Human Cathepsin G Lacking Functional Glycosylation Site Is Proteolytically Processed and Targeted for Storage in Granules after Transfection to the Rat Basophilic/Mast Cell Line RBL or the Murine Myeloid Cell Line 32D*

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Daniel Garwicz, Anders Lindmark, and Urban Gullberg‡
From the Division of Hematology, Department of Medicine, Lund University, Lund, Sweden

The neutral protease cathepsin G belongs to a family of hematopoietic serine proteases stored in the azurophil granules of the neutrophil granulocyte. To investigate the function of asparagine-linked carbohydrates in neutrophil serine proteases, we constructed a mutant cDNA, coding for human cathepsin G deficient of a functional glycosylation site, for use in a transgenic cellular model. Wild type and mutant cDNA were stably expressed in the rat basophilic/mast cell line RBL and in the murine myeloblast-like cell line 32D. Biosynthetic labeling, followed by immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and fluorography, showed that carbohydrate-deficient cathepsin G was synthesized as a 29-kDa proform in both cell lines. The proform was proteolytically processed into a stable form with an apparent molecular mass of 27.5 kDa, indicating removal of the carboxy-terminal prodomain. The mutant cathepsin G was enzymatically activated as determined by acquisition of affinity to aprotinin, a serine protease inhibitor. As for wild type cathepsin G, small amounts of the unprocessed form of the mutated enzyme were released from the cells, while the major part was transferred to a granular compartment as demonstrated by subcellular fractionation. Thus, neither processing leading to enzymatic activation nor granular sorting was obviously affected by the lack of oligosaccharides on the mutant cathepsin G. Our results therefore indicate that glycosylation is not essential for these processes. In addition to the previously utilized cell line RBL, we propose the 32D cell line as a suitable cellular model for transgenic expression of human neutrophil serine proteases.

The azurophil granules of neutrophil granulocytes contain lysosomal hydrodases and can therefore be regarded as specialized forms of lysosomes (1, 2). The azurophil granules also store bactericidal proteins which are unique for the myeloid lineage (3-5). Among them are the neutral serine proteases cathepsin G and leukocyte elastase, which belong to a superfamily of hematopoietic serine proteases also including granzymes of cytotoxic T lymphocytes and certain mast cell proteases (6-8). Hematopoietic serine proteases are stored in cytosolic granules as active enzymes but are transiently present as inactivezymogens; activation is likely to follow the post-translational removal of an amino-terminal dipeptide in a pregranular compartment (9-13).

Early processing of lysosomal enzymes involves modifications of carbohydrate side chains with phosphorylation of mannose residues followed by translocation to lysosomes mediated by receptors for mannose 6-phosphate (14-16). In cells from patients with I cell disease phosphotransferase activity is defective, and consequently mannose 6-phosphate cannot be added during processing. Therefore, lysosomal enzymes are constitutively secreted from the cells with resulting intracellular deficiency of most lysosomal enzymes (17). The packaging of serine proteases into azurophil granules is different from that of typical lysosomal enzymes and seems to be consistently independent of the mannose 6-phosphate receptors (18). Specific sorting mechanisms involved in transfer of proteins for storage in azurophil granules have not been identified. Human cathepsin G contains one consensus site for asparagine-linked glycosylation (Asn64, numeration according to Ref. 19). Accordingly, biochemical characterization of cathepsin G has demonstrated asparagine-linked carbohydrates which are processed into complex forms (20). Similarly, the other members of the neutrophil serine protease family, i.e. leukocyte elastase, proteinase 3, and azurocidin, also contain asparagine-linked carbohydrates (20-23). In general, functions of asparagine-linked oligosaccharides include effects on folding and conformation as well as a role in recognition events (24, 25). For example, glycosylation of the lysosomal enzyme β-glucosidase is required for catalytic activity (26), and carbohydrates of the cysteine protease propapain A are important for subcellular transport and secretion of the enzyme (27). However, a functional role for the carbohydrates of the serine proteases of the azurophil granules has not been demonstrated.

