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Lysosomal Segregation of a Mannose-Rich Glycoprotein Imparted by the Prosequence of Myeloperoxidase

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Abstract The role of the N-terminal sequence of myeloperoxidase in the intracellular targeting was examined by using glycosylated lysozyme as a reporter. A fusion protein was constructed in which the presequence residues −18 through −6 of the lysozyme moiety had been replaced by residues 1–158 of prepromyeloperoxidase. Expression of the fusion protein in Chinese hamster ovary cells demonstrated its partial secretion and partial intracellular retention. The latter was accompanied by trimming the myeloperoxidase prosequence off the lysozyme moiety. The rate of the retention of the lysozyme fusion protein was higher than that of glycosylated lysozyme that had been expressed in cells transfected with cDNA of glycosylated lysozyme. The retention was insensitive to NH_4Cl. In the secreted protein, lysozyme contained predominantly complex oligosaccharides as demonstrated by a proteolytic fragmentation in vitro and resistance to endo-β-N-acetylglucosaminidase H. In contrast, when targeted to lysosomes, the lysozyme moiety of the fusion protein contained predominantly mannose-rich oligosaccharides. In baby hamster kidney cells, the trimming of the oligosaccharides in the lysozyme fragment was less vigorous, and a selective targeting of molecules bearing mannose-rich oligosaccharides to lysosomes was more apparent than in Chinese hamster ovary cells. In the presence of monensin, the formation of complex oligosaccharides in the fusion protein and its secretion were strongly inhibited, whereas the intracellular fragmentation was not. We suggest that the prosequence of myeloperoxidase participates in the intracellular routing of the precursor and that this routing operates on precursors bearing mannose-rich rather than terminally glycosylated oligosaccharides and diverts them from the secretory pathway at a site proximal to the monensin-sensitive compartment of the Golgi apparatus. J. Cell. Biochem. 71:158–168, 1998.

Key words: glycosylation; lysosomal targeting; lysozyme; monensin; myeloperoxidase

Myeloperoxidase is a major constituent of azurophilic granules. In promyelocytic cells it is synthesized in the rough endoplasmic reticulum as a larger precursor with five N-linked oligosaccharides. A portion of the oligosaccharides becomes phosphorylated. The phosphorylation does not seem to be involved in the targeting to the granules because it is insensitive to NH_4Cl, an inhibitor of the mannose 6-phospate receptor targeting [Hasilik et al., 1984; Nauseef et al., 1988]. Owing to the long residency of the precursor in the endoplasmic reticulum [Nauseef et al., 1995], the phosphorylation is probably adventitious. It has been suggested that myeloperoxidase exits the endoplasmic reticulum or *cis*-Golgi prior to the site of Golgi mannosidases [Nauseef et al., 1995]. The mechanism of the segregation has not been elucidated. With segregation into the granules, the precursor is subjected to a proteolytic maturation, in which a prosequence of 164 amino acids is removed and the remainder is fragmented to polypeptides of 60 and 13 kDa, with an excision of six residues between the two chains and a removal of the C-terminal serine [Hashinaka et al., 1988]. To examine the role of the prosequence in the targeting, we prepared a recombinant cDNA, in which a 5′-fragment of the myeloperoxidase cDNA [Morishita et al., 1987] of human myeloperoxidase was joined to cDNA of a glycosylated mutant human lysozyme, thus replacing its presequence-encoding portion, and examined the intracellular targeting and secretion of the fusion protein in cultured cells as compared with those of glycosylated lysozyme. We report

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that the prosequence causes about a third of the protein to be targeted to the lysosomal compartment and that a large portion of the targeted molecules bear mannose-rich oligosaccharides, whereas the secreted glycoprotein is endowed with complex oligosaccharides.

MATERIALS AND METHODS

Tran^{35}S-Label, specific activity 40 GBq/mmol, was obtained from ICN Biomedicals GmbH (Eschwege, Germany). The restriction endonucleases *Eco*RI, *Pst*I, and *Xba*I were obtained from MBI Fermentas (St. Leon-Rot, Germany). *Kpn*I and other enzymes for the recombinant DNA reactions and endo-β-N-acetylglucosaminidase H and glycopeptidase F were purchased from Boehringer-Mannheim (Mannheim, Germany). Monensin was obtained from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). Other reagents were analytical grade chemicals obtained from different commercial sources.

Cell Culturing

Chinese hamster ovary (CHO) cells ATCC CCL61 were cultured in α-minimal essential medium and baby hamster kidney (BHK) cells ATCC CCL10 in Dulbecco's modified Eagle's medium (both from Gibco-BRL, Eggenstein, Germany), with 10% (v/v) fetal bovine serum under air/CO_2 (95/5, v/v). U937 cells were cultured as described previously [Gupta et al., 1984]. The cells used were free of mycoplasma.

