Human lysosomal α -glucosidase: functional characterization of the glycosylation sites

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N-linked glycosylation is one of the important events in the post-translational modification of human lysosomal α -glucosidase. Phosphorylation of mannose residues ensures efficient transport of the enzyme to the lysosomes via the mannose 6-phosphate receptor. The primary structure of lysosomal α -glucosidase, as deduced from the cDNA sequence, indicates that there are seven potential glycosylation sites. We have eliminated these sites individually by site-directed mutagenesis and thereby demonstrated that all seven sites are glycosylated. The sites at Asn-882 and Asn-925 were found to be located in a C-terminal propeptide which is cleaved off during maturation.

Evidence is presented that at least two of the oligosaccharide side chains of human lysosomal α -glucosidase are phosphorylated. Elimination of six of the seven sites does not disturb enzyme synthesis or function. However, removal of the second glycosylation site at Asn-233 interferes dramatically with the formation of mature enzyme. The mutant precursor is synthesized normally and assembles in the endoplasmic reticulum, but immunoelectron microscopy reveals a deficiency of α -glucosidase in the Golgi complex and in the more distal compartments of the lysosomal transport pathway.

INTRODUCTION

Lysosomal α -glucosidase (EC 3.2.1.3) is a glycoprotein like the other acid hydrolases that perform their function in the lysosomes. The function of α -glucosidase is degradation of glycogen to glucose. Enzyme deficiency leads to glycogenosis type II, an inherited glycogen-storage disorder (Hers, 1963). Several patients have been described with a defect in the synthesis or post-translational processing of lysosomal α -glucosidase (Reuser and Kroos, 1982; Beratis et al., 1983; Reuser et al., 1985, 1987; Martiniuk et al., 1986, 1990a; Van der Ploeg et al., 1989; Hermans et al., 1991a; Zhong et al., 1991).

During translation, lysosomal enzymes enter the endoplasmic reticulum where glycosylation is assumed to start even before the protein is completely folded (Rothman et al., 1978). Asparagine residues in the sequence Asn-Xaa-Ser/Thr (Xaa all but Pro) are the potential sites for attachment of N-linked carbohydrate side chains which are transferred *en bloc* from dolichol pyrophosphate (Marshall, 1972; Kornfeld and Kornfeld, 1985). However, not all potential glycosylation sites are used. Studies by Bause and Legler (1981) indicate that a proper protein conformation is required for recognition and glycosylation of a site. After removal of the three terminal glucose residues of the oligosaccharide precursor chain in the endoplasmic reticulum (Kornfeld et al., 1978; Hubbard and Robbins, 1979), most lysosomal enzymes obtain a mannose 6-phosphate recognition marker as lysosomal targeting signal (Creek and Sly, 1984). This is accomplished by the transfer of N-acetylglucosamine 1-phosphate from UDP-N-acetylglucosamine to particular mannose residues (Reitman and Kornfeld, 1981; Waheed et al., 1981), and the subsequent uncovering of the phosphate by a phosphodiesterase which is probably localized in the mid-Golgi (Lazzarino and Gabel, 1988). Transport to the lysosomes continues via the trans-Golgi cisternae to the trans-Golgi reticulum. Binding to the mannose 6-phosphate receptor in this part of the transport pathway is essential for lysosomal targeting. Lysosomal enzymes are then

transported to the late endosomes (Griffiths and Simons, 1986), where a fall in pH causes the ligand to dissociate from the receptor. The enzymes continue their way to the lysosomes and the receptor cycles back to the Golgi complex (Brown et al., 1986; Von Figura and Hasilik, 1986).

Lysosomal α -glucosidase is known to follow this transport route and is subject to several post-translational modifications involving both the carbohydrate chains and the protein backbone. The enzyme is synthesized as a glycosylated precursor of approximately 110 kDa, which is phosphorylated. The amino acid sequence of lysosomal α -glucosidase, as derived from the cloned cDNA, indicates that there are seven potential glycosylation sites (Hoefsloot et al., 1988; Martiniuk et al., 1990b). Proteolytic processing gives rise to a 95 kDa intermediate form and results finally in the formation of two lysosomal enzyme species of 76 kDa and 70 kDa (Hasilik and Neufeld, 1980; Reuser et al., 1985). The latter two forms of lysosomal α -glucosidase have been purified and analysed with respect to their sugar content and carbohydrate chain structure. It was estimated that lysosomal α -glucosidase from human placenta and liver contains an average of four to five carbohydrate chains (Belenky et al., 1979; Mutsaers et al., 1987).

