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DOI
10.1111/j.1432-1033.1997.00279.x

Publication date
1997

Published in
European Journal of Biochemistry

Eur. J. Biochem. *244*, 279–285 (1997)

Synthesis, sorting, and processing into distinct isoforms of human macrophage chitotriosidase

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(Received 2 October/9 December 1996) – EJB 96 1463/1

Chitotriosidase, the human analogue of chitinases from non-vertebrate species, has recently been identified. The macrophage-derived enzyme is remarkably heterogeneous in molecular mass and isoelectric point. The synthesis and modification of the enzyme in cultured macrophages is reported. Chitotriosidase is synthesized as a 50-kDa protein with a pI of about 6.5 and 7.2. It is predominantly secreted, but in part processed into a 39-kDa form with a pI of 8.0 that accumulates in lysosomes. In the C-terminal extension of the 50-kDa chitotriosidase, sialic-acid containing O-linked glycans are present, causing its heterogeneous acidic isoelectric point. Chitotriosidase lacks N-linked glycans and the mechanism of routing to lysosomes proves to be distinct from that of soluble, N-glycosylated, lysosomal enzymes. It was observed that, in macrophages, alternative splicing generates a distinct chitotriosidase mRNA species, encoding a 40-kDa chitotriosidase that is C-terminally truncated. This enzyme is almost identical to the 39-kDa chitotriosidase formed from the 50-kDa precursor by proteolytic processing. It is concluded that the C-terminus present in the 50-kDa chitotriosidase, but absent in the 39-kDa isoform, was found to mediate tight binding to chitin. In the blood stream the secretory 50-kDa chitotriosidase occurs predominantly, whilst in tissues the 39-kDa form is also abundant. These findings are consistent with the data on the synthesis and processing of chitotriosidase in the cultured macrophage model.

Keywords: chitotriosidase; macrophage; sorting; processing; chitinase.

In Gaucher disease (glucosylceramidosis), the glycolipid glycosylceramide accumulates in lysosomes of macrophages due to a deficiency of the enzyme glucocerebrosidase [1]. An intriguing marker enzyme for manifestation of this disorder has recently been identified. It has been designated chitotriosidase on the basis of its ability to hydrolyse the artificial fluorogenic substrate 4-methylumbelliferyl (MeUmb)-chitotrioside. Chitotriosidase is produced in large amounts by the lipid-laden macrophages in Gaucher patients, and its activity is on average 600-fold increased in the plasma of symptomatic patients [2].

Recently we reported the purification and characterization of chitotriosidase from Gaucher spleen. The purified enzyme was found to hydrolyse chitin. The amino acid composition of the N terminus and an internal sequence of chitotriosidase indicate similarity with members of the family of chitinases [3, 4]. The nucleotide sequence of cloned chitotriosidase cDNA further substantiated that chitotriosidase has to be considered as the human analogue of chitinases from several non-mammalian species [5]. Until recently it has generally been assumed that such an enzyme is absent in man and other mammals. Previous studies have revealed that chitotriosidase is almost exclusively produced by macrophages. When isolated human peripheral blood monocytes are cultured *ex vivo*, spontaneous differentiation into activated macrophages occurs within a few days. After about 7 days, chitotriosidase mRNA is detectable, in combination with secretion of active enzyme [2, 5]. The expression of chitotriosidase increases with time, and after three weeks of cell culture chitotriosidase is one of the major secretory proteins.

Human chitotriosidase is known to be heterogeneous with respect to molecular mass and isoelectric point [3]. Two major isoforms of the enzyme are present in spleen: a 39-kDa isoform with a pI of 8.0 and a 50-kDa isoform with a pI of 7.2 and pI of about 6.5. In serum the pI-8.0 form is virtually absent. The information about the relationship between the various forms of chitotriosidase is still limited [3]. The pI-8.0 and pI-7.2 forms isolated from spleen have an identical N-terminal amino acid sequence. All forms of chitotriosidase appear to be absent in individuals with an inherited chitotriosidase deficiency. An antibody raised against the pure 39-kDa chitotriosidase is capable of precipitating all enzyme activity. On the basis of these findings it seems likely that post-translational modification of chitotriosidase contributes to the occurrence of distinct isoforms.

The present study describes the synthesis, post-translational modification and routing of chitotriosidase in cultured macrophages. The results obtained with the macrophage cell model provide information on the heterogeneous nature of chitotriosidase as encountered in various locations in the body.

MATERIALS AND METHODS

Materials. MeUmb-β-D-*N*,*N*′,*N*″-triacetylchitotriose, MeUmb-*N*-acetyl-β-D-glucosaminide, brefeldin A_1, bafilomycin A_1, leupeptin, tunicamycin, Histopaque-1077, crab-shell chitin, and RPMI-1640 were obtained from Sigma. Percoll and protein-

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Abbreviations. TIM, triose phosphate isomerase; MeUmb, 4-methylumbelliferyl.