Recombinant DNA, Vectors, and Transfection

Human myeloperoxidase cDNA [Morishita et al., 1987] that had been inserted into the expression vector pBEH [Cully et al., 1989] was cut with the endonucleases *Cla*I and *Kpn*I and a 2-kbp fragment including the SV40 promotor sequence of the vector, and a proximal portion of the myeloperoxidase cDNA was isolated. The fragment was treated with *Hin*PI, its ends were filled with Klenow fragment DNA polymerase, and the product was cut with *Eco*RI. The resulting 620-bp fragment encoding the preprosequence of the myeloperoxidase was isolated after agarose gel electrophoresis. From human lysozyme cDNA encoding a Gly22Asn replacement mutation in the vector pBEHpac18 [Horst et al., 1991, 1993], two fragments were prepared. First, a 300-bp *Eco*RI–*Pst*I fragment was isolated and treated with *Mnl*I to obtain a fragment of approximately 270 bp. Second, the plasmid was cut with *Pst*I and *Bam*HI to obtain the distal portion of lysozyme cDNA. The proximal fragment of the myeloperoxidase cDNA and the two from the mutant lysozyme cDNA were ligated to a dephosphorylated pBEHpac18 vector that had been cut with *Eco*RI and *Bam*HI. With the ligated DNA, competent *Escherichia coli* DH5α cells were transfected and DNA was examined in a number of clones. Approximately 10% of the clones contained inserts of the expected 2.1-kbp size, and their restriction with *Pst*I and *Xba*I produced fragments of the expected sizes of 1.2 and 1.0 kbp, respectively. The insert was released by digestion with *Xba*I and cloned in phage M13mp19. The ssDNA was prepared and sequenced according to Sanger et al. [1977]. In three clones, in-frame ligation was confirmed and had guanosine nucleotide 623 of myeloperoxidase cDNA [Morishita et al., 1987] joined to thymidine nucleotide 54 of lysozyme cDNA [Yoshimura et al., 1988]. The recombinant cDNA encoded residues 1–158 of prepromyeloperoxidase [Morishita et al., 1987] followed by residues −5–130 of the Gly22Asn mutant lysozyme [Horst et al., 1991] and N-glycosylation signals at residues Asn139 and Asn185. The cDNA was cloned into the vector pBEHpac18. Transfection of CHO and BHK cells and isolation of clones were performed as described previously [Horst et al., 1991, 1993].

Characterization of the Fusion Protein in Cultured Cells and Subcellular Fractions

The metabolic labeling was performed with Tran^{35}S-Label, and lysozyme-containing polypeptides were isolated by immunoprecipitation and sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [Horst et al., 1991]. The radioactive polypeptides were visualized by fluorography [Laskey and Mills, 1975]. For quantitave purposes, phosphor imaging was performed (Model SI Molecular Dynamics, Krefeld, Germany). Proteolytic fragmentation of the fusion protein was performed by resuspending the immunoprecipitates in 1% (w/w) bovine serum albumin, 2 mM EDTA, and 5 mM sodium phosphate buffer, pH 5.0, and incubating the samples in aliquots of 25 µl without or with 2 µg human cathepsin D or 15 µg bovine cathepsin B at 37°C for 15 h [Horst et al., 1993].

Homogenates of labeled cells were prepared by N_2-cavitation [Gupta et al., 1984]. A postnuclear supernatant fraction was prepared and

mixed with 2.88 vol 67.2% (w/w) sucrose. The mixture, 1.5 ml, was introduced to the bottom of a 18–47% (w/w) sucrose gradient, and the sample was subject to centrifugation at 220.000g_{av} for 18 h in a TH641 rotor (DuPont, Bad Nauheim, Germany). The fractions were collected from the top, and the residue was taken up in 1 ml of homogenization buffer as fraction 12. The concentration of sucrose was determined by refractometry. The activity of β-hexosaminidase was determined with *p*-nitrophenyl-β-N-acetylglucosaminide as described elsewhere [Gupta et al., 1984]. The activity of α-glucosidase was determined by the method of Burns [1982] and that of galactosyl transferase according to Rome et al. [1979].

Magnetic separation of the organelles was performed as follows. The transfected cells were cultured for 6 h in the presence or absence of superparamagnetic particles [Rodriguez-Paris et al., 1993]. Postnuclear supernatant fractions were prepared as above. The supernatants, 0.4 ml, were applied to 450-µl magnetic columns that were suspended between magnets according to the supplier's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). After a 20-min incubation at 18°C, column contents were displaced by 2 vol of the buffer and collected as the unbound fraction. The column was washed by an additional volume in the magnetic field. In the collected fractions, the activities of β-hexosaminidase and galactosyl transferase were determined (see above). The concentration of lysozyme was determined by a quantitative enzyme-linked immunosorbent assay using human milk lysozyme, 10 pg to 2 ng [Gupta et al., 1984], as the standard, a 3,000-fold diluted rabbit anti-human lysozyme antiserum [Gupta et al., 1984], and 0.5 µg/ml goat anti-rabbit immunoglobulin (H + L) conjugated to horse raddish peroxidase (BioRad Laboratories, München, Germany) as the first and the second antibodies, respectively.

RESULTS

Biosynthesis, Secretion, and Intracellular Processing in CHO Cells

We constructed a cDNA encoding the prepro-sequence of human myeloperoxidase and the mature portion of a glycosylated mutant human lysozyme. In transfected CHO cells, a fusion protein was synthesized with an apparent molecular mass of 33 kDa. Its transport resulted in sorting and a maturation (Fig. 1).

