The biosynthesis of human acid ceramidase (hAC) starts with the expression of a single precursor polypeptide of ~53–55 kDa, which is subsequently processed to the mature, heterodimeric enzyme (40 + 13 kDa) in the endosomes/lysosomes. Secretion of hAC by either fibroblasts or acid ceramidase cDNA-transfected COS cells is extraordinarily low. Both lysosomal targeting and endocytosis critically depend on a functional mannose 6-phosphate receptor as judged by the following criteria: (i) hAC-precursor secretion by NH2-Cl-treated fibroblasts and I-cell disease fibroblasts, (ii) inhibition of the formation of mature heterodimeric hAC in NH2-Cl-treated fibroblasts or in I-cell disease fibroblasts, and (iii) blocked endocytosis of hAC precursor by mannose 6-phosphate receptor-deficient fibroblasts or the addition of mannose 6-phosphate. The influence of the six individual potential N-glycosylation sites of human acid ceramidase on targeting, processing, and catalytic activity was determined by site-directed mutagenesis. Five glycosylation sites (sites 1–5 from the N terminus) are used. The elimination of sites 2, 4, and 6 has no influence on lysosomal processing or enzymatic activity of recombinant ceramidase. The removal of sites 1, 3, and 5 inhibits the formation of the heterodimeric enzyme form. None of the mutant ceramidases gave rise to an increased rate of secretion, suggesting that lysosomal targeting does not depend on one single carbohydrate chain.
An intriguing functional concept for ceramide-mediated signaling postulates changes in membrane fluidity through the formation of ceramide microdomains (18, 19). Structural changes in membrane morphology triggered by the action of sphingomyelinase may then allow rapid and efficient signaling inside the cell.

Recently, the purification of AC from human sources has led to the identification of the genomic and the full-length cDNA sequences of hAC (20–22). These results enabled us to identify and characterize several mutations in the hAC gene causing different subtypes of Farber disease (21–23).

hAC is a heterodimeric glycoprotein. Under reducing conditions, hAC is cleaved into two subunits designated α (molecular mass ~13 kDa) and β (molecular mass ~40 kDa). Complete deglycosylation of hAC with protein N-glycanase (PNGase F) reduces the apparent molecular mass of the β-subunit to 28 kDa, whereas the α-subunit is not glycosylated. Purified AC from human urine or placenta has optimal enzymatic activity at pH 4.0. In our detergent-based hAC assay system, it is most active toward N-lauroylphosphinosine as substrate (20).

Both neutral and alkaline ceramidase activities have also been found in various mammalian tissues. A neutral, membrane-bound nonlysosomal ceramidase has been purified to apparent homogeneity from rat brain (24). This neutral ceramidase displays a broad pH optimum in the range from 7 to 10 and is also capable of hydrolyzing dihydroceramide to a limited extent. Two different alkaline ceramidases were purified from guinea pig epidermis, which are most active toward long chain ceramides such as N-lineoloylphosphinosine. In addition, neutral and alkaline ceramidase activity have been found in cultured skin fibroblasts, white blood cells, cerebellum, kidney, and small intestine, where it seems to play an important role in the hydrolysis of dietary ceramides (25–28).

In this paper, we focus on the processing, targeting, and glycosylation of lysosomal hAC. Lysosomal enzymes are usually translocated into the lumen of the endoplasmic reticulum and then transported further to the early Golgi compartment. In the Golgi compartment, they are subjected to a variety of post-translational modifications (i.e., modification of carbohydrate structures with mannose 6-phosphate) before being routed to the acidic organelles of the cell. In fibroblasts, most of the soluble lysosomal proteins are targeted to endosomes and lysosomes via N-linked glycosylation sites by metabolic labeling studies.

**EXPERIMENTAL PROCEDURES**

**Materials—**[35S]methionine/cysteine (>1000 Ci/mmol) was purchased from ICN, and [32P]phosphate was from Hartmann Analytics (Braunschweig, Germany). Protease inhibitors were from Roche Molecular Biochemicals; PNGase F and endo-β-N-acetylgalactosaminidase H (Endo H) were from New England Biolabs; and anti-IgY-agarose was from Promega. DMEM as well as methionine/cysteine-deficient and phosphate-free MEM were purchased from Sigma, and Opti-MEM was from Life Science. All other chemicals were of the highest purity available and purchased from Sigma, Merck, or Roth GmbH.