Enzymes. Cathepsin D (EC 3.4.23.5); hexosaminidase (EC 3.2.1.52); lysozyme (EC 3.2.1.17); chitinase (EC 3.2.1.14).

A–Sepharose CL-4B were from Pharmacia. Methionine-free minimal essential medium and macrophage-serum-free medium were from Gibco BRL. [^{35}S]methionine was obtained from Amersham.

Enzyme assays. Chitotriosidase activity was measured with the fluorogenic substrate MeUmb-chitotrioside as described previously [3]. For the measurement of hexosaminidase activity MeUmb-*N*-acetyl-β-D-glucosaminide was used as substrate, according to Galjaard [6] with slight modification (the buffer used was 50 mM/100 mM McIlvain buffer, pH 4.0).

Antibodies. Purified 39-kDa chitotriosidase [3] and purified cathepsin D from Gaucher spleen were used to raise polyclonal antisera in rabbit.

Macrophage isolation and culture. Peripheral blood monocytes were isolated from healthy volunteers as follows. Heparized blood was diluted 1:1 with NaCl/P_i (140 mM NaCl, 2.7 mM KCl, 5 mM K_2HPO_4, 1 mM NaH_2PO_4, pH 7.4), and 25 ml was layered on 12.5 ml Histopaque (1.077 g/ml) and subjected to density-gradient centrifugation (20 min, 400×*g*, room temperature). The mononuclear cells were harvested from the interface and washed once with NaCl/P_i, and once with RPMI-1640 supplemented with 2% foetal calf serum, and suspended in culture medium (RPMI-1640 with 10% human AB-serum, 10 mM Hepes, 2 mM L-glutamine). Cells were cultured in plastic flasks at 0.5–1×10^6 cells/ml. After 5–7 days culture, the medium was changed to macrophage-serum-free medium and changed weekly. Macrophages from control persons and Gaucher disease patients produced comparable amounts of chitotriosidase. Cells used for experiments were always cultured for 3 weeks.

Macrophage extracts. After washing in NaCl/P_i, cells were harvested by scraping in either 50 mM Tris, pH 7.5, 0.5% Triton X-100, 5 mM MgCl, 20 mM NaCl including the protease inhibitors leupeptin and phenylmethylsulfonyl fluoride for immunoprecipitation, or 50 mM potassium phosphate pH 6.5, for other experiments, and extracted by brief sonication, followed by centrifugation to remove insoluble material.

Metabolic labeling. Macrophages were incubated for 1–2 h in methionine-free minimal essential medium. The cells were labeled for 10 min in the same medium supplemented with 5% dialysed foetal calf serum and 100 μCi [^{35}S]methionine. The cells were harvested immediately or chased for the indicated times in culture medium. Immunoprecipitations were carried out with extracts of cells as well as with the media. Labeled proteins were visualized by autoradiography after SDS/PAGE [7].

The additions made in the metabolic-labeling experiments were as follows: brefeldin A_1 (5 μg/ml), monensin (1 μM), methylamine (25 mM), bafilomycin A_1 (2 ng/ml), tunicamycin (10 μg/ml). All compounds were present at the indicated concentrations during the preincubation, the pulse, and the chase.

Subcellular fractionation by Percoll-gradient centrifugation. Macrophages were labeled for 16 h with [^{35}S]methionine as described above. Harvested cells were gently disrupted using a Dounce homogenizer in isotonic buffer (250 mM mannitol, 5 mM Mops/Tris, pH 7.0, 1 mM EGTA) and after low-speed centrifigation (2 min, 500×*g*) the post-nuclear supernatant (0.7 ml) was layered on top of an isotonic Percoll solution (7.5 ml) with a starting density of 1.065 g/ml in the same isotonic buffer. After centrifugation at 33000×*g* for 1 h, fractions of 250 μl were taken from the top. Enzyme activities were measured after three cycles of freeze/thawing. Chitotriosidase was immunoprecipitated from the fractions, electrophoretically separated on SDS/PAGE and visualized by autoradiography.

Immunoelectron microscopy. After 3 weeks in culture, macrophages were fixed with 1% paraformaldehyde/0.1% glutaraldehyde in Ringer buffer. Frozen cell samples were cryosectioned and immunolabeled, as described in reference [8], and examined using a Phillips 420 transmission electron microscope at an accelerating voltage of 80 kV.