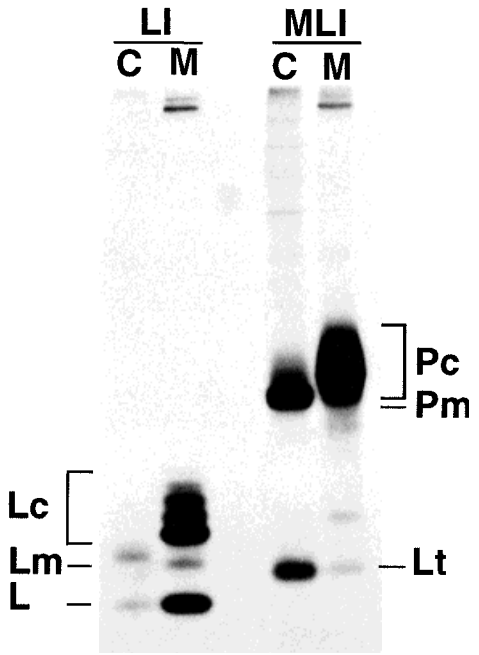

Fig. 1. Biosynthesis of glycosylated lysozyme in transfected Chinese hamster ovary (CHO) cells. CHO cells transfected with Gly22Asn lysozyme (LI) and myeloperoxidase prosequence–lysozyme fusion protein (MLI) cDNAs were cultured with Tran^{35}S-Label for 16 h. Immunoprecipitates were prepared from extracts of cells (C) and the medium (M). After solubilization in the presence of sodium dodecylsulfate (SDS) and dithiothreitol, the radioactive polypeptides were separated by SDS–polyacrylamide gel electrophoresis and visualized by fluorography. The labeled polypeptides that were obtained reproducibly with the antiserum from the former cells were nonglycosylated lysozyme (L), lysozyme bearing complex (Lc), and mannose-rich oligosaccharides (Lm) and those from the fusion protein were lysozyme with trimmed oligosaccharides (Lt) and the precursor forms with complex (Pc) and mannose-rich oligosaccharides (Pm). These tentative assignments were substantiated in the subsequent experiments.

Unlike the control, the transfected cells possessed lysozyme activity (not shown). One portion of the precursor was secreted and the other remained associated with the cells. In the cells, the maturation produced a fragment (Lt in Fig. 1) that had the apparent size of a lysozyme form bearing a short oligosaccharide of 15.8 kDa and could be collected with antilysozyme antiserum. Lysozyme has been shown to be released from a fusion protein containing cathepsin D [Horst et al., 1993]. In electrophoresis, the intracellular fragment appeared to represent a considerable fraction of the fusion protein produced in the cells. In contrast, glycosylated lysozyme that had been expressed from cDNA encoding the glycosylated lysozyme alone had been predominantly secreted, and little of

it had remained within the cells [Horst et al., 1991]. The difference in the sorting efficiency is illustrated in Figure 1.

In a previous study, pulse-chase experiments showed that the stability of the intracellular lysozyme is similar for the glycosylated and unglycosylated forms.

In pulse-chase experiments, it was observed that within 16 h most of the precursor was either fragmented or secreted (see below). In the cells, besides the 33-kDa form (Pm), a small amount of a larger species (Pc) was found, ranging from 35 to 41 kDa. These larger molecules were secreted into the medium. The protein ultimately remaining with the cells was smaller than the precursor and by its size resembled glycosylated forms of lysozyme. A major form of the fragment (Lt in Fig. 2) corresponded to 15.8 kDa. After digestion with glycopeptidase F, it was converted to a 14-kDa form (Ld). By the criteria of the apparent size, this form could not be distinguished from the form of the wild-type lysozyme. The deglycosylation of the fusion protein from either the cells or the medium produced a 28-kDa polypeptide. We considered the possibility that the lysozyme moiety is subject to a significant degradation and examined the effect of 10 mM NH_4Cl, which is assumed to cause a neutralization of acidic compartments, on the apparent labeling of the polypeptides (Fig. 2). In this and other experiments, we observed that in the presence of NH_4Cl the apparent labeling of the lysozyme fragment was increased. Therefore, in experiments examining the retention of lysozyme, NH_4Cl was used to impede the possible degradation.

Besides the 15.8-kDa form (Lt), larger species of glycosylated lysozyme (Lm in Fig. 3) were found, which were characterized by their sensitivity to *endo*-β-N-acetylglucosaminidase H. During the chase period, concomitantly with the fragmentation the proportion of the sensitive form (Lm) decreased (Fig. 3). The metabolic labeling of the fusion protein, its fragmentation, and the partial hydrolysis of the oligosaccharides linked to the lysozyme fragment, concomittant with the loss of sensitivity to endo-β-N-acetylglucosaminidase, as shown in this presentation, is representative of a larger number of similar experiments.

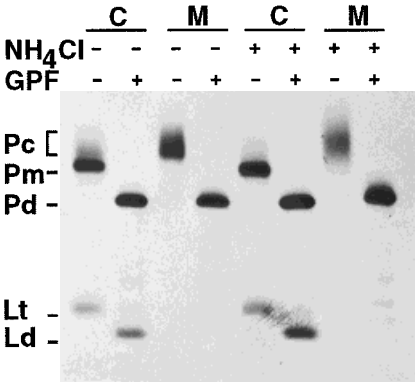

Fig. 2. Deglycosylation of lysozyme-containing polypeptides from cells that were metabolically labeled in the absence or presence of 10 mM NH_4Cl. The polypeptides were isolated from cells (C) and the medium (M) after 18 h of culturing with Tran^{35}S-Label. The solubilized immunoprecipitates were halved and incubated without (−) or with (+) glycopeptidase F (GPF). The radioactive polypeptides were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis and fluorography. The symbols Pd and Ld refer to the deglycosylated precursor and lysozyme polypeptides, respectively. The other symbols are explained in the caption to Figure 1.