Diploid human skin fibroblasts and fibroblasts of a patient with I-cell disease were established from biopsies submitted to us for diagnosis. Mannose 6-phosphate receptor knockout mouse fibroblasts (35) were kindly provided by Dr. von Figura (Göttingen, Germany).

**Materials and Methods**

**Cell Culture—**Human skin fibroblasts and COS-1 cells were grown in monolayers at 37 °C in a 5% CO2 atmosphere in DMEM supplemented with antibiotics and 10% fetal calf serum.

**Preparation and Characterization of Chicken Anti-hAC Antiserum—** Specific pathogen-free chickens (VALO, Lohmann, Cuxhaven) were hatched and raised in isolators under filtered negative air pressure. Prior to the immunization with hAC, preimmune sera were obtained from these birds to serve as hAC antibody negative controls. Pure preparation of hAC (approximately 1.2 mg/ml) was solved in phosphate-buffered isotonic saline, pH 7.0, containing 0.05% β-octyl glucoside. In total, 0.5 ml of this preparation was emulsified with two parts of complete Freund’s adjuvant (Difco) and administered at multiple intramuscular and intradermal parts of two 6-week-old chicken. After 6 weeks, the chicken were Boostered and exsanguinated 14 days later. This experiment was performed with permission of the Lower Saxony Authorities (permit number 99A 884).

**Metabolic Labeling and Immunoprecipitation—** Subconfluent (80–90% confluence) fibroblast cell cultures (21-cm2 dishes) were starved for 2 h in methionine/cysteine-free MEM supplemented with 4% dialyzed (with TRIS) and heat-inactivated FCS. Labeling was initiated by the addition of 100 μCi/ml [35S]methionine/cysteine to the deficient medium. Various chase periods were started by substituting the pulse medium with methionine/cysteine-free MEM supplemented with 4% dialyzed and heat-inactivated FCS and 3 mM methionine, 0.2 mM cysteine final concentration.

Prior to [35S]phosphate labeling, cells were washed twice with phosphate-buffered MEM supplemented with 4% dialyzed (with TRIS) and heat-inactivated FCS and incubated for 10 min. Labeling was conducted by the addition of 250 μCi/ml [32P]phosphoric acid for the indicated time. For some experiments, cells were preincubated with 10 mM NH4Cl 1 h prior to [35S]methionine/cysteine labeling, and the treatment continued throughout the pulse and chase periods.

Labeled cells were washed once with cold PBS, harvested by scraping, and lysed in radioimmuno precipitation buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, and protease inhibitor mixture mini-table/tube/10 ml (Complete™; Roche Molecular Biochemicals) in PBS, pH 7.2, 4 °C, 20 min, unless otherwise stated. Lysis buffer for [35S]phosphate-labeled cells was additionally supplemented with 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and 1 mM orthovanadate.

Subsequently, cell lysates were cleared by centrifugation, and the supernatant was preadsorbed with protamine sulfate for an additional 30 min on ice. The insoluble precipitate was sedimented by centrifugation. The cleared supernatants were carefully removed and incubated with 2 μl of chicken anti-human hAC antiserum for >4 h. The immune complexes were precipitated by adding anti-IgY-agarose for an additional 2 h. The immunoprecipitates were washed 10 times for 30 min at 4 °C with radioimmune precipitation buffer.

Immunoprecipitation from the cell culture media was carried out by the addition of 2 μl of chicken anti-hAC-antiserum and subsequent precipitation with anti-IgY-agarose as outlined above.

**Deglycosylation Experiments—** Deglycosylation with Endo H and PNGase F were essentially performed as suggested by the manufacturers except that instead of 1% Triton X-100, as recommended for PNGase F digestion, 0.25% β-octyl glucoside was used. Deglycosylation was generally performed at 37 °C overnight.

**Endocytosis Studies—** Confluent human skin fibroblasts were labeled with [35S]methionine/cysteine in the presence of NH4Cl for 16 h. The radiolabeled culture medium was dialyzed extensively against DMEM and added either to unlabeled fibroblasts for 12 h in the presence of 10 mM mannose 6-phosphate or to M6PR-deficient mouse fibroblasts (35). For immunoprecipitation, cell lysates were prepared and treated as described above.