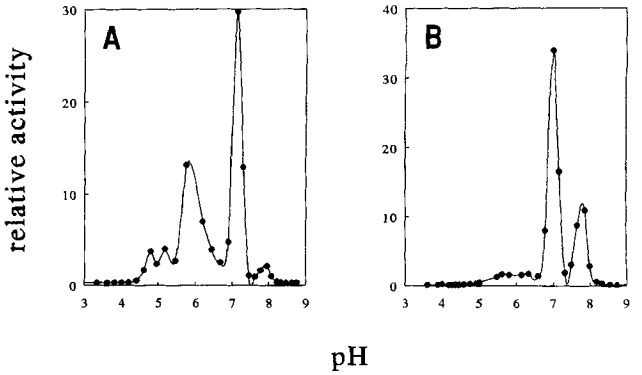

Fig. 1. Isoelectric point of chitotriosidase. Samples were subjected to preparative isoelectric focussing. pH and chitotriosidase activity were measured after fractionation of the gel. (A) Human-macrophage-conditioned medium; (B) human-macrophage cell extract. The enzyme activity present in the different fractions is expressed as a percentage of the total activity present in all fractions.

Determination of molecular mass. Purified 39-kDa spleen chitotriosidase was used for mass spectrometry. Electronspray spectra were obtained with a Fisons Bio Q triple quadra pole instrument, using 50% acetonitrile/water and 1% (by vol.) formic acid as a solvent. The Macintosh MacVector programme was used for molecular-mass predictions.

Chitin-binding assay. Chitin from crab shells was prepared by boiling in 1% SDS, 1% 2-mercaptoethanol, and extensively washed with water according to Kuranda et al. [9]. Chitotriosidase isoforms isolated from Gaucher spleen as previously described [3] were incubated with different amounts of chitin at 4°C for 16 h. After centrifugation, residual chitotriosidase activity was measured in the supernatant.

RESULTS

Chitotriosidase isoforms produced by cultured macrophages. Chitotriosidase is produced in large amounts by human macrophages upon prolonged culture. To study the presence of different chitotriosidase isoforms, extracts of cultured macrophages and their medium were subjected to isoelectric focussing [3] (Fig. 1). Forms with pI of 7.2 and 8.0 are abundant in the cells, whereas the medium contains the pI-7.2 form and an isoform with a pI of 6.0.

Synthesis and processing. The possibility that the different isoforms of chitotriosidase in cultured macrophages and their medium are the result of post-translational processing was investigated by metabolic pulse labeling of newly synthesised proteins produced by macrophages cultured for 3 weeks.

Fig. 2 shows the processing of chitotriosidase and cathepsin D, a lysosomal protease. After a 10-min pulse with [^{35}S]methionine, the amounts of radiolabeled chitotriosidase and cathepsin D were comparable, being about 1% of the total radioactively labeled protein formed by the cells. In macrophages from different individuals the production of chitotriosidase is variable, in contrast to that of cathepsin D.

In macrophages, cathepsin D is formed as a high-molecular-mass precursor (47 kDa in our electrophoretic system) that undergoes a two step proteolytic processing (Fig. 2A). Via a short-lived intermediate form that is generated in a prelysosomal com-

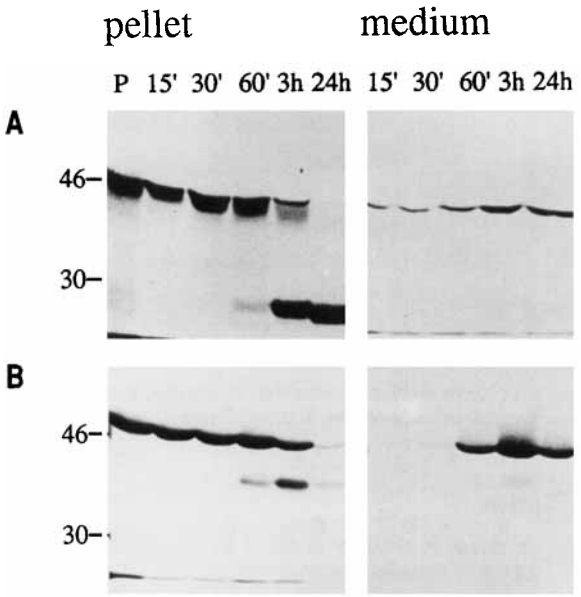

Fig. 2. Synthesis and processing of chitotriosidase. Cultured macrophages were subjected to metabolic-labeling experiments as described in Materials and Methods. Cells were harvested at the indicated chase periods; P represents a 10-min pulse. Immunoprecipitations are shown for pellets and media. (A) cathepsin D; (B) chitotriosidase. Molecular-mass markers (kDa) are indicated.

partment, the enzyme is processed after about 60 min to the mature protein of 31 kDa by a proteolytic step in the lysosome that requires low pH. The cathepsin D precursor is partly secreted into the medium after 15 min chase. These findings are similar to earlier observations made for cathepsin D in fibroblasts [10].

