Accumulation of Sialic Acid in Endocytic Compartments Interferes with the Formation of Mature Lysosomes

IMPAIRED PROTEOLYTIC PROCESSING OF CATHEPSIN B IN FIBROBLASTS OF PATIENTS WITH LYOSOMAL SIALIC ACID STORAGE DISEASE*

(Received for publication, November 30, 1998, and in revised form, April 20, 1999)

Johannes A. Schmid‡§, Lukas Mach‡, Eduard Paschke**, and Josef Glössl‡

From the ‡Centre of Applied Genetics, University of Agricultural Sciences, Vienna A-1190, the §Department of Vascular Biology and Thrombosis Research, University of Vienna, Vienna A-1235, Austria, the ¶Biochemistry Department, University of Western Australia, Nedlands W.A. 6907, Australia, and **Children’s Hospital, Graz A-8036, Austria

The impact of an altered endocytic environment on the biogenesis of lysosomes was studied in fibroblasts of patients suffering from sialic acid storage disease (SASD). This inherited disorder is characterized by the accumulation of acidic monosaccharides in lysosomal compartments and a concomitant decrease of their buoyant density. We demonstrate that C-terminal trimming of the lysosomal cysteine proteinase cathepsin B is inhibited in SASD fibroblasts. This late event in the biosynthesis of cathepsin B normally takes place in mature lysosomes, suggesting an impaired biogenesis of these organelles in SASD cells. When normal fibroblasts are loaded with sucrose, which inhibits transport from late endosomes to lysosomes, C-terminal cathepsin B processing is prevented to the same extent. Further characterization of the terminal endocytic compartments of SASD cells revealed properties usually associated with late endosomes/prelysosomes. In addition to a decreased buoyant density, SASD “lysosomes” show a reduced acidification capacity and appear smaller than their normal counterparts. We conclude that the accumulation of small non-diffusible compounds within endocytic compartments interferes with the formation of mature lysosomes and that the acidic environment of the latter organelles is a prerequisite for C-terminal processing of lysosomal hydrolases.

The functional role of lysosomes in mammalian cells is not yet fully understood, although some unique features clearly distinguish them from other intracellular compartments. Dense, or secondary, lysosomes receive their structural and functional components from other organelles involved in the endocytic and biosynthetic pathways and, therefore, share some but not all characteristics with pre-lysosomal compartments. Lysosomes have been originally defined as terminal degradative compartments, since macromolecules, once transferred into these organelles, are usually not released in the intact state (1). However, it has been recently questioned whether lysosomes really act as final traps in the endocytic pathway, since some substances apparently escape from this location via retrograde transport systems (2, 3). Moreover, degradative processes are at least initiated already in endosomes that have been shown to contain substantial amounts of some lysosomal hydrolases (4). Indeed, current studies suggest that proteolytic digestion of endocytosed substances occurs in endosomes rather than in secondary lysosomes (5).

The biogenesis of lysosomes involves the biosynthesis and the transport of acidic hydrolases to these organelles as well as the formation of the lysosomal membranes. Intracellular trafficking of soluble lysosomal enzymes, which constitute the majority of hydrolases within these compartments, is mediated by mannose 6-phosphate receptors (1). Lysosomal membrane glycoproteins are retrieved from the trans-Golgi network (TGN)1 or the plasma membrane by mechanisms involving the interaction of a polypeptide motif in their cytoplasmic domains with cytosolic receptors (6). Although the transport of membrane proteins and soluble enzymes implies some membrane flow from the TGN to lysosomes, an important portion of the lysosomal membrane originates from the plasma membrane after endocytosis. Two different models have been proposed to explain the delivery of endocytosed ligands and membrane components from the plasma membrane to late locations in the endocytic pathway. Experimental support has been provided for vesicular transport events between pre-existing endocytic compartments (7–10) as well as for the maturation hypothesis, which postulates that plasma membrane-derived vesicles gradually convert into late endosomes (11–15). Many of the conflicts on the relative contribution of the two concepts for transport from the plasma membrane to late endosomes have now been resolved, and it is generally accepted that the mode of endocytic membrane traffic may vary between individual cell types (16).

Remarkably less knowledge is available on the molecular mechanisms underlying the transfer of internalized substances from late endosomes to lysosomes. As in the case of the passage from early to late endosomes, this process could be either mediated by maturation and/or fusion events. Initially, any involvement of fusion processes appeared unlikely since dense lysosomes and late endosomes display quite substantial differences in the composition of their membranes. However, content mixing by fusion between endosomes and lysosomes has now been demonstrated both in vitro (17) and in vivo (15). These findings would be in good agreement with a recently postulated

---

* This work was supported by the Austrian Science Foundation Projects P-6574 and P-8435-Med and by the “Hochschuljubiläumsstiftung” of the City of Vienna. The costs of publication of this article were therefore be hereby marked “advertisement”.