The aim of this work was to determine whether the asparagine-linked oligosaccharide has a critical role for folding, processing, or granular targeting of cathepsin G. We have recently used the rat basophilic leukemia cell line RBL-1 as a transgenic model for studying the processing of transfected human cathepsin G and leukocyte elastase (28, 29). Therefore, we have utilized site-directed mutagenesis for elimination of the glycosylation site by substitution of Asn64 with Gln followed by transfection of the mutated cDNA to RBL cells in order to investigate the consequences of absent glycosylation. To further strengthen our data, we have extended the studies by transfecting wild type and mutated cathepsin G to murine myeloid 32D cells. Our results demonstrate that, after transfection to RBL or 32D cells, cathepsin G lacking asparagine-
linked carbohydrates is expressed as a proform which is processed into active enzyme. The oligosaccharide-deficient protein is also targeted for granular storage, thus questioning the importance of asparagine-linked carbohydrates of cathepsin G.

**EXPERIMENTAL PROCEDURES**

Materials—The eukaryotic expression vector pCDNA3 was from Invitrogen, British Biotechnology, Oxford, UK. The vector provides a cytomegalovirus promoter-driven expression of introduced cDNA. The plasmid also confers resistance to geneticin, allowing selection of recombinant cell lines. The pBluescript plasmid was from Stratagene, La Jolla, CA. [35S]Methionine and [35S]cysteine (cell labeling grade) was from Amersham, Amersham, UK. Percoll and protein A-Sepharose CL-4B were from Pharmacia, Uppsala, Sweden. Protein G-Sepharose and aprotinin-agarose were from Sigma. Geneticin was from Boehringer Mannheim.

cDNA, Mutagenesis and Construction of Expression Vector—For site-directed mutagenesis cDNA of human cathepsin G (generously provided by Dr. G. Selvesca, Duke University, Durham, NC) was used as template in a two-step “spliced overhang extension” polymerase chain reaction (PCR) (30) in the following way. In the first reaction two separate amplifications with 100 ng of DNA template in 10-cycle PCRs produced two fragments of cathepsin G cDNA overlapping the glycosylation-site (Asn47). By design of the primers, Asn47 was replaced with glutamine and the Kozak consensus leader sequence for maximum translational efficiency (31) was introduced 5’ to the ATG initiation codon, and the flanking restriction enzyme sites KpnI and NotI were included for subsequent cloning into plasmid. The PCR primers in the two amplifications were upstream 5’-GATTCCAGTACCCCGGCGACATG (no. 1) plus downstream 5’-TAGGGTACCTGAGGCTGTACG-3’ (no. 2) and upstream 5’-AGCACATGAGGATCTGCTGCTGG-3’ (no. 1) plus downstream 5’-CAGCCACTCCTGCTTCTGCTGG-3’ (no. 4), respectively (start and stop codons in bold, restriction enzyme sites underlined, Gln46 in italics). The PCR products were isolated on agarose gel, mixed, and subjected to a second 20-cycle splicing PCR amplification with primer nos. 1 and 4, thus creating full-length cathepsin G cDNA with Gln replacing Asn47. The resulting PCR product was digested by KpnI and NotI, followed by isolation on an agarose gel and cloning into pBluescript plasmid. Individual clones were isolated and sequenced to verify the mutation and the integrity of the reading frame. cDNA with correct sequence was cloned into pCDNA3 to create the expression vector pCDNA3/cathepsinG/Gln46. All PCRs were performed in a Perkin-Elmer 480 Thermal Cycler using ULMa polymerase (Perkin-Elmer) according to the manufacturer’s instructions. The expression vector pCDNA3/CatG, encoding the expression of wild type protein, was analogously created using a single PCR amplification with primer nos. 1 and 4.