The metabolic-labeling experiment (Fig. 2B) shows that chitotriosidase is initially synthesised as a 50-kDa protein. During the chase period, part of the 50-kDa precursor is processed into a 39-kDa protein after about 60 min. The majority of the 50-kDa protein is secreted within a few hours into the medium and during more prolonged chase small amounts of a 39-kDa protein are released also in the medium. It has been documented that cultured macrophages can extrude some of their lysosomal content [11].

Upon SDS/PAGE, the 39-kDa isoform of chitotriosidsae sometimes migrated as a doublet rather than a single band (e.g. Figs 4B, 5B). This was due to incomplete reduction of disulfide bridges. Chitotriosidase, like other chitinases [12], contains disulfide bridges that are relatively resistant to reduction. In cells of some individuals minor amounts of a radiolabeled 40-kDa chitotriosidase were detected after 10 min pulse labeling (Fig. 2). Since such rapid formation of 40-kDa chitotriosidase could not be due to post-translational modification of the protein, the phenomenon was further investigated. It was found to be due to alternative splicing of the pre-mRNA, resulting in an mRNA coding for a C-terminally truncated 40-kDa protein (details to be published elsewhere). RNase protection experiments (data not shown) revealed that in cultured macrophages of these individuals only very little of the alternatively spliced mRNA is present compared with normal mRNA.

Intracellular localization and processing. The presence of chitotriosidase was studied by immunoelectron microscopy (Fig. 3). Comparably to cathepsin D, chitotriosidase in macrophages was detected in lysosomes. Indirect immunofluorescence microscopy rendered similar results (data not shown).

A lysosomal localization for chitotriosidase is also indicated by subcellular fractionation of [^{35}S]methionine-labeled macrophages by means of Percoll-gradient-density centrifugation. Chitotriosidase activity distributes with that of β-hexosaminidase, a lysosomal enzyme (Fig. 4A). The 39-kDa isoform of chitotriosidase is predominantly present in the dense fractions (fraction 23-26), which are considered to represent mature lysosomes, whereas the 50-kDa isoform is present in fractions with a lower density (Fig. 4B), which are considered to contain pre-lysosomal compartments.

Manipulation of processing. A variety of components that are known to interfere with the routing and processing of lysosomal enzymes were tested on their effects on chitotriosidase. In Fig. 5, the processing of chitotriosidase is compared with that of cathepsin D.

The presence of brefeldin A_1, a fungal antibiotic that inhibits transport of secretory proteins from the endoplasmic reticulum to the Golgi apparatus [13], results in inhibition of processing and secretion of both cathepsin D and chitotriosidase, presumably due to accumulation of the enzymes in the endoplasmic reticulum (Fig. 5, panels b).

Addition to the culture medium of pH-gradient-disturbing agents, such as monensin, methylamine and bafilomycin A_1, (partially) inhibits processing of cathepsin D and causes increased secretion of its precursor form (Fig. 5A, panels c, d and f). The effects of these agents on chitotriosidase are different. The presence of monensin, methylamine and bafilomycin A_1 does not effect secretion of chitotriosidase. The carboxylic ionophore monensin increases the stability of the intracellular enzyme (Fig. 5B, panel c). Methylamine, an acidotrophic agent that raises the pH of acidic compartments, particularly inhibits the processing of 50-kDa chitotriosidase to the 39-kDa form (Fig. 5B, panel d). Bafilomycin A_1, an inhibitor of the lysosomal protein pump [14], has little effect on the processing (Fig. 5B, panel f). The presence of the inhibitor results in a stabilization of intracellular chitotriosidase similar to the effect of monensin.

Also of interest is leupeptin, a powerful inhibitor of thiol-proteases, which are responsible for intralysosomal proteolytic processing of most lysosomal enzymes. No inhibition of conversion of 50-kDa chitotriosidase to the 39-kDa enzyme was seen in the presence of leupeptin (data not shown), suggesting that this step is not catalysed by these proteases.

Glycosylation of chitotriosidase. Previously it was shown that the pI-7.2 and pI-8.0 isoforms have molecular masses of 50 kDa and 39 kDa, respectively [3]. The pI-6.0 isoform of chitotriosidase, which is secreted by cultured macrophages, shows a mobility similar to the pI-7.2 isoform on gel filtration. Heterogeneity in glycan composition, in particular sialic acid content, is known to underly the heterogeneity in isoelectric points of lysosomal hydrolases [15].