1 The abbreviations used are: TGN, trans-Golgi network; CCCP, carbonyl cyanide chlorophenylhydrazone; FITC, fluorescein isothiocyanate; ISSD, infantile sialic acid storage disease; MPR300, 300-kDa mannose 6-phosphate receptor; SASD, sialic acid storage disease; PAGE, polyacrylamide gel electrophoresis; TRITC, tetramethylrhodamine isothiocyanate; Z-Arg-Arg-NHNap, benzoylcarbonyl-arginyl-arginine-β-naphthylamide.
model, where lysosomes transiently fuse with endosomes thereby transferring luminal molecules without significant mixing of the surrounding membranes ("kiss and run model") (18). Nevertheless, we still lack information on important aspects of the formation of dense lysosomes such as the mechanism(s) leading to their inherent high buoyant density. It has been suggested that this unique feature is due to osmotic shrinking and depends on aggregation of the luminal contents upon acidification (19). Inversely, internalization of indigestible small compounds induces osmotic swelling of lysosomes (20).

We have now addressed the impact of an altered endocytic environment on the function and the biogenesis of these organelles in fibroblasts from patients suffering from sialic acid storage disease (SASD). This inherited disorder is characterized by an accumulation of free sialic acid in compartments of the lysosomal pathway. It occurs in a mild form called Salla disease (21) and a more severe variant termed infantile sialic acid storage disease (SSSD; see Ref. 22), with a close correlation between the intracellular sialic acid concentration and the severity of the disease. SASD is caused by negligible egress of sialic acid and other acidic monosaccharides from (pre)lysosomal compartments due to a defective carrier protein, with a concomitant decrease in the buoyant density of the affected organelles (23–25). However, it remains to be established as to how this phenotype results in impaired lysosomal functions in SASD cells.

The proteolytic maturation of the lysosomal marker enzyme β-N-acetylgalactosaminidase is incomplete in SASD fibroblasts, although this particular hydrolase does not depend on processing to exhibit its enzymatic activity (26). In the present study, we have investigated the biosynthesis of the cysteine proteinase cathepsin B in SASD cells, since this lysosomal enzyme undergoes a series of well-established proteolytic processing steps during maturation that are required to generate its proteolytic activity (27). Our results demonstrate that C-terminal processing of cathepsin B is prevented in SASD fibroblasts, apparently due to inhibition of the exopeptidase activity of the enzyme itself. Inhibition of C-terminal cathepsin B processing can be induced in normal fibroblasts by loading with the non-degradable disaccharide sucrose, which inhibits the delivery of internalized substances from late endosomes to lysosomes. Since the terminal endocytic compartments of SASD cells exhibit some characteristics of endosomes, we propose that in general the accumulation of small non-diffusible molecules in endocytic compartments prevents the formation of mature dense lysosomes and hence impairs the functions of the lysosomal system.

### EXPERIMENTAL PROCEDURES

#### Materials—
The human skin fibroblast lines GM 5520 (SSSD-1), GM 8496 (Salla-1), GM 5521, and GM 5522 (from the unaffected parents of three different SASD patients) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The cell lines B.B. (SSSD-2) and F 8471 (Salla-2) as well as normal fibroblasts (control) were established at the Children’s Hospital, Graz, Austria. All fibroblasts were maintained in continuous culture essentially as described (28).