The aim of this study was to establish the actual number of glycosylation sites and to determine the role of each site in the transport and catalytic function of lysosomal α -glucosidase. The approach that was taken was to eliminate the potential sites by site-directed mutagenesis and to study the effect by expression of the mutant cDNA constructs *in vitro* and in transiently transfected COS cells.

MATERIALS AND METHODS

Construction of mutants

The Muta-Gene *in vitro* mutagenesis kit from Bio-Rad (Richmond, CA, U.S.A.) was used to carry out site-directed mutagenesis, as described by Kunkel (1985). The oligonucleotides

Table 1 Oligonucleotides used for mutagenesis

The altered nucleotides are underlined.

Mutant	Oligonucleotide
ΔG1	5'-AAGCTGGAGCAGCTGAGCTCC-3'
ΔG2	5'-GTGCTGCTGCAGACGACGGTG-3'
ΔG3	5'-GTGGTGGAGCAGATGACCAGG-3'
ΔG4	5'-TTCATCACCCAGGAGACCGGC-3'
ΔG5	5'-TTCCTGGGCCAGACCTCAGAG-3'
ΔG6	5'-CTGGCCAGGCAGAACACGATC-3'
ΔG7	5'-CCTGTCTCCAGTTCACCTAC-3'

used to alter the seven potential glycosylation sites are listed in Table 1. They were synthesized on an Applied Biosystems 381A DNA synthesizer.

Site-directed mutagenesis and cloning of the mutant cDNAs in the eukaryotic expression vector pSG5 (Green et al., 1988) were performed exactly as described previously (Hermans et al., 1991b).

Transient expression in COS cells

COS-1 cells (Gluzman, 1981) were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics at 37 °C. The transfection protocol was as described previously (Hoefsloot et al., 1990). The culture medium was collected and the cells were harvested 90 h after transfection. Cell homogenates were made by repeated freezing and thawing of cell pellets in distilled water.

The activity of lysosomal α -glucosidase in the cell homogenate and the culture medium was measured with 4-methylumbelliferyl α -D-glucopyranoside (4-MU) as described previously (Reuser et al., 1978). The protein concentrations of cell homogenates were determined with the use of the BCA protein assay kit (Pierce).

Lysosomal α -glucosidase was immunoprecipitated from culture media using a rabbit polyclonal antiserum against human lysosomal α -glucosidase in combination with *Staphylococcus aureus* membranes (Bethesda Research Laboratories) and analysed by immunoblotting (Reuser et al., 1987). To characterize the intracellular forms of lysosomal α -glucosidase, COS cells were labelled for 2 h with [3 H]leucine (190 μ Ci/mmol) (Amersham U.K.) 65 h after transfection, and lysosomal α -glucosidase was immunoprecipitated either directly (pulse) or after 16 h of chase. The different molecular species of lysosomal α -glucosidase were separated by SDS/PAGE (8% acrylamide, 1% cross-link unless indicated otherwise) as described (Reuser et al., 1985).

Phosphorylation of lysosomal α -glucosidase

COS cells were transfected as described above. After 90 h the cells were preincubated for 1 h in phosphate-free DMEM to which FCS, dialysed against 0.9% NaCl, was added in a final concentration of 4%. The medium was then replaced with fresh medium and carrier-free [32 P] P_i (Amersham) was added at a concentration of 80 μ Ci/ml. The cells were pulse-labelled for 6 h and harvested either directly or after a subsequent chase of 12 h. Lysosomal α -glucosidase was immunoprecipitated from cell

extracts as described (Reuser et al., 1985) and analysed by SDS/PAGE.