Cell Culture—The rat basophilic leukemia cell line RBL-1 (32) was a generous gift from Dr. L. Heldman, Uppsala, Sweden. The cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Inc.) (complete medium). 32D cells (33), kindly provided by Dr. G. Rovera (Philadelphia, PA), were grown in complete medium consisting of Iscove’s modified Dulbecco’s medium supplemented with 10% heat-inactivated fetal calf serum and 20–30% WEHI-conditioned medium as a source of interleukin 3 (34). The cell cultures were kept in 5% CO2 at 37°C in a fully humidified atmosphere. Exponentially growing cells were used in all experiments.

Transfection Procedure—RBL cells were transfected using the Bio-Rad Electroporation Apparatus (Bio-Rad) with electrical settings of 960 μF and 300 V as described previously (28). After electroporation, genetically unaltered cells were selected for recombinant clones expressing the geneticin-resistant gene of pCDNA3. Similarly, transfected 32D cells were selected in the presence of 1 mg/ml geneticin. Individual clones growing in the presence of antibiotic were isolated, expanded into mass cultures, and screened for expression of cathepsin G by biosynthetic labeling. Clones with the most pronounced expression were chosen for further experiments.

Biosynthetic Labeling—Biosynthetic labeling of newly synthesized proteins was performed as described previously (28). Unless otherwise indicated, cells were starved for 30 min followed by pulse labeling with [35S]methionine and [35S]cysteine for 30 min. In chase experiments, following pulse labeling, cells were resuspended in complete medium. At timed intervals, cells were withdrawn and subjected to extraction of whole cells or homogenization and subsequent subcellular fractionation.

Subcellular Fractionation—Subcellular fractionation was performed as described previously (28). Briefly, the cell homogenate was fractionated in a Percoll density gradient after which nine fractions were collected with all cytosol in fraction no. 9. The distribution of lysosomes and Golgi elements in the density gradient was determined by assaying β-hexosaminidase and galactosyl transferase as described elsewhere (35, 36). Peak activities of β-hexosaminidase and galactosyl transferase in subcellular fractions from RBL cells were localized in fractions 1–2 and 5–8, respectively, and from 32D cells in fractions 2 and 6 (data not shown).

Immunoprecipitation—For immunoprecipitation, whole cells or Percoll-containing subcellular fractions were solubilized, and biosynthetically labeled cathepsin G was immunoprecipitated and subjected to electrophoretic analysis (SDS-PAGE) followed by fluorography as described previously (28).

Adsorption to Aprotinin-Agarose—Adsorption to aprotinin-agarose was performed essentially as described by Salvesen and Enghild (9) and Gullberg et al. (28). Briefly, cells were lysed, and the lysate was allowed to react with a suspension of aprotinin-agarose. The aprotinin with bound material was washed followed by incubation with elution buffer to obtain release of bound material. Cathepsin G in the eluate or remaining in the lysate after adsorption to aprotinin-agarose (lacking affinity to aprotinin) was immunoprecipitated and subjected to SDS-PAGE and fluorography.

**RESULTS**

The Proform of Cathepsin G in RBL Cell Lysates—Proteolytically Processed—An expression vector for human cathepsin G lacking glycosylation site (pCDNA3/cathepsin G/Gln46) was prepared by site-directed mutagenesis as described under “Experimental Procedures.” The mutated cDNA (pCDNA3/cathepsin G/Gln46) was transfected to RBL cells, and the stable expression was investigated by biosynthetic labeling followed by immunoprecipitation, SDS-PAGE, and fluorography. Fig. 1 shows biosynthesis and processing of cathepsin G (Gln46) in RBL cells (RBL/CatG-Gln46 cells), demonstrating a proform of 29 kDa. This should be compared to the earlier demonstrated proform of 32.5 kDa of wild type cathepsin G transfected to RBL cells (28). The difference in apparent mo-
lecular mass (3.5 kDa) between mutant and wild type cathepsin G corresponds well to the amount of asparagine-linked carbohydrates of transgenic cathepsin G in RBL cells, thus confirming the absence of carbohydrates on cathepsin G/Gln64.