**Construction of Mutagenized hAC Expression Vectors—** The wild-type cDNA was cloned into the pSV-Sport-1 vector (Life Science) commonly used for transient protein expression in COS-1 cells. Site-directed mutagenesis of the wild-type hAC-cDNA was performed using the QuikChange kit from Stratagene. Two complementary PCR primers for...
each of the six potential N-glycosylation sites were generated with a sequence mutation that results in the substitution for the normal asparagine by glutamine within the N-glycosylation consensus sequence. The Pfu polymerase replicates both strands of the plasmid without replacing the two oligonucleotides. A mutagenized plasmid with staggered nicks was generated by incorporation of the oligonucleotide primers. In a subsequent step, the wild-type plasmid strands were digested by the endonuclease DpnI, which specifically acts on methylated and hemimethylated DNA strands. The PCR-derived strands carrying the desired mutation were not cleaved by DpnI, since they are not methylated.

The polymerase chain reaction primers used for mutagenesis are as follows (changed nucleotides indicated in boldface type): ΔGS1 (first glycine-containing site from the 5'end), primer 1A (5'-TGGCAACATAATCAGATACCTGG-3') and primer 1B (5'-CCAGGTATCTTGATTATGTTCCAAGACTGTT-3'); ΔGS2, primer 2A (5'-TTCCAAGAAACCACAAACTGCTCTC-3') and primer 2B (5'-GAAGACAGTTTTTTGTCCAGAGCT-3'); ΔGS3, primer 3A (5'-ACAGTCTGGAACAGCACAAGTTAT-3') and primer 3B (5'-ATAACTGTGTCTTGTTCCAGAAACTGTT-3'); ΔGS4, primer 4A (5'-CCTGGGAGGCAGAAGTCTGGG-3') and primer 4B (5'-CCAGACTCTGTGCTCCAGAG-3'); ΔGS5, primer 5A (5'-AGA-TGTGTTGCTGGCCACTCACT-3') and primer 5B (5'-GCCTGGTGCCTGGCCAGACACATCT-3'); ΔGS6, primer 6A (5'-CCAGCCAAAGACAAATCTCATGTTGA-3') and primer 6B (5'-TCAAATGAGATTGCTCTTGGGCTGG-3').

**RESULTS**

**Processing and Glycosylation of hAC in Normal Fibroblasts**—Cultured human fibroblasts were pulse-labeled with [35S]methionine/cysteine and harvested after various time periods. The hAC protein was immunoprecipitated using a polyclonal chicken anti-hAC serum raised against recombinant hAC produced by a baculovirus/insect cell expression system (not shown) and separated by SDS-PAGE. Fluorographic images of the respective gels indicate that hAC is synthesized as a precursor protein with an apparent molecular mass of 53–55 kDa (Fig. 1a). Time resolution of this early process by pulse/chase experiments revealed the initial biosynthesis of a precursor polypeptide pair of 53 and 55 kDa, respectively (Fig. 1b). Within approximately 2 h, the intensity of the 53-kDa form increases at the expense of a decreasing amount of 55-kDa precursor, and the mature heterodimeric form of hAC becomes detectable (Fig. 1, a and b). As formerly reported on hAC cDNA-transfected COS cells (21), proteolytic cleavage of the common precursor polypeptides results in the formation of both α-subunit (∼13 kDa) and β-subunits (∼40 kDa). Both subunits of mature hAC are linked by disulfide bridges as depicted by SDS-PAGE under nonreducing conditions (Fig. 2a, right panel). Complete processing of the hAC precursor is accomplished ∼12 h after the beginning of biosynthesis, and the half-life of mature heterodimeric hAC is estimated to be above 20 h (not shown). Different from most other soluble lysosomal proteins, secretion of hAC from fibroblasts and even transfected COS-cells (as indicated below) was significantly delayed and almost negligible. Only small amounts of the hAC precursor with reduced apparent molecular mass (46–48 kDa) were observed after an initial lag phase of up to 8 h from biosynthesis (Fig. 2b). Mature heterodimeric hAC was not detectable in the culture medium of radiolabeled normal fibroblasts.

Analysis of the N-linked carbohydrate structures on different hAC forms from fibroblasts was carried out by deglycosylation of hAC immunoprecipitates with either Endo H or PNGase F. Complete removal of oligosaccharide chains reduces the apparent molecular mass of the hAC precursor from 53–55 to 42 kDa and that of the β-subunit from 40 to 28 kDa (Fig. 3). The α-subunit, which misses any potential N-glycosylation site is,
as expected, not glycosylated. The apparent molecular mass of 13 kDa remains unchanged after PNGase F treatment. Removal of high mannose/hybrid carbohydrate chains by Endo H treatment reduces the apparent molecular weight of the hAC precursor to 44 kDa and that of the \( \beta \)-subunit to 30 kDa (not shown) identical to our results from hAC-transfected COS cells (see Fig. 9).