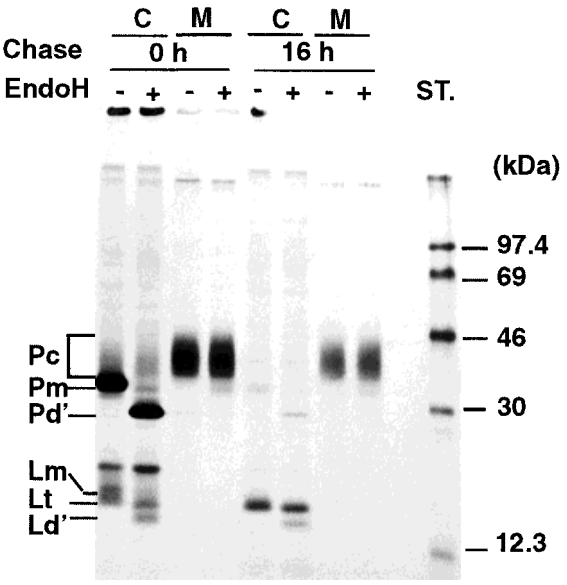

Fig. 3. Biosynthesis and maturation of the fusion protein in transfected Chinese hamster ovary cells. The cells were cultured with Tran^{35}S-Label for 8 h and either harvested or supplemented with a mixture of nonradioactive methionine and cysteine and incubated for 16 h. Aliquots of the solubilized immunoprecipitates from cells (C) and the medium (M) were incubated without (−) or with (+) *endo*-β-N-acetylglucosaminidase H (EndoH). This enzyme cleaves mannose-rich and hybrid oligosaccharides within the di-N-acetylchitobiose linking region. The radioactive polypeptides were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis and fluorography. The endoglycosidically cleaved, i.e., partly deglycosylated forms of the precursor fusion protein (Pd′) and lysozyme (Ld′), are indicated. Other symbols are explained in the caption to Figure 1. The band observed in the intracellular material in the pulse-labeled sample above the Lm form is considered to be a contamination because it was not found in any other experiments with similarly labeled cells.

From the differential sensitivity to *endo*-β-N-acetylglucosaminidase H and the apparent size, it was concluded that the Lm and Lt forms of glycosylated lysozyme contained mannose-rich and trimmed oligosaccharides, respectively. The trimming accompanied and followed the proteolytic fragmentation. As determined by densitometry, after the pulse labeling (Fig. 3) the proportion of molecules bearing mannose-rich oligosaccharides in the intracellular fraction of lysozyme (Ld′ vs. the sum of Ld′ and Lt) corresponded to 38%. After the chase incubation, this proportion decreased to 18%.

Carbohydrate Processing in the Intracellularly Retained Reporter and the Secreted Fusion Protein in BHK Cells

When expressed in BHK cells, similarly to CHO cells, the fusion protein was partly retained and partly secreted. However, the secreted protein was subject to a fragmentation (Fig. 4). The formation of the secreted lysozyme fragment during or rather after the secretion was indicated by the fact that the protein moiety of the lysozyme fragment found in the medium was larger than that found in the intracellular fraction. Thus, different proteinases appeared to be responsible for the fragmentation of the fusion protein in the different compartments. By removing the myeloperoxidase moiety bearing one of the two oligosaccharides from the secreted form of the fusion protein, it was possible to compare the type of the oligosaccharides in the lysozyme moiety in the secreted and the intracellular molecules. Most of the carbohydrate in extracellular lysozyme fragments behaved as a complex oligosaccharide. From the resistance to *endo*-β-N-acetylglucosaminidase, it was concluded that in the secreted fusion protein both the myeloperoxidase and lysozyme moieties contain mainly complex oligosaccharides. As determined by densitometry, the extracellular lysozyme fragment bore 90% complex and 10% mannose-rich oligosaccharides. In contrast, within the cells the lysozyme fragment bore either mannose-rich (*endo*-β-N-acetylglucosaminidase-sensitive) or trimmed (*endo*-β-N-acetylglucosaminidase-resistant) oligosaccharides.

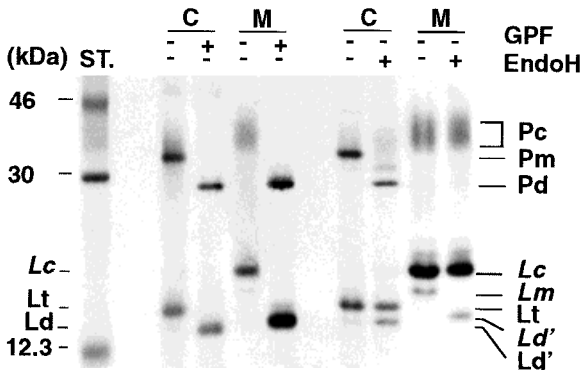

Fig. 4. Biosynthesis and processing of the fusion protein in transfected baby hamster kidney cells. The cells were cultued in the presence of Tran^{35}S-Label for 22 h. Aliquots of the solubilized immunoprecipitates were treated with glycopeptidase F (GPF) or *endo*-β-N-acetylglucosaminidase H (EndoH), and the radioactive polypeptides were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis and visualized by fluorography. The symbols of the polypeptides are explained in the captions to Figures 1 and 3. Symbols in italics represent lysozyme fragments that were formed in the medium or derived from these.