Following translation, the proform of cathepsin G/Gln64 was converted into a 27.5-kDa form which was stable during 4 h of chase (Fig. 1). This reduction of molecular mass most likely represents carboxy-terminal proteolytic processing, as demonstrated for endogenous and transgenic cathepsin G and leukocyte elastase (9, 28, 29). No further processing was evident during the time of the experiment. Thus the absence of carbohydrates on cathepsin G apparently did not affect proteolytic processing or the stability of the protein. Small amounts of the unprocessed proforms of both mutant, hardly visible in Fig. 1, and of wild type cathepsin G (data not shown) were released into the medium, as commonly seen with lysosomal enzymes (17). No obvious difference between the extracellular release of cathepsin G/Gln64 and wild type cathepsin G was evident.

Cathepsin G/Gln64 Transfected to RBL Cells Is Enzymatically Activated—The enzymatic activation of cathepsin G follows the removal of an amino-terminal dipeptide and the acquisition of catalytic activity is paralleled by the appearance of affinity to the serine protease inhibitor aprotinin (9, 28, 29). To determine whether the lack of carbohydrates on cathepsin G/Gln64 interferes with enzymatic activation, we utilized pulse-chase experiments followed by adsorption to aprotinin-agarose. Newly synthesized procathepsin G/Gln64 (29 kDa) did not reveal any affinity to aprotinin (Fig. 2), indicating the absence of enzymatic activity. After 30 min of chase, however, small amounts of the processed enzyme (27.5 kDa) did bind to the protease inhibitor, suggesting that enzymatic activation of the proenzyme had been initiated. After 5 h of chase, roughly half of the labeled enzyme showed an affinity for aprotinin (Fig. 2). These results are analogous to those from experiments with wild type cathepsin G and leukocyte elastase (28, 29), indicating that the asparagine-linked glycosylation of cathepsin G is nonessential for enzymatic activation.

Cathepsin G/Gln64 Transfected to RBL Cells Is Translocated to Granules—Endogenous cathepsin G and leukocyte elastase are targeted for storage in the azurophil granules of the neutrophil granulocyte. Similarly, cathepsin G and leukocyte elastase transfected to RBL cells are translocated to a granular compartment (28, 29). The mechanisms by which subcellular sorting is mediated are, however, unknown. Consequently it is unclear whether the asparagine-linked carbohydrates of cathepsin G are necessary for transport to granules. Therefore, pulse-chase labeling experiments, followed by subcellular fractionation of RBL/CatG-Gln64 cells, were performed. Fig. 3 shows that labeled cathepsin G/Gln64 was transferred, within 90 min, to dense fractions corresponding to lysosomes, as judged by the lysosomal marker b-hexosaminidase. After 5 h of chase, most of the labeled cathepsin G/Gln64 was found in the two fractions of highest density. Concurrently with transfer to the high density compartment, processing of the 29-kDa proform into the mature 27.5-kDa form was seen (Fig. 3). Thus, as for the wild type enzyme (28), transfected cathepsin G/Gln64 is efficiently translocated to dense fractions in RBL cells, indicating that carbohydrates are not necessary for sorting to granules in these cells.