Transcription and translation *in vitro*

Wild-type and mutant cDNAs cloned in the expression vector pSG5 were linearized with *Bg*II and used as a template in the transcription reaction. The T7 promoter was used for transcription. The reaction was allowed to proceed for 1.5 h at 40 °C in 40 mM Tris/HCl, pH 7.5, containing 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 1 unit of RNAase inhibitor (Promega), 0.8 mg/ml BSA, 500 μ M ATP, CTP and UTP, 50 μ M GTP, 500 μ M dGpppG and 1 μ g of DNA template with 20 units of T7 polymerase (Boehringer Mannheim). The template was removed by adding 1 unit of RNAase-free DNAase (Boehringer Mannheim). The excess of nucleotides was removed by Sephadex G-50 filtration.

A 0.2 μ g sample of RNA was used for translation *in vitro* in a rabbit reticulocyte lysate system (Promega) containing 54 μ Ci of [35 S]methionine (1130 Ci/mmol; Amersham International) in the presence of canine pancreatic microsomal membranes, according to the instructions of the manufacturer. Radioactively labelled lysosomal α -glucosidase was immunoprecipitated and separated by SDS/PAGE as described above.

Immunocytochemistry

Immunocytochemistry on transiently transfected COS cells was performed exactly as described previously (Hoefsloot et al., 1990).

RESULTS

In lysosomal α -glucosidase, asparagine residues in the recognition sequence for N-linked glycosylation, Asn-Xaa-Thr/Ser (Xaa can be any residue except Pro), are found at seven positions, namely at Asn-140, -233, -390, -470, -652, -882 and -925 (Hoefsloot et al., 1988; Martiniuk et al., 1990b) (EMBL entry number Y00839). These potential glycosylation sites were eliminated one by one to determine which sites were actually used, and to examine the importance of individual sites for enzyme function and lysosomal targeting. To this end the recognition consensus sequence of

Figure 1 Translation *in vitro* of human lysosomal α -glucosidase

Wild-type (Wt) and mutant (Δ G2) cDNAs were transcribed *in vitro* and the mRNAs were translated in a rabbit reticulocyte lysate in the presence of canine pancreatic microsomes. The proteins were labelled with [35 S]methionine, immunoprecipitated and separated by SDS/PAGE. Lane 1, wild type; lane 2, mutant Δ G2. The molecular masses (kDa) of the unglycosylated and glycosylated lysosomal α -glucosidase precursors are indicated.

Figure 2 Synthesis and processing of wild-type and mutant lysosomal α -glucosidase in COS cells

COS cells transfected with wild-type (Wt) and mutant cDNA constructs were labelled for 2 h with [3 H]leucine and harvested directly (Pulse) or after a subsequent period of 16 h (Chase). Lysosomal α -glucosidase was immunoprecipitated and analysed by SDS/PAGE. The molecular masses (kDa) of wild-type and mutant lysosomal α -glucosidase species are indicated.

Figure 3 Secretion of wild-type and mutant lysosomal α -glucosidase by transiently transfected COS cells

Lysosomal α -glucosidase was immunoprecipitated from the culture media at 90 h after transfection and analysed by Western blotting after SDS/PAGE. The molecular masses (kDa) of wild-type (W) and mutant lysosomal α -glucosidase species are indicated. PI, placenta.

each potential site was altered by site-directed mutagenesis of the lysosomal α -glucosidase cDNA inserted in M13mp19. The codon for Asn was replaced by one coding for the very similar amino acid residue Gln, using the oligonucleotides listed in Table 1. The mutants, each missing a different glycosylation site, were

designated Δ G1– Δ G7 in order of appearance from the N-terminus.

Transcription and translation *in vitro*

The use of glycosylation sites was tested by cloning the wild-type and mutant cDNAs in the eukaryotic expression vector pSG5 allowing *in vitro* transcription and subsequent translation of the mRNAs in the presence of dog pancreatic microsomes. Translation of wild-type mRNA resulted in the formation of two molecular species, a translocated and glycosylated precursor of 112 kDa and an unglycosylated precursor of 97 kDa (Figure 1; and Van der Horst et al., 1987). The glycosylation sites are easily identified by this method. Figure 1, for instance, shows that the substitution of Asn-233 by Gln-233 (Δ G2) results in a 2 kDa size decrease in the glycosylated precursor, whereas the apparent size of the unglycosylated wild-type and mutant precursor remains the same (97 kDa).