Intracellular Retention and Targeting

Additional experiments were performed with transfected CHO cells. Cells expressing glycosylated lysozyme [Horst et al., 1991] or its fusion protein with cathepsin D [Horst et al., 1993] have been shown to secrete or process these glycoproteins in lysosomes within 2 h of labeling. In contrast, the 33-kDa fusion protein appeared to persist in its precursor form, containing mannose-rich oligosaccharides several hours longer. In transfected cells that were incubated with the radioactive medium for up to 18 h, a great portion of the fusion protein remained associated with the cells in the precursor form (Figs. 1–3). After a 16 h-chase (Fig. 3), most of the precursor was either secreted or processed. It has been reported that the proteolytic fragmentation of promyeloperoxidase in promyelocytes takes on average more than 6 h [Hasilik et al., 1984], and this has been explained by a long residence in the endoplasmic reticulum and an interaction of the apoprotein with a 60-kDa calreticulin [Nauseef et al., 1995]. Such an interaction may be responsible for the slow intracellular transport of the studied fusion protein versus that of lysozyme.

The subcellular localization of the processed glycosylated lysozyme fragment of the fusion protein was examined in subcellular fractionation experiments in which organelles were subjected to flotation in a sucrose gradient. When the labeling period was followed by an incubation that allowed for maturation of the newly synthesized proteins, the distribution of

the lysozyme fragment in the flotating portion of the gradient approached that of the activity of the lysosomal marker β-hexosaminidase. This is shown in a representative experiment using cells that had been subjected to a 12-h pulse, 16-h chase labeling (Fig. 5). In several experiments, the homogenization and adjusting the sample to 50% (w/w) sucrose resulted in the release of up to 40% β-hexosaminidase and lysozyme activity into the soluble fraction. This was represented by fractions 10–12. In the experiment shown in Figure 5, 43% of the activity of β-hexosaminidase and 41% of lysozyme were associated with the nonflotating material in fractions 11 and 12. The preferential association of lysozyme with the sediment (fraction 12) was likely due to the tendency of lysozyme to bind to particles containing nucleic acids.

Independent evidence for the lysosomal localization of the lysozyme fragment was obtained in experiments in which the cells were allowed to endocytose dextran-coated magnetizable particles and a portion of the labeled endosomal/lysosomal organelles were separated from the other compartments in a magnetic field. In the magnetically adsorbed fraction, the yield of the lysosomal marker β-hexosaminidase was 20% and that of lysozyme was practically the same (Table 1). In contrast, galactosyl transferase was not associated with the magnetically separable fractions. The eluate contained $<1\%$ of protein that was applied to the magnetic column (not shown).

To compare the quantity of lysozyme retained after the segregation within the cells and secreted as the fusion protein, the cells were labeled for 16 h in the presence of 10 mM NH_4Cl and the radioactivity associated with the 15.8-kDa intracellular lysozyme form and the 35–41-kDa secreted fusion protein was quantitated and corrected for the trimming of the myeloperoxidase prosequence from the intracellular lysozyme. The cleavage of the prosequence of the myeloperoxidase moiety is compatible also with the lysosomal targeting of the intracellular fusion protein. The proportion of the radioactivity associated with the lysozyme and the nonlysozyme moiety could not be calculated from the amino acid sequence data because the protein was labeled by a mixture of radioactive methionine and cysteine and there was a possibility that the pools of these amino acids in the cells could differentially affect the incorporation. To solve this problem, we assumed that the selective trimming of the myeloperoxidase portion of the molecule should also be achieved in vitro in the presence of lysosomal enzymes. Figure 6 shows that incubation of the immunoprecipitates of the fusion protein with either cathepsin B or cathepsin D resulted in a fragmentation of the polypeptides. By their apparent size the fragments resembled lysozyme bearing complex oligosaccharides. The apparent size of fragments L*c and L*m obtained with cathepsin D was significantly larger than that with cathepsin B. This result indicated that both cathep-

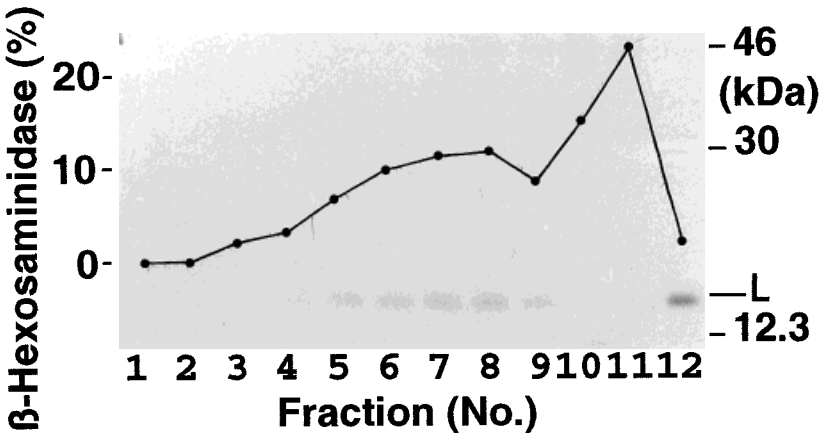

Fig. 5. Subcellular fractionation of Chinese hamster ovary cells expressing the fusion protein. The cells were metabolically labeled with Tran^{35}S-Label for 10 h and subjected to a 16-h chase. A postnuclear supenatant was adjusted to 50% (w/w) sucrose, and the organelles were separated by flotation in a sucrose gradient. The fractions were collected from the top and analyzed for β-hexosaminidase activity. The lysozyme fragment (L) was immunoprecipitated and analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis and fluorography. The peak of galactosyl transferase corresponded to fraction 6 and neutral α-glucosidase as a marker of the endoplasmic reticulum peaked in fractions 7–8.