The occurrence of oligosaccharide chains in chitotriosidase has been studied. The 39-kDa (pI 8.0) form has been previously shown not to be glycosylated [3]. Experiments with tunicamycin, an inhibitor of N-linked glycosylation, suggested the absence of N-linked glycans in 50-kDa chitotriosidase (data not shown). Furthermore, the nucleotide sequence of chitotriosidase cDNA reveals no consensus sequence for such modification in the protein. In chitinases from other species, O-linked glycosylation has been noted [12]. To investigate the possibility that chitotriosidase contains O-linked glycans, the 50-kDa enzyme was incubated with O-glycanase. No reproducible shifts in molecular mass were observed upon the incubation. Since it is known that O-glycanase digestion is not effective for several glycoproteins with O-linked glycans, attention was focussed to the demonstra-

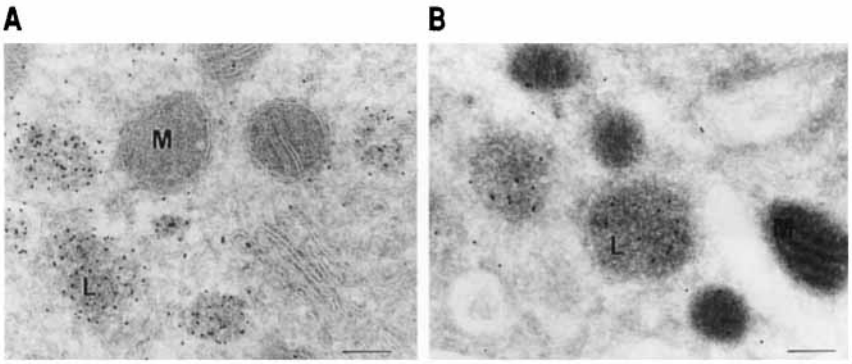

Fig. 3. Detection of chitotriosidase by immunoelectron microscopy. Human macrophages were processed for immunoelectron microscopy as described in Materials and Methods. (A) cathepsin D; (B) chitotriosidase. L, lysosome; M, mitochondrion. Bar = 0.2 μm.

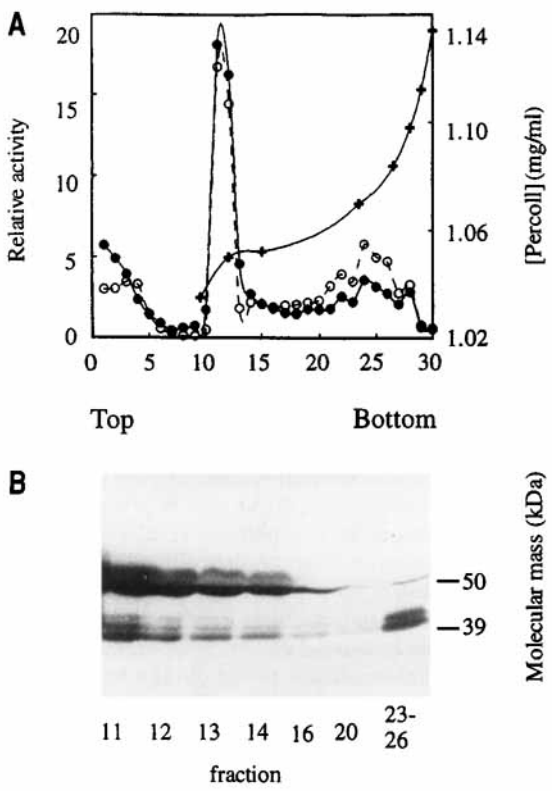

Fig. 4. Subcellular localisation of chitotriosidase. Macrophages were metabolically labeled with [^{35}S]methionine, and subcellular fractionation by Percoll-gradient centrifugation was performed as described in Materials and Methods. (A) Distribution of enzyme activity. Chitotriosidase (●), hexosaminidase (○). The density of the fractions is indicated (+). (B) Immunoprecipitated chitotriosidase after SDS/PAGE and autoradiography. The apparent molecular masses (kDa) of chitotriosidase are indicated. The enzyme activity present in the different fractions is expressed as the percentage of the total activity present in all fractions.