Transient expression in COS cells

The functional consequence of the removal of glycosylation sites was investigated by following the synthesis and maturation of α -glucosidase in transiently transfected COS cells. [3 H]Leucine-labelled wild-type and mutant lysosomal α -glucosidase species were immunoprecipitated from cell homogenates after a 2 h pulse period or after a subsequent chase of 16 h, and analysed by SDS/PAGE (Figure 2). The secreted form of lysosomal α -glucosidase was immunoprecipitated from the culture media and analysed by Western blotting (Figure 3). In cells transfected with wild-type cDNA the lysosomal α -glucosidase precursor of 110 kDa is synthesized (pulse) and converted into a 95 kDa intermediate and a 76 kDa mature species (chase) (Figure 2). The culture medium contains only the secreted 110 kDa precursor (Figure 3). All mutant precursor proteins (isolated from cells and media) missing one potential glycosylation site appeared to have a slightly lower molecular mass (108 kDa) than the wild-type precursor. This indicates that all seven potential glycosylation sites of lysosomal α -glucosidase are used. For the mutants lacking the first, third, fourth or fifth glycosylation site (Δ G1, Δ G3, Δ G4 and Δ G5), the lower molecular mass is maintained during the maturation process leading to the formation of a 93 kDa processing intermediate and a 74 kDa mature enzyme species (Figure 2, Chase). A different effect was observed with the mutants Δ G6 and Δ G7. The precursor and the processing intermediate were smaller (93 kDa) than the comparable wild-type species, but the final maturation products of Δ G6 and Δ G7 (76 kDa) and wild-type lysosomal α -glucosidase were of the same size (Figure 2, Chase). This indicates that a C-terminal peptide containing the glycosylation sites at Asn-882 and Asn-925 is cleaved off when lysosomal α -glucosidase matures from 95 to 76 kDa.

The only glycosylation site which has a severe effect on the biosynthesis of α -glucosidase when eliminated is Asn-233 (Figure 2). The mutant precursor of 108 kDa is evidently formed during the 2 h pulse, but the 93 kDa processing intermediate and the 74 kDa mature enzyme are clearly deficient in the chase. The Δ G2 precursor is not secreted into the culture medium, in contrast with all other mutant precursors which are secreted normally (Figure 3).

To investigate the effect of glycosylation on the catalytic activity of lysosomal α -glucosidase, cell homogenates and culture media of transfected COS cells were assayed for enzyme activity. COS cells transfected with *Escherichia coli* β -galactosidase cDNA were taken as a reference. The data of a typical transfection

Table 2 Activity of the glycosylation mutants of lysosomal α -glucosidase in transiently transfected COS cells measured 90 h after transfection

The activity is expressed as nmol of 4-MU/h per mg of protein. The activities compared with wild-type are given within parentheses as percentages $\pm$ S.E.M. ($n = 1-4$).

Type of mutation	Catalytic activity (nmol/h per mg)	
	Cells	Medium
Wild-type	960 (100)	406 (100)
Δ G1	665 (61.7 $\pm$ 7.4)	175 (42.6 $\pm$ 5.5)
Δ G2	116 (5.0 $\pm$ 2.3)	15 (2.1 $\pm$ 0.9)
Δ G3	789 (77.9 $\pm$ 9.1)	307 (89.2 $\pm$ 15.7)
Δ G4	671 (69.8)	275 (67.4)
Δ G5	645 (66.8 $\pm$ 6.6)	152 (89.1 $\pm$ 30.1)
Δ G6	919 (90.1 $\pm$ 7.7)	304 (75.9 $\pm$ 5.2)
Δ G7	1027 (109.2 $\pm$ 6.9)	473 (116.6 $\pm$ 0.1)
β -Gal construct	50 (0)	3 (0)

Figure 4 Phosphorylation of wild-type and mutant lysosomal α -glucosidase in COS cells

COS cells transfected with wild-type (Wt) and mutant cDNA constructs were labelled for 6 h with [32 P]P_i and harvested directly (pulse) or after a subsequent period of 12 h (chase). Lysosomal α -glucosidase was immunoprecipitated from the cell homogenates and separated by SDS/PAGE (10% acrylamide, 1% cross-link). The molecular masses of the wild-type lysosomal α -glucosidase species are indicated.

experiment and the average outcome of the different experiments are given in Table 2. When compared with wild-type, all glycosylation-site-defective mutants except Δ G2 exhibited intracellularly a similar activity of lysosomal α -glucosidase. Δ G2 was different in that only 5% of wild-type activity was measured in the cell homogenates and less than 3% in the medium. Near-normal levels of lysosomal α -glucosidase were secreted by COS cells transfected with the other mutant constructs (Table 2).