TABLE I. Endocytosis-Dependent Separation of Lysozyme[a]

Fraction[b]	Lysozyme (%)[c]	β-Hexosaminidase (%)[c]	Galactosyltransferase (%)[d]
Without magnetic particles			
Unbound	95 ± 10	98 ± 0.9	98
Wash	3.5 ± 7	1.4 ± 0.8	0.5
Eluate	1.5 ± 3	0.3 ± 0.5	1
With magnetic particles			
Unbound	73 ± 7	83 ± 4	98
Wash	3 ± 6	2 ± 2	1
Eluate	24 ± 3	18 ± 4	0.5

[a]The transfected cells were incubated without or with superparamagnetic particles for 6 h.
[b]Postnuclear supernatant fractions were prepared and subjected to magnetic subcellular separation. In fractions the concentration of lysozyme and the activities of two enzymes marking lysosomal and Golgi compartments were determined.
[c]Four independent experiments.
[d]Average of two experiments.

sins removed a large portion of the myeloperoxidase prosequence from the lysozyme moiety and that the removal was less complete with the more specific proteinase cathepsin D. Previously, we have shown that exposure of a fusion protein of lysozyme with cathepsin D to lysosomal proteinases both in transfected cells and in vitro produces fragments that closely resemble normal lysozyme [Horst et al., 1993]. Because of the limited amount of the material, the N-terminus of the lysozyme fragment that was obtained in vitro was not determined. Nevertheless, from the small difference between the apparent size of the fragment and the authentic glycosylated lysozyme of about 0.25 kDa, it was assumed that the cleavage occurred within the sequence Val-Thr-Val-Gln-Gly that links Cys158 of the myeloperoxidase moiety to Lys1 of lysozyme (Lys164 in the fusion protein). Considering the apparent size of the product and the preference of cathepsin D for hydrophobic residues at the substrate site S1, a cleavage after Val161 leaving two residues attached to the N-terminus of lysozyme was likely to occur. Therefore, the cleavage in vitro was expected to reduce the radioactivity in the recovered lysozyme to the same extent as that within the cells. After the cleavage with cathepsin B and cathepsin D, the radioactivity recovered in the lysozyme-containing material dropped to 74% and 65%, respectively. In the subsequent calculation of the proportion of the intracellularly retained fusion protein from the radioactivity associated with the lysozyme fragment, the average of the two values was used.

In additional experiments, the cells were labeled in the presence of 10 mM NH_4Cl. The radioactivity associated with the lysozyme fragment in the cells and with the fusion protein in the medium was determined, and by using the above calculated proportion value, it was estimated that 35% (mean of two measurements) of the fusion protein was retained and processed to lysozyme. The data are compatible with the distribution of the protein presented in Figures 1 and 2 and show that a large proportion of the fusion protein is sorted into the lysosomal compartment.

The glycosylation pattern in the lysozyme fragment from the secreted fusion protein of CHO cells (Fig. 6, lanes 5 and 6) was more complex than in the medium from BHK cells (Fig. 4). It has been shown that the glycosylation pattern of lysozyme differs in the two cell types because of the ability of CHO cells to elongate the carbohydrate with N-acetyllactosamine repeats [Horst et al., 1991; Hummel et al., 1997].

Routing of the Intracellularly Retained Fusion Protein

The selective occurence of mannose-rich oligosaccharides in the lysozyme fragment suggested that a fraction of the fusion protein molecules avoided the processing of mannose-rich oligosaccharides to the complex type and was targeted to lysosomes. This could be due to a selective retention of nonprocessed mannose-rich ligands. It was not due to a phosphorylation and binding to mannose 6-phosphate receptors because the lysosomal targeting was not inhibited by NH_4Cl. To examine an alternative possibility that the segregation from the secretory pathway was an early event preceding the trimming and/or the terminal glycosylation in the Golgi apparatus, we treated the cells with monensin, an ionophore that inhibits the transport through the Golgi apparatus and the acidification-dependent traffic to lysosomes [Tartakoff, 1983]. Figure 7 shows that monensin inhibited the secretion of the fusion protein

