is reduced more efficiently than that of the  $\beta$  subunit by alanine substitution of Lys183 and Lys214, as indicated by the reduced  $\alpha/\beta$  phosphorylation ratio in both the cells and the medium. Although the conformation of the oligosaccharide shows a high degree of variation, it can be estimated that the distance of the mannose of the  $\alpha$  subunit glycan to Lys183 is  $\sim 25$  Å and to Lys214  $\sim 20$  Å. The corresponding distances to the  $\beta$  subunit glycan are 40 and 15 Å respectively. Lys177, whose substitution did not alter the  $\alpha/\beta$  phosphorylation ratio, is within the same  $\sim 40$  Å distance from both glycosylation sites. The location of the *N*-glycosylation site with respect to the phosphotransferase recognition determinant thus seems to affect the efficiency of phosphorylation of the oligosaccharide chain to some extent. However, the distance between the oligosaccharide and the determinant cannot be exact, since the glycan can adopt a range of different conformations. Consistent with these results, Cantor and Kornfeld (1992) have shown that oligosaccharides located on novel glycosylation sites generated in the cathepsin D molecule were efficiently phosphorylated. However, the degree of phosphorylation was dependent on the distance between the oligosaccharide and the phosphotransferase recognition site, oligosaccharides residing closer to the targeting determinant being better phosphorylated.

### **Interactions with the mannose 6-phosphate receptor**

A single high mannose oligosaccharide can acquire one or two Man 6-P markers (Varki and Kornfeld, 1983) and it has been shown that oligosaccharide chains tagged with only a single Man 6-P are poorer ligands for the Man 6-P receptors than those containing two Man 6-P (Creek and Sly, 1982; Varki and Kornfeld, 1983; Hoflack *et al.*, 1987). The mutant AGA polypeptides in which either Lys183 together with Lys214 or all three lysines and the Tyr of the recognition determinant were substituted by alanines are both still phosphorylated to a low but detectable degree. However, all of the phosphorylated polypeptides are secreted from the cells into the medium and are incapable of being endocytosed by Man 6-P receptors back into the cells. Substitutions of Lys183 and Lys214 are associated with dramatically increased secretion as well as low lysosomal targeting efficiency of the mutant AGA polypeptides. These mutants exhibit a reduced phosphorylation ratio of the subunits, which thus seems to be correlated with increased secretion and reduced lysosomal targeting and is likely to reflect the affinity of the phosphorylated oligosaccharides for the Man 6-P receptor.

Alanine substitutions of Lys177 or Tyr178 that clearly affect overall phosphorylation of AGA do not significantly alter the  $\alpha/\beta$  phosphorylation ratio and the mutant polypeptides exhibit lysosomal targeting and secretion comparable with wild-type AGA. From this data it is evident that a mere reduction in overall phosphorylation of the oligosaccharides is not necessarily sufficient to result in a targeting defect, whereas the degree of phosphorylation of individual oligosaccharides seems to be more important for lysosomal targeting efficiency.

Interestingly, Baranski *et al.* (1992) obtained similar results when analyzing phosphorylation of chimeric molecules containing parts of cathepsin D and the secretory protein glycopepsinogen fused together. When the amino lobe of cathepsin D is replaced by the corresponding sequence from glycopepsinogen, the resulting chimeric molecules are efficiently phosphorylated, but over half of the polypeptides are secreted, as compared with 9% secretion of wild-type cathepsin D. However, the authors were unable to provide any explanation for this phenomenon.

### **Lysosomal targeting as an example of molecular recognition**

In many biological processes specific molecular recognition is a prerequisite for formation of a protein–protein complex, such as that between a lysosomal enzyme and the phosphotransferase. Other examples of specific protein–protein interactions are found in antigen–antibody and receptor–hormone recognition and in the formation of oligomeric protein complexes. The protein surface area required for this type of interaction varies, but is typically 1000 Å<sup>2</sup>. In the case of recognition of lysosomal enzymes, the phosphotransferase must specifically recognize and bind a large variety of proteins whose three-dimensional structures are quite different from each other. This recognition event must be specific enough to identify the correct ligands and the binding strong enough for a productive interaction to occur, but it should still allow for the large variation in overall structure of the ligand molecules. This

kind of recognition could be achieved either by a single well-defined large determinant common to all ligands or by the use of two or more distinct sites, in each of which the recognition area as such is small. It is unlikely that lysosomal enzymes would all possess a distinct targeting sequence or even a universal structural determinant for phosphotransferase recognition. It is more plausible that the lysosomal targeting signal would be based on one or more lysine residues, their mutual position and the distance between them, which would allow for structural variation of the ligand enzyme molecules.