**Materials—**The human skin fibroblast lines GM 5520 (SSSD-1), GM 8496 (Salla-1), GM 5521, and GM 5522 (from the unaffected parents of GM 5520, controls 1 and 2, respectively) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The cell lines B.B. (SSSD-2) and F 8471 (Salla-2) as well as normal fibroblasts (control) were established at the Children’s Hospital, Graz, Austria. All fibroblasts were maintained in continuous culture essentially as described (28). **Materials—**The human skin fibroblast lines GM 5520 (SSSD-1), GM 8496 (Salla-1), GM 5521, and GM 5522 (from the unaffected parents of GM 5520, controls 1 and 2, respectively) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The cell lines B.B. (SSSD-2) and F 8471 (Salla-2) as well as normal fibroblasts (control) were established at the Children’s Hospital, Graz, Austria. All fibroblasts were maintained in continuous culture essentially as described (28). **Materials—**The human skin fibroblast lines GM 5520 (SSSD-1), GM 8496 (Salla-1), GM 5521, and GM 5522 (from the unaffected parents of GM 5520, controls 1 and 2, respectively) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The cell lines B.B. (SSSD-2) and F 8471 (Salla-2) as well as normal fibroblasts (control) were established at the Children’s Hospital, Graz, Austria. All fibroblasts were maintained in continuous culture essentially as described (28). **Materials—**The human skin fibroblast lines GM 5520 (SSSD-1), GM 8496 (Salla-1), GM 5521, and GM 5522 (from the unaffected parents of GM 5520, controls 1 and 2, respectively) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The cell lines B.B. (SSSD-2) and F 8471 (Salla-2) as well as normal fibroblasts (control) were established at the Children’s Hospital, Graz, Austria. All fibroblasts were maintained in continuous culture essentially as described (28). **Materials—**The human skin fibroblast lines GM 5520 (SSSD-1), GM 8496 (Salla-1), GM 5521, and GM 5522 (from the unaffected parents of GM 5520, controls 1 and 2, respectively) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The cell lines B.B. (SSSD-2) and F 8471 (Salla-2) as well as normal fibroblasts (control) were established at the Children’s Hospital, Graz, Austria. All fibroblasts were maintained in continuous culture essentially as described (28).
ol (5 min) and acetone (2 min). The samples were then incubated with rabbit antibodies to cathepsin B, its C-terminal extension, MPR300, rab7, or IgpL10. Non-immune rabbit immunoglobulins were used as a negative control. In single labeling experiments, bound primary antibodies were visualized with FITC-conjugated swine anti-rabbit IgG immunoglobulins. When the respective cells had been preloaded with FITC-dextran, TRITC-labeled secondary antibodies were used for immunostaining. The immunostained cells were examined using Zeiss Axiosvert or Olympus AH-2 microscopes with the appropriate filter combinations.

Digital Image Analysis of Terminal Endocytic Compartments—To determine the size of the terminal endocytic compartments, fibroblasts were labeled with FITC-dextran as described above, fixed with 3% paraformaldehyde in phosphate-buffered saline, and examined by fluorescence microscopy. The digital images thus obtained were analyzed using VIDAS 2.1 software (Kontron, Germany). The circle equivalent diameter of the labeled compartments was determined according to Equation 1,

\[ d = 2 \sqrt{A/\pi} \]  

where \( A \) is area of the labeled compartment in \( \mu \text{m}^2 \).

Immunoblotting—Cell homogenates from SASD and normal fibroblasts (50 \( \mu \text{g} \) of total cellular protein) were subjected to SDS-PAGE and Western blotting as described earlier (29). The membranes were probed with affinity purified rabbit antibodies to human cathepsin B or its C-terminal extension and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG immunoglobulins. When the respective cells had been preloaded with FITC-dextran, TRITC-labeled secondary antibodies were used for immunostaining. The immunostained cells were examined using Zeiss Axiosvert or Olympus AH-2 microscopes with the appropriate filter combinations.

Our previous studies indicated that the carbohydrate moiety of cathepsin B is partially cleaved at a later stage into the two-chain enzyme (28). Both proteolytic processing steps appear to rely primarily on the action of cathepsin B itself (29, 30).

As in control cells, the first detectable form of newly synthesized cathepsin B in SASD fibroblasts was the 45-kDa proteinase which is targeted to lysosomes via mannose 6-phosphate receptors, with only a minor fraction being secreted. Upon delivery to the lysosomal pathway, procathepsin B is initially converted into the single-chain 33-kDa form of the mature proteinase, which is partially cleaved at a later stage into the two-chain enzyme consisting of subunits of 27 kDa (heavy chain) and 5 kDa (light chain; Fig. 1 (28)). Both proteolytic processing steps appear to rely primarily on the action of cathepsin B itself (29, 30).

As in control cells, the first detectable form of newly synthesized cathepsin B in SASD fibroblasts was the 45-kDa proteinase, which was partially secreted into the culture medium and exhibited the same apparent molecular mass as its normal counterpart. However, the fully processed mature form of cathepsin B appeared to be larger in SASD cells than in normal fibroblasts. Most strikingly, the heavy chain of mature two-chain cathepsin B exhibited a molecular mass of 28 kDa in SASD cells, whereas a 27-kDa polypeptide was observed in normal fibroblasts. In contrast, the electrophoretic mobility of the single-chain enzyme was apparently not affected, but any small difference between the SASD and the normal forms would be masked by the consistently rather diffuse nature of the 33-kDa bands. Furthermore, it should be pointed out that proteolytic conversion of single-chain into two-chain cathepsin B was significantly retarded in SASD fibroblasts (Fig. 1 and Table 1).