Mutant and Wild Type Cathepsin G Transfected to 32D Cells Are Processed and Sorted to Granules—Transfected cathepsin G and leukocyte elastase are adequately processed, enzymatically activated and translocated to granules in RBL cells (28, 29). The sorting mechanisms in the basophilic/mast cell line RBL may, however, not be identical to those of the myeloid promyelocyte. In order to use a myeloid cellular model, we therefore adopted the murine myeloblast-like cell line 32D (33, 34). cDNA of wild type cathepsin G and cathepsin G/Gln64 was transfected to 32D cells and biosynthetic labeling of the proteins was performed. Fig. 4 shows processing of wild type cathepsin G (Fig. 4A) and cathepsin G/Gln64 (Fig. 4B) in 32D cells. As seen with RBL cells, proforms of 32.5 and 29 kDa, respectively, were processed into products with molecular masses of 31 and 27.5 kDa. The 31-kDa form of wild type cathepsin G was to a minor extent further processed into a 30 kDa form, which is analogous to what is seen in RBL cells (28).
Small amounts of the proforms were released into the extracellular medium with no obvious difference between wild type and mutant cathepsin G. Targeting of wild type cathepsin G and cathepsin G/Gln64 to granules in 32D cells was investigated by use of subcellular fractionation. Fig. 5 shows translocation of processed forms of cathepsin G (Fig. 5A) and cathepsin G/Gln64 (Fig. 5B) to dense fractions. No obvious difference between the subcellular transfer of wild type cathepsin G and that of cathepsin G/Gln64 was evident. These results from myeloblast-like cells further support the notion that glycosylation of cathepsin G is not obligatory for synthesis, stability, processing or granular sorting of the enzyme.

DISCUSSION

The aim of the present work was to investigate the role of asparagine-linked carbohydrates in the cellular processing of cathepsin G. For this purpose we utilized site-directed mutagenesis to create a mutant form of cathepsin G, lacking a functional glycosylation site. Mutant or wild type cDNA was transfected into target cells and the processing of wild type and mutant protein was compared. Our results demonstrate that the mutant, carbohydrate-deficient, form of cathepsin G was synthesized in RBL and 32D cells as a proform with apparent molecular mass of 29 kDa. This is close to the calculated molecular mass of the protein core (26.9 kDa) and consistent with the previous characterization of wild type cathepsin G in RBL cells, which showed an apparent proform of 32.5 kDa, containing asparagine-linked oligosaccharides of approximately 3.5 kDa (28). The proform of the carbohydrate-deficient mutant was processed into a 27.5-kDa form, indicating proteolytic removal of a 1.5-kDa prodomain, again similar to the processing of the wild type protein. The transfer of mutant cathepsin G to a granular compartment in RBL and 32D cells, as demonstrated by subcellular fractionation experiments, was not obviously

**FIG. 4. Processing of transgenic cathepsin G and cathepsin G/Gln64 in 32D cells.** A, 32D/cathepsin G cells and B, 32D/cathepsin G/Gln64 cells were pulse-labeled with [35S]methionine/[35S]cysteine for 30 min followed by chase for up to 4 h. At indicated points, 20 x 10^6 cells were withdrawn and subjected to solubilization, immunoprecipitation, and analyses as described in the legend to Fig. 1. The fluorograms were exposed for 7 days. The positions of the proforms of 32.5 and 29 kDa, respectively, and the processing forms are indicated with arrows to the right.

**FIG. 5. Granular targeting of transgenic cathepsin G and cathepsin G/Gln64 in 32D cells.** A, 32D/cathepsin G cells and B, 32D/cathepsin G/Gln64 cells were pulse-labeled for 30 min, followed by chase for 90 min and 5 h. At times indicated, 100 x 10^6 cells were homogenized after which subcellular fractionation, immunoprecipitation, and subsequent analyses were performed as described in the legends to Figs. 1 and 3. The fluorograms were exposed for 7 days. The positions of the proforms of 32.5 and 29 kDa, respectively, and the processing forms are indicated with arrows to the right.
affected by the lack of oligosaccharides. We also demonstrated that mutant cathepsin G was enzymatically activated in RBL cells, as judged by the acquisition of affinity to aprotinin, which indicates that carbohydrates are dispensable for efficient processing of cathepsin G.