Phosphorylation of lysosomal α -glucosidase

To determine which of the seven oligosaccharide chains of lysosomal α -glucosidase were phosphorylated, we transfected COS cells with the wild-type and mutant cDNA constructs and

Figure 5 Localization of glycosylation-site-deficient lysosomal α -glucosidases in transfected COS cells studied by light microscopy

Transiently transfected cells were fixed and incubated with a rabbit polyclonal antiserum against human lysosomal α -glucosidase. Immune complexes were visualized with goat anti-rabbit IgG conjugated to fluorescein. (a) Δ G4, (b) Δ G2. Magnifications: (a) $\times 227$, (b) $\times 567$.

labelled the cells with [32 P]P_i. After a 6 h labelling period, phosphorylated precursor and intermediate forms of lysosomal α -glucosidase were observed in cells expressing either wild-type or mutant enzyme (Figure 4). The mature forms of lysosomal α -glucosidase became detectable after a subsequent chase of 12 h, except in cells expressing Δ G2. The 32 P-labelling procedure appeared sensitive enough to demonstrate conversion of the Δ G2 precursor into the 93 kDa intermediate but the formation of mature (74 kDa) enzyme remained undetectable. When [32 P]P_i-labelled wild-type lysosomal α -glucosidase was incubated with endoglycosidase F, before SDS/PAGE, no phosphorylated protein could be detected, indicating that the [32 P]P_i was linked to the mannose residues of the carbohydrate side chains (results not shown).

Intracellular transport

The intracellular localization of the mutant α -glucosidase species missing one glycosylation site was investigated using immunocytochemistry. As observed in earlier studies, the formation of mature enzyme was in all instances correlated with a typical punctate lysosomal labelling pattern. Figure 5(a), for instance, illustrates the lysosomal localization of Δ G4. Transfection of COS cells with Δ G2, showing an apparent maturation defect, resulted in a diffuse network of labelled structures spreading from the nucleus into the cytoplasm (Figure 5b). The exact intracellular localization of Δ G2 was revealed by immunoelectron microscopy (Figure 6). Labelling of the nuclear envelope and the endoplasmic reticulum was obtained but the enzyme could not be detected in the Golgi complex nor in the trans-Golgi reticulum or the lysosomes. The latter compartments were labelled after transfection with the wild-type cDNA construct (results not shown).

Figure 6 Intracellular localization of Δ G2 in transfected COS cells studied by immunoelectron microscopy

Ultrathin cryosections were incubated with rabbit polyclonal antiserum against human lysosomal α -glucosidase and subsequently with goat anti-rabbit IgG coupled to colloidal gold. Mutant lysosomal α -glucosidase was found at the nuclear envelope (a) and in the endoplasmic reticulum (b), but not in the Golgi complex (c), or the lysosomes (d). N, nucleus; M, mitochondrion; R, rough endoplasmic reticulum; G, Golgi complex; L, lysosomes. Magnifications: (a) $\times 37\,400$, (b) $\times 34\,160$, (c) $\times 51\,935$, (d) $\times 26\,550$.

DISCUSSION

Site-directed mutagenesis was applied to determine the glycosylation sites of lysosomal α -glucosidase and to define their significance for enzyme function and lysosomal targeting. The removal of one high-mannose type of carbohydrate chain is expected to result in a molecular mass decrease of approximately 2 kDa and to lead to a comparable increase in electrophoretic mobility on SDS/PAGE. This effect was observed on deletion of each of the seven potential glycosylation sites of the 110 kDa precursor. The mobility shift in the more mature forms of α -glucosidase is informative with respect to the sites of proteolytic processing. The 2 kDa size decrease is maintained after formation of the 95 kDa processing intermediate irrespective of the deleted glycosylation site. This implies that the peptides that are cleaved off during the conversion of 110 kDa into 95 kDa do not contain a glycosylation site. Since the seventh glycosylation site is located just 31 residues from the C-terminus of the 110 kDa precursor (Hoefsloot et al., 1988), we conclude that the proteolytic conversion occurs mainly at the N-terminus of the precursor. On further maturation of the enzyme to 76 kDa, the mutants lacking the sixth and the seventh glycosylation sites at Asn-882 and Asn-925 are no longer smaller than the wild-type species whereas the