Our previous studies indicated that the carbohydrate moiety frequently accounts for cell type-specific cathepsin B forms (29). However, enzymatic deglycosylation of the immunoprecipitates with peptide N-glycosidase F prior to SDS-PAGE analysis did not eliminate the difference in the electrophoretic mobility between the heavy chains of the SASD and normal enzymes, although removal of the attached oligosaccharides shifted the apparent molecular masses of the large subunits to 25 and 24 kDa, respectively (data not shown). Altogether, these results rule out that a different N-glycosylation pattern causes the observed discrepancy between the molecular forms of cathepsin B in SASD and normal fibroblasts and suggest that the proteolytic maturation of this proteinase is not complete in the former cells.
the presence of 10 mM ammonium chloride to stimulate the secretion of lysosomal enzymes. The conditioned donor media were offered to the indicated recipient cell lines (ISSD, B.B.; Salla, F8471; control, Kl.) for receptor-mediated endocytosis over a period of 48 h. Cathepsin B was immunoprecipitated from recipient cell extracts and analyzed as described in the legend of Fig. 1.

The SASD-specific Forms of Cathepsin B Are Not Due to a Modified Precursor—To assess the impact of the proenzyme itself on the altered maturation of cathepsin B in SASD cells, metabolically labeled procathepsin B secreted by normal fibroblasts was offered to unlabeled SASD and normal recipient cells for receptor-mediated endocytosis. Exogenous radiolabeled procathepsin B was taken up in a mannose 6-phosphate-dependent manner (results not shown (29)) and processed into mature forms indistinguishable from the respective endogenous enzymes, demonstrating that the 28-kDa heavy chain typically associated with SASD cells can be generated from the normal proenzyme. Analogous incubation of normal fibroblasts with procathepsin B secreted by labeled SASD cells confirmed the independence of the processing products from the source of the cathepsin B precursor (Fig. 2), emphasizing the unique phenotype of cathepsin B processing in SASD fibroblasts and indicating that the defect in the maturation of this enzyme occurs both in the endocytic and biosynthetic pathways. However, it should be noted that proteolytic processing of endocytosed procathepsin B progressed slower in SASD cells than in normal fibroblasts as already observed for the biosynthetic route and that less procathepsin B was endocytosed (Fig. 2 and Table I), supporting the idea that SASD-specific cathepsin B forms are created at a late stage in the biosynthesis of the enzyme.

Stage in the Biosynthesis of the Enzyme—To assess whether early events in (pro)cathepsin B processing were affected in SASD cells, both normal and SASD fibroblasts were metabolically labeled in the presence of the protonophore CCCP, which prevents the transport of lysosomal enzymes from secretory proteins in distal Golgi areas (41). When the biosynthesis of cathepsin B was studied in cells maintained at 19 °C, any SASD-specific forms of the proteinase are produced in the endoplasmic reticulum to the Golgi apparatus (40). When intracellular trafficking to the Golgi apparatus was thus inhibited, proteolytic processing of newly synthesized procathepsin B was completely prevented. Both the SASD proenzymes and their normal counterparts migrated under these conditions as diffuse 45-kDa bands. Removal of N-linked oligosaccharides with peptide N-glycosidase F converted each cathepsin B precursor species into a discrete 39-kDa polypeptide without any pronounced difference between SASD and normal cells (Fig. 3a). Similar results were obtained when the respective cell lines were pulse-labeled with [35S]methionine for 10 min and immediately subjected to immunoprecipitation analysis (data not shown), thus unanimously ruling out that the SASD-specific forms of the proteinase are produced in the endoplasmic reticulum. The ionophore monensin, which dissipates Na⁺ and H⁺ gradients across intracellular membranes, inhibits the segregation of lysosomal enzymes from secretory proteins in distal Golgi areas (41). When SASD and control fibroblasts were treated with this drug, procathepsin B processing was affected in a similar manner as upon the addition of CCCP (not shown).

Incubation of mammalian cells at reduced temperatures has been reported to abolish vesicular trafficking events. At 18–20 °C, the exit of lysosomal enzymes from the trans-Golgi network (TGN) is hindered (42, 43). When the biosynthesis of cathepsin B was studied in cells maintained at 19 °C, any proteolytic processing of the 45-kDa proenzyme was prevented...
in both SASD and normal fibroblasts. Concomitantly, residual procathepsin B secretion into the medium was slightly reduced (Fig. 3b). Decreased temperature also exerts effects on the endocytic route, since exogenously added markers endocytosed at 18–20 °C accumulate in endocytic compartments and fail to reach late prelysosomal compartments or lysosomes (42, 44). When radiolabeled procathepsin B was added to unlabeled recipient cells at 19 °C, the heterologous proenzyme was internalized and almost quantitatively converted into the mature 33-kDa single-chain enzyme. The apparent molecular mass of this polypeptide appeared to be the same in SASD and control samples. Further processing of endocytosed single-chain cathepsin B into the two-chain form was completely inhibited at 19 °C, even after incubation for 48 h, in both SASD and normal fibroblasts (Fig. 3c). These results imply that the processing step that is impaired in SASD cells takes place in a post-Golgi compartment after convergence of the endocytic and biosynthetic pathways at a late endosomal or lysosomal stage where single-chain cathepsin B has already been converted into the two-chain form.