A minor portion of the mature forms of wild type cathepsin G and elastase in RBL cells is slowly processed with further reduction in molecular mass of approximately 1 kDa (28, 29). This late process occurring at a point of time when transfer to granules is accomplished, was also visible when wild type cathepsin G was expressed in 32D cells, seen as a tight double band after four hours of chase (Fig. 4A) and could involve further proteolytic processing or late trimming of carbohydrates. However, contrary to what was seen with wild type cathepsin G, the mutant form of cathepsin G (cathepsin G/Gln64) did not show any late reduction in molecular mass, whether expressed in RBL or 32D cells (Figs. 1 and 4B). Our results therefore indicate that the late processing of wild type cathepsin G is the result of trimming of carbohydrates in granular structures rather than further proteolytic processing. Different structures of the sugar chains of cathepsin G and leukocyte elastase in neutrophil granulocytes have been reported (37); typical biantennary chains, commonly found on secreted glycoproteins (38), were present in a small fraction of the enzymes, while the major forms had truncated oligosaccharide chains. Since similar short structures of sugar chains are found on several lysosomal enzymes, it was hypothesized that the biantennary chain-containing cathepsin G and leukocyte elastase constitute a minor fraction of the enzymes, processed in a separate pathway, with specific functions involving extracellular secretion (37). However, our results which indicate that late trimming of the carbohydrates is possible in the milieu of the maturing granules, rather support the alternative interpretation that the truncated forms arise by degradation of the complex chains by glycosidases present in the granules.

A major concern when studying transgenically expressed proteins, is the relevance of the target cell chosen for transfection. The granules of the basophilic/mast cell like RBL cell line contain lysosomal enzymes and mast cell serine proteases (32, 39, 40) and transfected cathepsin G and leukocyte elastase are adequately processed in this cell line (28, 29). However, mechanisms for granular targeting in RBL cells could be different from those of promyelocyte-like cells, in which cathepsin G and leukocyte elastase are normally formed and stored. Therefore, we extended our study of processing and granular targeting of carbohydrate-deficient cathepsin G (cathepsin G/Gln64) to include myeloblastic 32D cells. The 32D cell line has successfully been employed for transfection of defenses, another component of azurophil granules (41). Similar to the results from RBL cells, a stable expression of cathepsin G and cathepsin G/Gln64 was seen in 32D cells. Furthermore, processing of the proforms and targeting of processed forms to granules were also distinguishable from what was seen in RBL cells. Thus also in myeloblast-like cells, the carbohydrates of cathepsin G do not seem to be important for stability or subcellular transport.

Most lysosomal hydrolases carry phosphorylated mannose residues enabling a mannose 6-phosphate receptor-mediated transfer to a prelysosomal compartment (14–16). The sorting of cathepsin G and leukocyte elastase, on the other hand, seems to be independent of the mannose 6-phosphate receptors (17). Alternative targeting mechanisms to azurophil granules have not been demonstrated. A transient mannose 6-phosphate-independent membrane association can possibly be involved in the subcellular trafficking of some lysosomal enzymes (42–48). This binding to membranes in the endoplasmic reticulum or the Golgi apparatus in some cases involves homodimeric proteins. Similar mechanisms could play a role in the sorting of cathepsin G and leukocyte elastase as both proteins exhibit C-terminal extensions which are proteolytically removed after transfer to granules (7, 9, 28, 29). However, previous results from experiments with deletion mutants indicate that transfer to granules occurs independently of the C-terminal prodomain of cathepsin G and leukocyte elastase (29). Thus data argue against an important function of the carboxyl-terminal prodomains in sorting of these enzymes. Similarly, the present results indicate that the oligosaccharide structures are also dispensable for granular targeting of cathepsin G. The sorting mechanisms for these hemopoietic serine proteases therefore remain obscure.

In conclusion, the present investigation has demonstrated that cathepsin G lacking a functional glycosylation site can be stably expressed in both rat basophilic RBL cells and murine myeloid 32D cells. The absence of carbohydrates did not have any obvious consequence for stability, processing, activation, or granular targeting of the protein, thus strongly arguing against an important role for carbohydrates of cathepsin G in these processes.

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