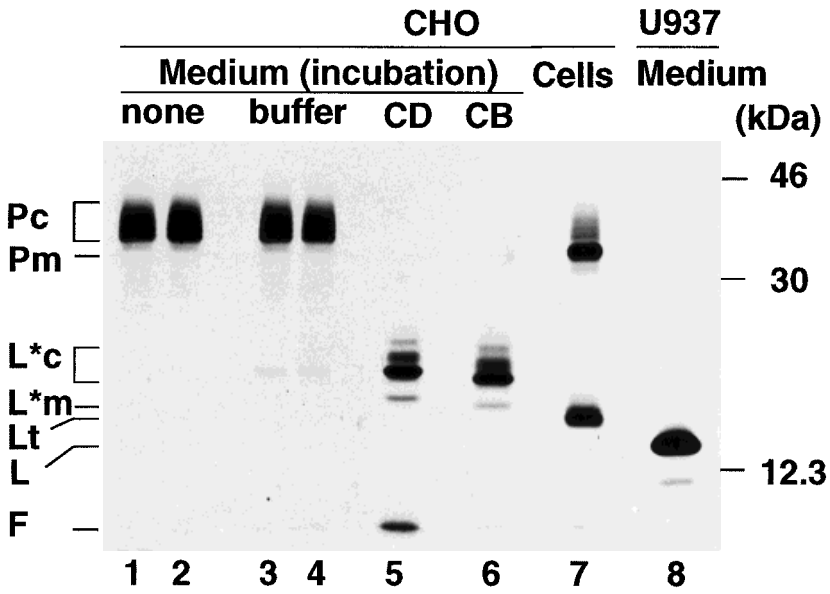

Fig. 6. Fragmentation of the fusion protein by in vitro proteolysis. Aliquots of the medium from metabolically labeled Chinese hamster ovary (CHO) cells expressing the myeloperoxidase preprosequence–Gly22Asn lysozyme fusion protein were incubated with buffer without or with cathepsin D (CD) or cathepsin B (CB). After boiling in the presence of sodium dodecylsulfate (SDS), the samples were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography. For comparison, immunoprecipitates from the intracellular fraction of the transfected CHO cells and from the medium of U937 cells that contained the nonglycosylated lysozyme are shown. F refers to fragments found in the salt boundary in the gel. L*c and L*m refer to the lysozymelike fragments containing complex and mannose-rich oligosaccharides, respectively. The other symbols are explained in the caption to Figure 1.

during both the pulse and the chase incubations but appeared not to inhibit its intracellular fragmentation. Removal of monensin at the beginning of the chase period (Fig. 7) resulted in a partial restoration of the terminal glycosylation and in secretion of the precursor. The amount of the intracellular lysozyme fragment did not increase much after the chase incubation in the absence of monensin. Thus, monensin strongly inhibited the carbohydrate processing and the secretion but had little effect on the intracellular targeting and the proteolysis of the precursor.

DISCUSSION

Glycosylation of the Fusion Protein

Glycosylated mutant lysozyme has been used as a reporter molecule to indicate phosphorylation and lysosomal targeting in a fusion protein with cathepsin D [Horst et al., 1993]. In the present study, fusion of the glycosylated lysozyme to a major portion of the prosequence of human myeloperoxidase had a profound effect on its processing and targeting. In CHO cells, glycosylated lysozyme is synthesized with biantennary oligosaccharides containing up to five N-acetyllactosamine repeats that can be recognized in SDS-PAGE as a ladder of bands [Hummel et al., 1997]. The elongation of the antennae in the lysozyme moiety of the fusion protein (Fig. 6, lanes 5 and 6) was less proficient than that in the nonextended glycosylated lysozyme (Fig. 1). From the apparent size of the lysozyme fragment of the secreted fusion protein, it could be judged to contain a maximum of three N-acetyllactosamine repeats, with the major form being bare of them. A rather complete inhibition of the elongation of N-acetyllactosamine chains has been reported due to a fusion of glycosylated lysozyme with cathepsin D [Horst et al., 1993]. Thus far, protein signals for the elongation have not been found. The data obtained with glycosylated lysozyme in the previous and present studies suggest that the elongation depends on the accessibility of the antennae, which may be decreased in the fusion proteins.

In BHK cells during or after the secretion, the fusion protein was subjected to a fragmentation and the released lysozyme moiety contained predominantly complex oligosaccharide chains. In contrast, the fragmentation of the intracellu-