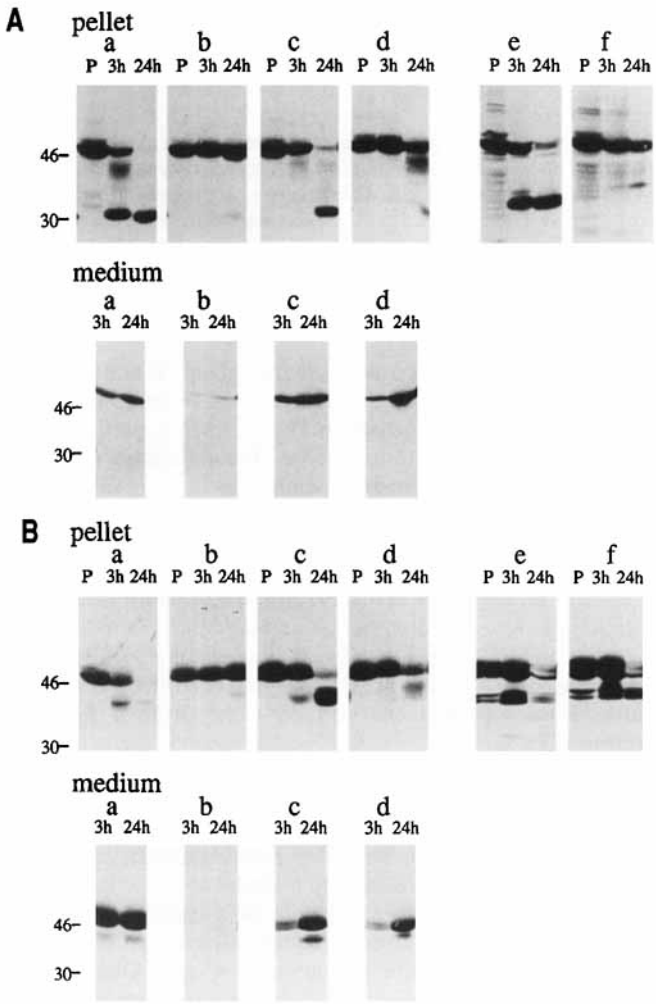

Fig. 5. Manipulation of processing of chitotriosidase by agents that affect lysosomal enzymes. Macrophages were metabolically labeled as described in Materials and Methods. Immunoprecipitations are shown for pellets and media. (A) cathepsin D; (B) chitotriosidase. The following additions were made: a, none; b, brefeldin A_1; c, monensin; d, methylamine; e, none; f, bafilomycin A_1. Incubations a–d were performed at the same time; e and f are from a separate experiment. Molecular-mass markers (kDa) are indicated.

tion of the presence of sialic acid in chitotriosidase. For this purpose, 50-kDa chitotriosidase secreted by human macrophages was incubated with and without neuraminidase, as described in [16]. The neuraminidase treatment results in a shift of the pI-6.0 form towards pI 7.2 (Fig. 6), suggesting that the presence of sialic acid contributes to the acidic isoelectric point. On the basis of this finding, it seems probable that chitotriosidase contains O-linked glycans with variable amounts of sialic acid.

Analysis of proteolytic processing by mass spectrometry. The precise molecular mass of the purified 39-kDa protein from Gaucher spleen was determined by electrospray mass spectrometry. Molecular masses of 40638.57 ± 23.88 kDa and 40713.93 ± 29.84 kDa were found (Table 1). Since 39 kDa-chitotriosidase is not glycosylated and the N terminus of the en-

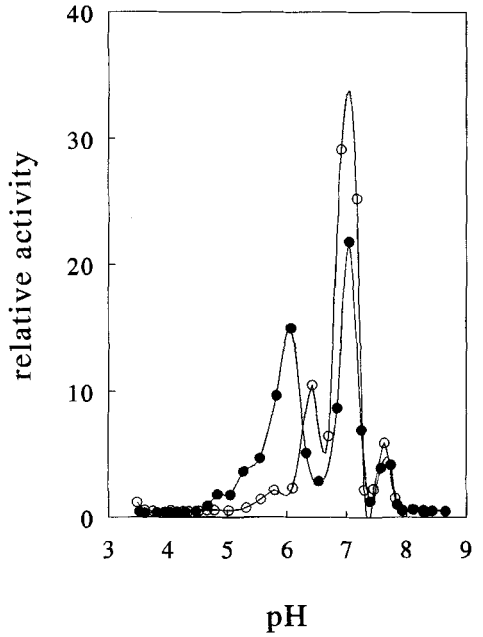

Fig. 6. Neuraminidase treatment of macrophage-conditioned medium. Macrophage-conditioned medium was incubated overnight with a neuraminidase preparation. The enzyme activity present in the different isoelectric-focussing fractions is expressed as percentage of the total activity present in all fractions. Control incubation (●), neuraminidase treated (○).

Table 1. C-terminal amino acid sequence of chitotriosidase.

Sequence	Predicted molecular mass	Experimental molecular mass
	Da	
...LRQELSE	40854.10	
...LRQELS	40740.95	40713.93 ± 29.84
...LRQEL	40653.88	40638.57 ± 23.88
...LRQE	40540.73	
...LRQ	40411.62	

zyme preparation was found to be homogeneous, as determined by protein sequencing, the variation in molecular mass is probably due to heterogeneity in the C terminus of the 39-kDa protein. On the basis of the observed molecular mass and the chitotriosidase amino acid sequence (predicted by the cDNA sequence), it is possible to tentatively assign the C-terminal amino acids in 39-kDa chitotriosidase as LRQELS or LRQEL. The predicted C terminus for the 40-kDa splice variant is LRQELNG, differing only in the last two amino acids. Apparently, two different mechanisms, alternative splicing and post-translational proteolytic modification, are used to generate comparable isoforms of chitotriosidase.