The C-terminal Extension of Cathepsin B Is Not Removed in SASD Fibroblasts—Whereas the molecular events involved in N-terminal (pro)cathepsin B processing have been characterized (27, 45), the cleavage of six amino acids from the C terminus of the proenzyme and its physiological significance are far less understood. In vitro, incubation of the enzyme at acidic pH results in the removal of this sequence with time, due to the sequential release of three dipeptides by the peptidyldipeptidase activity of cathepsin B itself (46). We have previously developed anti-peptide antibodies, which require the presence of the N-terminal dipeptide of the C-terminal extension for binding to cathepsin B, to demonstrate that the enzyme residing in lysosomes in vivo is, at least in human hepatoma HepG2 cells, in a fully processed state (30). When Western blots of SASD cell lysates were probed with these anti-peptide antibodies, single-chain cathepsin B (33 kDa) as well as the large 28-kDa subunit of the two-chain enzyme were detected (Fig. 4). Since the heavy chain contains the C-terminal region of the enzyme, these results indicate that C-terminal trimming of cathepsin B does not occur in SASD fibroblasts. The anti-peptide antibodies reacted also to some extent with cathepsin B from normal fibroblasts, which suggests that C-terminal processing of the enzyme is generally less efficient in this cell type than in liver cells and that at least a portion of normal fibroblast cathepsin B still contains the first two amino acids of the C-terminal extension which are part of the epitope. However, the heavy chain of the normal two-chain enzyme migrated faster than the corresponding polypeptide of SASD cells, indicating that the four most C-terminal amino acids have been removed and therefore most likely account for the observed 1-kDa difference between cathepsin B from SASD cells and the enzyme from normal fibroblasts. This is in agreement with studies demonstrating that these four amino acids are released from recombinant cathepsin B much faster under physiological conditions than the N-terminal dipeptide of the C-terminal extension (46).

Loading of Normal Fibroblasts with Sucrose Can Mimic the SASD Phenotype—A unique feature of SASD “lysosomes” is the accumulation of free, and therefore osmotically active, anionic monosaccharides. To mimic this situation in normal cells, control fibroblasts were cultured in the presence of the disaccharide sucrose, which is taken up by fluid phase endocytosis and accumulates in lysosomes since it is not cleaved by lysosomal hydrolases (20). When normal fibroblasts were incubated in the presence of sucrose prior to metabolic labeling, proteolytic processing of newly synthesized cathepsin B culminated in a 28-kDa polypeptide, indistinguishable from the heavy chain of the enzyme typically found in SASD cells (Fig. 5). Furthermore, the presence of sucrose significantly retarded the proteolytic conversion of single-chain cathepsin B into the two-chain enzyme in normal fibroblasts, as already observed in SASD cells (Fig. 1 and Fig. 5). As expected, the biosynthesis of cathepsin B in SASD fibroblasts was not significantly affected by the addition of sucrose to the culture medium.

Previous studies have indicated that extended culture of normal fibroblasts in the presence of N-acetylmannosamine, a precursor in the biosynthesis of sialic acids, increases the intracellular sialic acid concentration to a level reminiscent of SASD cells (24). However, loading with this compound exerted no significant impact on the biosynthesis of cathepsin B, as compared with control cells (Fig. 5). This finding is most likely related to the rapid egress of sialic acids from endocytic compartments of normal cells (24, 25), suggesting that elevated lysosomal sialic acid levels obtained by loading with N-acetylmannosamine cannot be maintained long enough to exert any pronounced effect on the morphology or functionality of these organelles. An additional polypeptide of 24 kDa was detected in the immunoprecipitates from N-acetylmannosamine-loaded cultures, which was also present in control cells in these experiments. This band is not commonly observed in fibroblast lysates, but is frequently detected in other cell lines, and has
been shown to correspond to the carbohydrate-free heavy chain of cathepsin B produced by the action of lysosomal endoglycosidases (29). However, the enzyme synthesized by SASD fibroblasts was fully glycosylated even in the presence of N-acetylmannosamine, suggesting that other lysosomal events besides C-terminal cathepsin B trimming are impaired in these cells (Fig. 5).