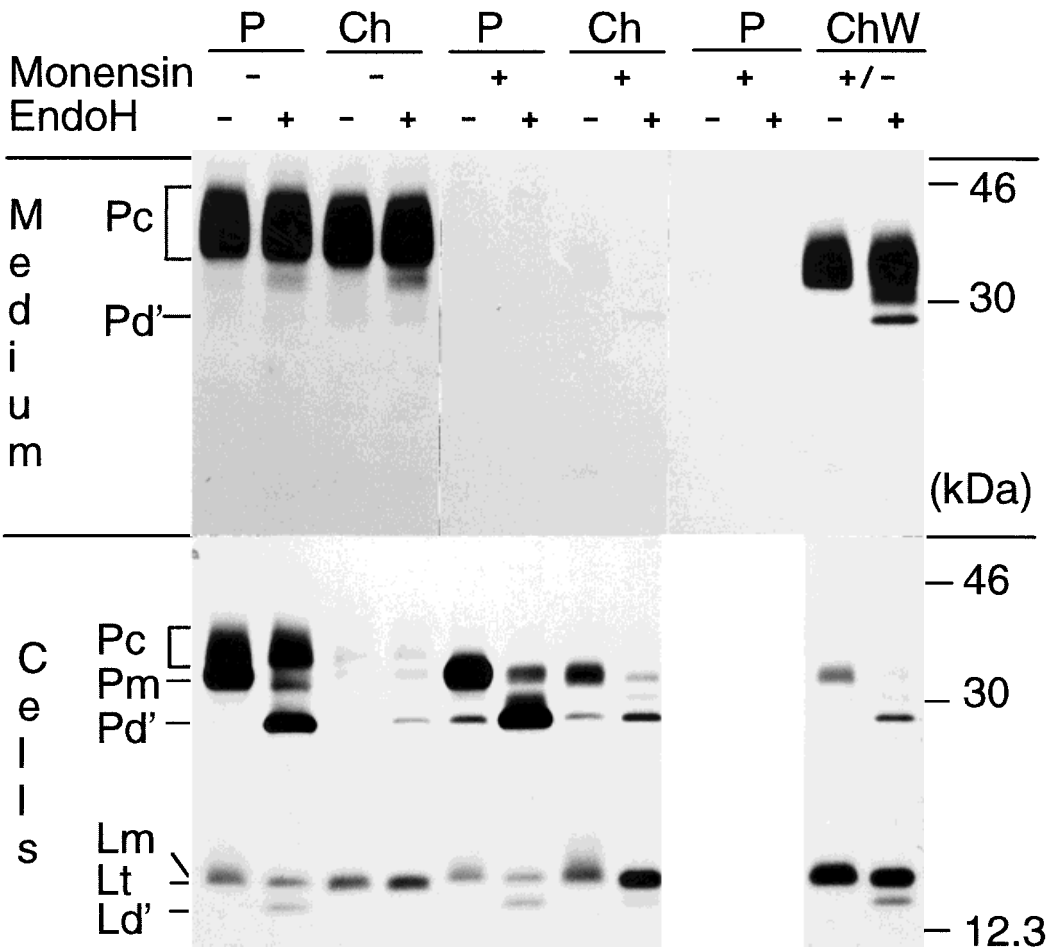

Fig. 7. Effect of monensin on secretion and intracellular processing of the fusion protein. The cells were labeled with Tran^{35}S-Label for 5 h in the absence or presence of 1.5 μM monensin. One pair of cultures was harvested after the labeling (P) and another after a 12-h chase incubation in the presence of an excess of cysteine and methionine (Ch). In a fifth culture, the labeling in the presence of monensin was terminated by washing, and the chase incubation was performed in a fresh medium. Lysozyme-containing proteins were immunoprecipitated and analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis and fluorography. In the washed culture, the medium after the pulse labeling and both the medium and cells after the wash and the chase incubation (ChW) were analyzed. The symbols of the polypeptides are explained in the captions to Figures 1 and 3.

larly retained fusion protein produced lysozyme fragments that contained mostly mannose-rich and trimmed oligosaccharides. The trimmed oligosaccharides could be formed from either the complex or the mannose-rich species. At earlier time points in the intracellularly processed molecules, more mannose-rich oligosaccharides were found at the expense of the trimmed chains. It is interesting that after a delivery to granules and maturation in promyelocytes HL-60 myeloperoxidase has been shown to contain predominantly mannose-rich oligosaccharides [Hasilik et al., 1984; Strömberg et al., 1986]. It has been suggested that this enzyme retains its mannose-rich oligosaccharides because it appears not to enter the compartment responsible for the terminal glycosylation [Nauseef, 1987]. This appears to be the case also for the the intracellularly processed fusion protein containing the prosequence of myeloperoxidase.

Intracellular Transport and Lysosomal Targeting of the Fusion Protein

Results on the subcellular fractionation and the degradation of the myeloperoxidase prosequence in the intracellularly retained fusion protein indicate that it is targeted to a lysosomal compartment. The targeting is insensitive to NH_4Cl. In the presence of this salt, the

yield of the lysozyme fragment is increased, probably due to an inhibition of the intralysosomal degradation. Nevertheless, NH_4Cl does not prevent the degradation of the myeloperoxidase prosequence. This resembles the processing of promyeloperoxidase in promyelocytes in the presence of NH_4Cl [Hasilik et al., 1984; Nauseef et al., 1988]. In HL-60 cells, the intracellular processing of myeloperoxidase is several times slower than that of the lysosomal marker cathepsin D [Hasilik et al., 1984]. The precursor of myeloperoxidase has been shown to be retarded in the endoplasmic reticulum [Nauseef, 1987; Bainton, 1988]. It is interesting that the apparent efficiency of the lysosomal targeting of the fusion protein to lysosomes as reported in the present study is higher than that of myeloperoxidase that has been studied previously [Cully et al., 1989]. We assume that in lysosomes of BHK cells myeloperoxidase is less stable than lysozyme.

Monensin inhibits the secretion and much less the maturation of myeloperoxidase [Nauseef et al., 1988]. Whereas Akin et al. [1987] reported that monensin inhibits the maturation of the myeloperoxidase precursor, our results with the fusion protein are in agreement with the findings of Nauseef et al. [1995] and with other reports on the impairment of the transport of proteins through the Golgi compartments [Tartakoff, 1983]. In the presence of monensin, maturation of the fusion protein continues, indicating that the transport to the lysosomal compartment is not interrupted.

The present findings that fusion protein consisting of the myeloperoxidase prosequence and glycosylated lysozyme resides in an early biosynthetic compartment for a longer period of time than does lysozyme and is lysosomally targeted in a form not experiencing the terminal glycosylation suggest that the prosequence itself is retarding the protein in the endoplasmic reticulum and perhaps also directing its packaging. In the precursor of myeloperoxidase, the enzyme moiety and its apo/holoenzyme status are likely to contribute to the retention in the endoplasmic reticulum and to the packaging competence of this hemoprotein [Nauseef et al., 1995]. The differential carbohydrate processing in the secreted and the lysosomally targeted fusion protein studied here suggests that the lysosomally targeted molecules bypass the *trans*-Golgi compartment and that the myeloperoxidase prosequence contains a corresponding segregation signal.

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