other mutants are still decreased in size. Thus the maturation from 95 to 76 kDa involves C-terminal processing at a site upstream of Asn-882. On the basis of the N-terminal sequence of the 76 kDa form of lysosomal α -glucosidase and the sequence of C-terminal tryptic peptides (Hoefsloot et al., 1988), we estimate that the site is located between amino acid residues 820 and 880.

According to the amino acid sequence of lysosomal α -glucosidase, the first glycosylation site is located between the N-termini of the 76 and 70 kDa species purified from human placenta. This leaves four sites occupied in the 70 kDa species. These findings are in line with the earlier estimates of Mutsaers et al. (1987) on the number of carbohydrate chains attached to human placental lysosomal α -glucosidase. A figure of four to five side chains per polypeptide molecule was obtained for a preparation containing equimolar amounts of the 76 and 70 kDa species.

Six of the seven mutants, each lacking a different glycosylation site, are transported to the lysosomes and are catalytically active. Also the proteolytic processing and secretion of these mutants is normal. However, elimination of the second glycosylation site at Asn-233 has a dramatic effect. The precursor (108 kDa) is synthesized normally but the intermediate and mature forms of α -glucosidase are severely decreased in amount. The relative persistence of the Δ G2 precursor during the 16 h chase (Figure 2) suggests a delayed transport of the mutant precursor from the endoplasmic reticulum to the Golgi. This suggestion is sustained by the fact that the immunogold labelling of the endoplasmic reticulum for lysosomal α -glucosidase is quantitatively normal, whereas the Golgi complex and the more distal compartments of the lysosomal transport route are clearly deficient in lysosomal α -glucosidase. This explains why transfection of COS cells with Δ G2 does not lead to a significant increase in the lysosomal α -glucosidase activity.

Mutation analyses of the glycosylation sites of a few other lysosomal proteins have been reported. For example, site-directed mutagenesis was used to determine the essential glycosylation sites of human β -hexosaminidase B (Sonderfeld-Fresco and Proia, 1989). In this enzyme four of the five potential sites were found to be glycosylated. Elimination of each site individually had no effect on lysosomal targeting or catalytic function. In addition to these studies, two naturally occurring glycosylation-site mutations in lysosomal proteins have been reported. One concerns arylsulphatase A. Normally, two of the three potential sites are glycosylated, but individuals carrying the pseudo-deficiency allele have lost one of the two utilized sites by a mutation changing Asn-350 to Ser (Gieselmann et al., 1989). However, the introduction of this mutation into wild-type cDNA did not affect the catalytic function or the stability of the encoded enzyme. The decreased arylsulphatase A activity leading to this pseudodeficiency is caused by a mutation abolishing the first polyadenylation signal. As a consequence, the mRNA species is labile which explains the severely diminished rate of enzyme synthesis (Gieselmann et al., 1989). The second mutation of a glycosylation site was found in saposin B. The mutation changes Thr-23 to Ile which eliminates the only possible site. The mutant protein is believed to be rapidly degraded (Rafi et al., 1990; Kretz et al., 1990). These examples and our own experiments demonstrate the differential function of glycosylation sites.

The incorporation of [32 P]P_i in the different biosynthetic forms of lysosomal α -glucosidase allows us to make an estimate on the minimal number of carbohydrate chains that are phosphorylated. Although each mutant is missing a different glycosylation site, it appears that all mutant precursor proteins are phosphorylated. From this we conclude that at least two of the seven carbohydrate chains contain the mannose 6-phosphate recognition marker.

Furthermore, it is evident that at least one of the five carbohydrate chains located within the boundaries of the 76 kDa polypeptide is phosphorylated, since the mature enzyme is also phosphate-labelled. The maturation defect of mutant $\Delta G2$ prevents the assessment of phosphorylation at site Asn-233 and thereby hampers a more detailed analysis of the phosphorylation sites by site-directed mutagenesis. Further identification of the phosphorylation sites via sequence analysis of phosphorylated peptides is in progress.

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