**SASD Lysosomes Exhibit Properties of Pre-lysosomal Compartments**—The accumulation of small molecules in intracellular compartments is expected to reduce their buoyant density due to the osmotic influx of water. To this end, postnuclear supernatants of SASD and control fibroblasts were subjected to density gradient centrifugation, a method conventionally used to separate dense lysosomes from more buoyant organelles of the endocytic and biosynthetic routes. When the obtained subcellular fractions were analyzed for the distribution of the lysosomal marker enzyme β-N-acetylhexosaminidase, most of its activity was associated in control cells with dense compartments (buoyant density $\geq 1.1$ g/ml), corresponding to mature lysosomes. In contrast, β-N-acetylhexosaminidase was located in SASD cells mainly in more buoyant fractions. The shift in density was more pronounced in fibroblasts from ISSD than Salla disease patients (buoyant density of 1.05 versus 1.065 g/ml, respectively), which correlates with the extent of sialic acid accumulation in these disease states. Interestingly, similar buoyant densities were determined in normal fibroblasts for endosomal/pre-lysosomal compartments (47). In all cell lines, the subcellular distribution of cathepsin B activity was identical to that of β-N-acetylhexosaminidase, indicating that this proteinase behaves like a classical soluble lysosomal hydrolase, even in SASD cells (Fig. 6). The fact that we could not detect endogenous lysosomal enzyme activity at a dense position of the centrifugal gradient points out that SASD cells contain virtually no mature lysosomal compartments. It is therefore highly unlikely that the impaired processing of cathepsin B in SASD cells is caused by the incompetence of pre-lysosomal compartments to fuse with pre-existing dense lysosomes.

A hallmark of mammalian lysosomes is the intrinsic low pH of these organelles. To estimate the lysosomal pH in SASD- and normal fibroblasts, the respective cell cultures were loaded with FITC-dextran, a fluid phase marker with pH-dependent fluorescence properties (35). The apparent lysosomal pH of control fibroblasts was 5.3 (S.D., ±0.15), which is in good agreement with data available for other mammalian cell types (48). However, lysosomes from ISSD patients exhibit a pH of 6.0 (S.D., ±0.1), which rather reflects the conditions in endosomal/pre-lysosomal compartments of normal cells (49). To compare the acidification properties of SASD and normal lysosomes *in vitro*, the respective cells were loaded with FITC-dextran as above, and the labeled terminal endocytic compartments were then isolated by differential centrifugation. The addition of ATP reduced the fluorescence of SASD lysosomes much less effectively than in the case of their normal counterparts, indicating a significantly lower *in vitro* acidification capacity of the former compartments (Fig. 7). The lumenal pH after addition of ATP was calculated to be 5.4 for lysosomes derived from control cells, whereas the pH in SASD lysosomes reached only 6.1, in line with the data obtained *in vivo*.

**SASD Lysosomes Are Smaller Than Normal Lysosomes**—It is generally believed that increased concentrations of osmotically active compounds in individual subcellular compartments induce an enlargement of the affected structures. Indeed, sucrose-loaded normal fibroblasts contained numerous large vesicles (data not shown). However, morphological analysis by fluorescence microscopy revealed that the terminal endocytic compartments in ISSD fibroblasts, tagged by internalization of

---

**Fig. 6. SASD lysosomes exhibit properties of pre-lysosomal compartments.** Homogenates of ISSD (□, GM 5520), Salla (○, GM 8496), and normal fibroblasts (●, control; GM 5521) were subjected to density gradient centrifugation as outlined under “Experimental Procedures.” The activities of cathepsin B and β-N-acetylhexosaminidase (lysosomal marker) were determined in each individual fraction of the gradient.

**Fig. 7. In vitro acidification of the terminal endocytic compartments in SASD and normal fibroblasts.** ISSD (□, GM 5520) and normal fibroblasts (●, control; GM 5521) were loaded for 18 h with FITC-dextran followed by incubation for 1 h in the absence of the marker. Microsomal fractions were prepared by differential centrifugation, and their ATP-driven acidification potential was assessed by dual spectrofluorimetry as described under “Experimental Procedures” and expressed as time course of the calculated lumenal pH. The figure shows one representative experiment.

FITC-dextran, are rather smaller than in control cells (Fig. 8a). Digital image analysis of the labeled compartments in paraformaldehyde-fixed SASD cells revealed a median diameter of 0.31 μm, whereas normal lysosomes exhibited a diameter...
of 0.88 μm (Fig. 8b). However, the difference was less obvious in living cells (not shown). Immunocytochemical detection of endogenous cathepsin B confirmed that those compartments, which contain mature lysosomal enzymes, are indeed smaller in SASD fibroblasts (Fig. 9). Similar results were obtained when the cells were stained with antibodies against the lysosomal membrane protein lgp120 (not shown). Double fluorescence analysis of internalized FITC-dextran and endogenous cathepsin B revealed a nearly complete co-localization in both SASD and normal fibroblasts, whereas the fluid phase marker did not co-localize with MPR300 to any significant extent in both cell types. Partial co-localization of FITC-dextran with the late endosome marker rab7 was observed for both control and SASD cells (data not shown).