Chitotriosidase isoforms and binding to chitin. The capacity of the 39-kDa and 50-kDa chitotriosidase isolated from tissue to bind to chitin particles was investigated. The 50-kDa chitotriosidase was precipitated with chitin particles, whereas the 39-kDa isozyme was not (Fig. 7). Analysis of the enzyme bound to chitin particles gave consistent results: only the 50-kDa chitotriosidase was recovered on chitin particles after extensive washing (data not shown). These findings suggest that a chitin interaction site is present in the C-terminal 11-kDa domain of 50-kDa chitotriosidase, and that this domain is absent in the 39-kDa enzyme.

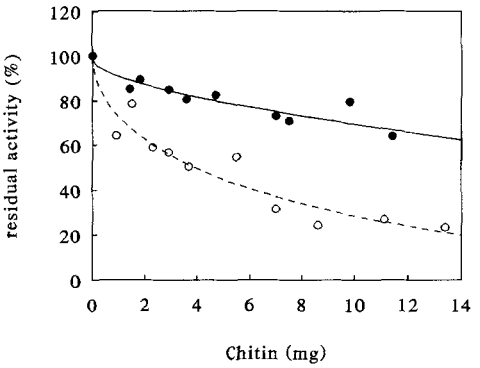

Fig. 7. Chitin-binding properties of the 39-kDa and 50-kDa chitotriosidase ioforms. Chitotriosidase isoforms, purified from Gaucher spleen extract, were incubated with increasing amounts of chitin particles at 4°C for 16 h. After centrifugation, the residual chitotriosidase activity in the supernatant was measured, and is expressed as a percentage of the activity in an incubation without chitin. 39-kDa chitotriosidase (●), 50-kDa chitotriosidase (○).

DISCUSSION

The unexpected discovery of a chitinase in man that is produced by macrophages [2] stimulated us to investigate in further detail the features of chitotriosidase. The aim of this study was to elucidate the synthesis, intracellular routing and post-translational modification of the enzyme. Using cultured human macrophages as a model, we found that chitotriosidase is initially synthesized as a 50-kDa protein, consistent with the prediction on the basis of the cloned cDNA [5]. Furthermore, although newly synthesized chitotriosidase is largely secreted, a significant proportion is efficiently routed to lysosomes, where it is present as a 39-kDa protein.

The routing and accompanying modification of chitotriosidase to lysosomes appear to be quite different from that known for lysosomal acid hydrolases. The sorting of the latter proteins is mediated by the presence of mannose-6-phosphate moieties in their N-linked glycans and interaction with mannose-6-phosphate–specific receptors [17]. This sorting event is known to be highly sensitive to perturbation of H^+ gradients, because the required uncoupling of ligand and receptor is dependent on a sufficiently acidic pH in a prelysosomal compartment. Agents, such as monensin, methylamine, and bafilomycin A_1, that can increase the pH of acidic compartments, result in increased secretion of newly synthesized acid hydrolases and reduced delivery to the lysosomes. In this study, the presence of monensin, methylamine and bafilomycin A_1 in the culture medium of macrophages exerted the above described effects on the acid hydrolase cathepsin D. The findings for chitotriosidase were in clear contrast. None of the agents promoted its secretion. Apparently, the mechanism involved in sorting of chitotriosidase is far less dependent on low pH. The underlying mechanism has to be fundamentally different, since chitotriosidase does not contain N-linked glycans and consequently lacks mannose-6-phosphate moieties. Chitotriosidase resembles lysozyme in this and other features. Lysozyme is also produced by macrophages, being partly secreted and partly routed to lysosomes. Lysozyme shows a high isoelectric point and lacks N-linked glycans. Its sorting mechanism is unknown. Further studies on the nature of the N-linked-glycan-independent routing of chitotriosidase is warranted. During proteolytic processing of the 50-kDa isoform of chitotriosidase an 11-kDa C-terminal domain is removed to give rise to the 39-kDa isoform of chitotriosidase. On the basis of the existing data, it is not possible to indicate the exact intracellular location where proteolytic processing of chitotriosidase

takes place. It may be that the proteolytic modification is a lysosomal event, or a prelysosomal process.