### **Interaction of lysosomal enzymes with the phosphotransferase**

The bovine phosphotransferase molecule has recently been purified and characterized and, although the exact subunit structure of this enzyme molecule remains to be verified, it is clear that the phosphotransferase is a large multisubunit complex which would be able to establish interactions over a large surface area of the substrate molecule (Bao *et al.*, 1996a,b). Binding of the lysosomal enzyme to the phosphotransferase could also trigger a conformational change in the phosphotransferase complex and result in establishment of enzymatic activity towards the oligosaccharides. Packing of the lysosomal enzyme against the phosphotransferase would thus allow for binding in the active site of the phosphotransferase and subsequent phosphorylation of only the oligosaccharides resident on the lysosomal enzyme itself. This kind of binding-induced activation of the phosphotransferase would efficiently prevent phosphorylation of non-lysosomal glycoproteins.

On the basis of the data presented here and in previous studies, it now seems probable that the phosphotransferase recognition signals of lysosomal enzymes are based on small contact points, on their mutual position and on the distance between them. The key elements in the contact seem to be one or more lysine residues on the surface of the lysosomal enzyme. In the case of lysosomal polypeptides with multiple oligosaccharide chains, such as AGA, it remains unresolved whether the phosphotransferase binds the lysosomal enzyme in two different orientations, phosphorylating different oligosaccharides, or if a single orientation allows for simultaneous phosphorylation of both oligosaccharides by the suggested two separate active sites of the phosphotransferase.

## **Materials and methods**

### ***In vitro* mutagenesis**

Mutagenesis was performed with a Chameleon Double-Stranded, Site-Directed Mutagenesis Kit (Stratagene) as suggested by the manufacturer. As a template, the full-length coding region of the AGA cDNA cloned into the *Bam*HI site of mammalian expression vector SV-poly was used (Tikkanen *et al.*, 1995). Restriction enzyme selection of single mutants was performed with a selection oligonucleotide located in the polylinker region of SV-poly which destroyed the *Eco*RV restriction enzyme cleavage site. In the combination mutants the substitutions were produced one after another in subsequent rounds of mutagenesis, except for the Lys177Tyr178Ala double substitutions, which were included in the same oligonucleotide. To create a second mutation in a construct, an oligonucleotide destroying the *Sac*I site and returning the *Eco*RV site was used for selection of mutated plasmids. The first selection oligonucleotide was then used again to produce a third mutation. Mutant clones were identified by sequencing.

**COS-1 and NIH 3T3 cell culture and DNA transfection**

COS-1 and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. For transfection, the cells were seeded on a 6-well plate at 400 000 cells/well and grown overnight. Transfection was performed with 7 µg plasmid construct by the DEAE dextran/chlorokin method (Luthman and Magnusson, 1983) and the cells analyzed on the third day after transfection.

**Metabolic labeling and immunoprecipitation**

The cells were incubated in cysteine-free medium for 30–60 min before labeling with 150 µCi/ml [<sup>35</sup>S]Cys (Amersham) using a 1 h pulse followed by a 1–6 h chase. Labeling with [<sup>32</sup>P]orthophosphate (Amersham) was carried out as described earlier (Tikkanen *et al.*, 1995) in phosphate-free medium with 400 µCi [<sup>32</sup>P]P<sub>i</sub>/ml with a 2.5 h pulse and a 3 h chase. The cells were harvested, lysed and the proteins immunoprecipitated and separated as described earlier (Peltola *et al.*, 1994). The media were concentrated to 100–200 µl with Centricon-30 microconcentrators (Amicon) before immunoprecipitation.

**Assay for AGA activity**

The AGA activity assay was performed as described earlier (Peltola *et al.*, 1996).

**Immunofluorescence**

COS-1 cells were transfected using lipofection (Felgner *et al.*, 1987) with the mutant Lys177Tyr178Lys183Lys214Ala or wild-type AGA construct. Immunostaining of AGA was performed with a rabbit anti-serum against human AGA as described earlier (Tikkanen *et al.*, 1995).

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