**DISCUSSION**

SASD is characterized by the aberrant accumulation of sialic acid in late endocytic compartments and a concomitant decrease of their buoyant density. This inherited disorder therefore provides an interesting model system to investigate the impact of an altered endocytic environment on the biogenesis of lysosomes. We have now found that the proteolytic processing of the lysosomal cysteine proteinase cathepsin B is severely affected in fibroblasts from SASD patients.

Our studies clearly demonstrate that in particular the removal of six amino acids from the C terminus of the heavy chain of cathepsin B is abolished in SASD fibroblasts. Similar results have been reported for two other lysosomal hydrolases, β-N-acetylhexosaminidase and α-fucosidase (26), indicating that late processing steps in the biosynthesis of lysosomal enzymes might be generally abrogated in these cells. The cellular events responsible for C-terminal cathepsin B processing in normal cells are only poorly understood, with both the involved enzyme(s) and the intracellular location of this event still awaiting unanimous identification. In vitro, cathepsin B itself has the capacity to process the C-terminal extension of the proteinase. Indeed, it is the ability of the enzyme to remove C-terminal dipeptides that accounts for C-terminal processing of recombinant cathepsin B in vitro (46).

Cathepsin B is unique within the lysosomal cysteine protease family due to its capacity to act both as an endopeptidase and as dipeptidylcarboxypeptidase. The recently determined three-dimensional structure of cathepsin B (50) has provided important clues to explain the rather acidic pH optimum of pH...
4.0–5.0 for the hydrolysis of peptidylaspartidase substrates by cathepsin B which is significantly lower than the optimum pH of its endopeptidase activity (51). It has been shown that the exopeptidase activity exerted by cathepsin B requires two critical histidines to be fully protonated, whereas this is not essential for its action as endopeptidase (52). Therefore, cathepsin B acts at the physiological pH of normal lysosomes both as endopeptidase and as exopeptidase. In contrast, the latter activity is probably fully repressed in SASD lysosomes (pH = 6.0). However, these conditions still allow cathepsin B, at least to some extent, to be active as endopeptidase (39, 53, 54).

Besides the inhibition of C-terminal cathepsin B processing, the endoproteolytic conversion of the single-chain enzyme into the two-chain form of the proteinase is also significantly retarded in SASD cells. In a previous study, we have demonstrated that the generation of the two-chain form of cathepsin B can be abolished in vivo by the cysteine proteinase inhibitor Z-Phe-Ala-CHN₂ (29). We concluded that this processing event is due to cathepsin B itself, since cathepsins L and S, the other major lysosomal cysteine proteinases inhibited by this compound, are not expressed at significant levels in the investigated cell lines (30). Recently, a novel lysosomal cysteine proteinase, legumain, has been purified and characterized (55). This enzyme apparently represents the mammalian homologue of a vacuolar processing enzyme previously found in plants, and it has been suggested that legumain is responsible for the internal cathepsin B cleavage in mammalian lysosomes. However, legumain is not inhibited by Z-Phe-Ala-CHN₂ which argues against a role of this enzyme in cathepsin B processing. Therefore, we believe that the reduced rate of the generation of two-chain cathepsin B in SASD fibroblasts rather reflects an effect of the elevated pH in the endocytic compartments of these cells on the endopeptidase activity of the enzyme, which is optimally active against protein substrates around pH 5.0 (34). Similarly, the observed decreased internalization rate of procathepsin B in SASD cells might be due to the slow dissociation of MPR300 and its ligands at elevated pH.

Cathepsin B is by far not the only lysosomal enzyme to undergo C-terminal maturation during its biosynthesis. A number of other acidic hydrolases are processed in a similar manner (56). However, the enzyme(s) responsible for these trimming reactions have not yet been identified. In the case of cathepsin D, the two C-terminal amino acids are removed at a late stage in the biosynthesis of the enzyme, which led to the proposal that the extension is involved in intracellular sorting (57). The removal of this domain in lysosomes is most likely executed by a cysteine protease(s), since all final steps in cathepsin D processing can be inhibited with specific inhibitors for these enzymes (58). The C termini of β-glucuronidase and α-fucosidase are also trimmed upon arrival in the lysosomes, which reduces the molecular mass of each enzyme by 3 kDa. At least for α-fucosidase, this processing event is abolished when human fibroblasts are cultured in the presence of cysteine proteinase inhibitors (56, 59). Since cathepsin B is unique among mammalian lysosomal cysteine proteinases in its capacity to act as dipeptidylcarboxypeptidase, the enzyme could be responsible not only for the removal of its own C-terminal extension but also for the processing of the C termini of other lysosomal enzymes such as cathepsin D, β-glucuronidase, and α-fucosidase. This is supported by the finding that β-N-acetylhexosaminidase and α-fucosidase are not properly processed in SASD fibroblasts (26). Although C-terminal trimming as part of the proteolytic maturation of β-N-acetylhexosaminidase remains a matter of dispute (60, 61), it is possible that the pH-induced inhibition of the exopeptidase activity of cathepsin B in SASD lysosomes plays a central role in the impaired biosynthesis of β-N-acetylhexosaminidase and α-fucosidase in these cells.