Based on sequence similarities, the 39-kDa form of chitotriosidase belongs to family 18 of glycosyl-hydrolases [18]. The three-dimensional crystal structures of some members of this protein family have been resolved and were found to have a triose phosphate isomerase (TIM)-barrel structure [19, 20]. On the basis of amino acid sequence similarities, it has been suggested that all members of the family have a similar TIM-barrel structure [21]. Such a structure consists of a cyclic eightfold repeat of a β-strand/loop/α-helix unit. Since 39-kDa chitotriosidase is highly similar to these proteins [5], it is expected to have a similar TIM-barrel structure. The C-terminal 11-kDa domain of chitotriosidase shows sequence similarities with the C-terminal part of chitinases of *Brugia malayi* [22] and *Manduca sexta [23]*. These domains contain conserved cysteine residues that probably form disulfide bridges. It is envisioned that, in analogy to the *B. malayi* chitinase, the C-terminal (11 kDa) domain in the 50-kDa chitotriosidase forms a separate domain that may be removed from the intact form by proteolytic processing. Different electrophoretic mobilities on SDS/PAGE under reducing and non-reducing conditions (data not shown) indicate that the C-terminal and catalytic domains of chitotriosidase are not linked via disulfide bridges. This finding is analogous with results obtained by Fuhrman et al. with V8 (Glu-C) protease proteolytic fragments of the similar *B. malayi* MF1 antigen chitinase [12]. Unfortunately, the 11-kDa fragment could not be visualized in our metabolic-labeling experiments, since the available antibody had been raised against the 39-kDa protein. Like the C-terminal domain in the chitinase of *B. malayi*, this domain in 50-kDa chitotriosidase probably contains O-linked glycans. The variable degree of sialylation of these O-linked glycans causes heterogeneity in the isoelectric point of the 50-kDa isoform, resulting in more acidic forms with increasing amounts of sialic acid in the oligosaccharide chains.

The present study has revealed an important functional difference between the 50-kDa chitotriosidase and the 39-kDa isoform generated by proteolytic processing. Only the 50-kDa enzyme is capable of binding strongly to chitin particles. Apparently, the presence of the C-terminal extension increases markedly the affinity for chitin. It has been reported for some chitinases of other species (such as *Bacillus circulans*, *Streptomyces olivaceoviridis*, *Urtica dioica* and *Saccharomyces cerevisiae*) that, in addition to a catalytic domain, a distinct chitin-binding domain is present [9, 24–26]. One might envision that chitinases such as 50-kDa chitotriosidase bind to chitin via their specific binding domain and subsequently hydrolyze the polysaccharide via their catalytic domain.

The investigation of chitotriosidase in cultured macrophages showed that these cells predominantly produce and secrete 50-kDa chitotriosidase. The 50-kDa chitotriosidase, which is routed to the lysosome, is converted into a 39-kDa protein with a homogeneous pI of 8.0. It is therefore attractive to speculate that the presence of the additional chitin-binding domain in the secretory (50 kDa) chitotriosidase mediates efficient targeting to the substrate. In the lysosomal apparatus, the presence of such a domain is not required since the (39 kDa) enzyme is in close contact with endocytosed substrate. Possibly, a tight binding of enzyme to substrate may even negatively influence the total degradation of the polysaccharide in lysosomes.

On the basis of our findings, the heterogeneity of chitotriosidase present in body fluids and tissues can be understood. Serum contains high levels of the 50-kDa secretory protein [3], whilst in organs, particularly kidney, the intracellular 39-kDa enzyme is also detected. In this connection, our finding concerning intravenously infused human 50-kDa chitotriosidase in the rat are of interest. The administered enzyme was found to be cleared from the circulation with a half-life of 5 min. 1 h after injection, uptake was noted for the liver (10%) and spleen (4%). Most enzyme activity was recovered in the kidney (26%), presumably due to uptake by epithelial cells. Furthermore, it was observed that MDCK cells (kidney epithelial cells) process endocytosed 50-kDa chitotriosidase to the 39-kDa isoform.

In conclusion, post-translational modification of macrophage-derived chitotriosidase, such as proteolytic processing and O-linked glycosylation, contribute to the heterogeneity in enzyme present in tissues and body fluids. Heterogeneity in chitotriosidase can also be generated by alternative splicing of the RNA, although this mechanism is quantitatively of minor importance. It is of great interest to establish the implications of the observed modifications in chitotriosidase with respect to the physiological role of the enzyme.

We like to thank Jessica Teeling for skillful assistance in some of the experiments. Dr M. Bijsterbosch is greatly acknowledged for performing the rat experiments. We like to thank Dr J. van Beeumen and B. Devreese for their help with the electronspray mass spectrometry. We also thank Dr S. van Weely and Prof. Dr J. M. Tager for helpful suggestions and discussion. All the blood donors are kindly acknowledged for their cooperation. Dr J. M. F. G. Aerts is a recipient of research grant 28-23131 from the *Praeventiefonds* (The Netherlands).

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