Intriguingly, the SASD phenotype of incomplete cathepsin B processing can be mimicked by the exposure of normal fibroblasts to sucrose. Sucrose accumulation has been postulated to induce an osmotic influx of water, resulting in the appearance of unique large vesicles ("sucrosomes"). Although the original reports have stated that sucrosomes represent swollen lysosomes (20, 62), new evidence was recently presented that these structures may be actually derived from late endosomes (3).

It has been proposed that the presence of sucrose interferes with fusion events between individual compartments of the endocytic pathway (62). However, sucrosomes are able to fuse with other endocytic compartments since subsequent internalization of invertase can revert the swelling. The accumulation of sucrose rather interferes with the formation of a dense lysosomal matrix, since prolonged incubation of cells in the presence of sucrose results in a decrease of the number of dense lysosomes (3). Similarly, accumulation of sialic acid might prevent the formation of mature, dense lysosomes rather than inhibiting the transport to them. This is also supported by the fact that we could not observe any completely processed cathepsin B even after prolonged chase periods.²

The formation of a dense lysosomal matrix appears to rely on the aggregation of resident lysosomal hydrolases that depends on the intrinsic acidic milieu of the lysosomal lumen (19). Thus, the decreased density of SASD lysosomes could be caused by the higher internal pH of these compartments. On the other hand, the accumulation of negatively charged monosaccharides, such as sialic acids and related sugars, might directly interfere with the aggregation of lysosomal components.

The increased lumenal pH of SASD lysosomes might be simply a consequence of the buffering capacity of the accumulated sialic acids, since the high intralysosomal concentration of these acidic sugars, which can reach at least 40 mM, could bind a considerable number of protons (24). However, an elevated internal pH has also been reported for sucrosomes (62) which argues against the outlined direct involvement of anionic monosaccharides in the perturbation of the intralysosomal pH in SASD cells. On the other hand, the accumulation of sialic acids (or sucrose) may directly affect the activity of the lysosomal H⁺-ATPase. Intriguingly, inhibition of the vacuolar proton pump has been reported to prevent transport from late endosomes to lysosomes (63), indicating that acidification may be a prerequisite for the formation of mature lysosomes.

Despite some similarities between sucrosomes and the lysosomal compartments of SASD fibroblasts, we could not observe any swollen vesicles in the latter cells. Actually, SASD lysosomes, as revealed by immunohistochemistry, appeared rather similar small relative to their normal counterparts. However, primary fibroblast cultures established from skin biopsies of SASD patients contain vacuoles reminiscent of sucrerosomes (21). This phenotype is apparently not maintained upon prolonged tissue culture, although the intracellular sialic acid concentration can reach at least 40 mM, could bind a considerable number of protons (24). However, an elevated internal pH has also been reported for sucrerosomes (62) which argues against the outlined direct involvement of anionic monosaccharides in the perturbation of the intralysosomal pH in SASD cells. On the other hand, the accumulation of sialic acids (or sucrose) may directly affect the activity of the lysosomal H⁺-ATPase. Intriguingly, inhibition of the vacuolar proton pump has been reported to prevent transport from late endosomes to lysosomes (63), indicating that acidification may be a prerequisite for the formation of mature lysosomes.

² J. A. Schmid, unpublished results.
we did not observe the presence of the cation-independent mannose 6-phosphate receptor (MPR300) in SASD lysosomes, although this protein often appears to be enriched in late endosomes (42). However, it was recently shown for HEP-2 cells that MPR300 resides at steady state predominantly within the trans-Golgi cisternae and the trans-Golgi network (65), indicating that this protein cannot be used as a universal marker for late endosomes in all cell types.

Since there is no evidence for the occurrence of functional dense lysosomes in SASD fibroblasts, we conclude that the formation of mature lysosomes must be impaired in these cells. It should be pointed out that most degradative processes, which have been classically attributed to lysosomes, can proceed in late endosomes (5). Hence, it is not surprising that the capacity of SASD fibroblasts to degrade internalized macromolecules is not significantly affected by the unique phenotype of their lysosomes (66).