**Processing, Glycosylation, and Transport in I-cell Fibroblasts**—Biosynthesis of hAC in I-cell disease fibroblasts was studied by \([\text{35S}]\)methionine/cysteine labeling followed by immunoprecipitation and SDS-PAGE analysis. No differences in the apparent molecular weight and amount of hAC precursor, synthesized during a 1-h pulse period, were detected in I-cell disease fibroblasts when compared with normal fibroblasts. However, immunoprecipitates obtained at different chase times indicate that there is a significant loss of intracellular hAC material in I-cell disease fibroblasts. As early as 5 h after biosynthesis, hAC polypeptides become undetectable in the cell homogenate (Fig. 4a). Furthermore, I-cell disease fibroblasts are not capable of processing the hAC precursor protein into the mature heterodimeric enzyme, suggesting that the uncoupling of the intracellular mannose 6-phosphate receptor targeting pathway leads to missorting and secretion of hAC precursor. The hAC is secreted in the form of the 53–55-kDa polypeptide and can be recovered from the culture medium of I-cell disease fibroblasts as early as 2 h after biosynthesis (Fig. 4b). The apparent molecular mass of the secreted hAC corresponds to the molecular mass of the intracellular precursor but not to that of the regularly secreted hAC (\(~46–48\) kDa). However, deglycosylation of both secretory forms with PNGase F results in molecular masses of 42 kDa (Fig. 4c). In contrast to the intracellular hAC from I-cell disease fibroblasts, small but notable amounts of mature heterodimeric hAC are detected in the cell culture medium of I-cell disease fibroblasts (Fig. 4c). This maturation might be due to extracellular proteolysis by acidic proteases, which are also secreted as a consequence of impaired lysosomal sorting in I-cell disease fibroblasts.

**Effect of \( \text{NH}_4\text{Cl} \) on hAC Processing and Secretion**—In order to uncouple the intracellular mannose-6-phosphate pathway, radiolabeled normal fibroblasts were preincubated with the lysosomotropic agent \( \text{NH}_4\text{Cl} \) (10 mM). Under these conditions, hAC processing closely resembled the one observed in I-cell disease fibroblasts: increased secretion of hAC at the expense of intracellular hAC precursor and failure to process hAC into the mature heterodimeric enzyme (Fig. 4, a and b). However, in contrast to secretory hAC from I-cell disease fibroblasts, \( \text{NH}_4\text{Cl} \)-induced secretory hAC had the same apparent molecular mass as the regularly secreted hAC (\(~46–48\) kDa; Fig. 4, b and c).

**Endocytosis of hAC via Mannose 6-Phosphate Receptor**—In order to investigate the potential role of the M6PR pathway for the internalization of extracellular hAC, radiolabeled hAC precursor was applied to the cell culture medium of normal fibroblasts with and without preincubation with mannose 6-phosphate. In addition to blocking the mannose 6-phosphate receptor with mannose 6-phosphate, we also analyzed the rate of hAC precursor endocytosis by using mannose 6-phosphate receptor-deficient mouse fibroblasts (35, 36). In these animals, both alleles encoding the two different receptors are deleted. Incubation of these fibroblasts with exogenous, radiolabeled hAC precursor revealed that endocytosis of the precursor is critically dependent on the presence of functionally active mannose 6-phosphate receptors. Internalization of hAC precursor is
observed only in those fibroblasts which carry the receptors and when the receptors are not blocked with mannose 6-phosphate (Fig. 5). Within the incubation period, hAC precursor is completely processed to the mature heterodimeric form of the enzyme, indicating that proper targeting of extracellular hAC precursor to the acidic organelles has occurred.

Phosphorylation of hAC — A common feature of many lysosomal enzyme in fibroblasts is the acquisition of oligomannosyl-linked mannose 6-phosphate residues (37). In order to define the phosphorylation state of both the precursor and the mature form of hAC, fibroblasts were metabolically labeled with [32P]phosphate for 5 h. This resulted in the formation of radiolabeled hAC precursor and α-subunit; the β-subunit was not detected by autoradiography and therefore obviously not radio-labeled (Fig. 6). A relatively poor labeling of the hAC β-subunit in comparison with the precursor protein might be due to the short pulse period during which only minor amounts of mature hAC are generated. Deglycosylation with PNGase F of [32P]phosphate labeled hAC immunoprecipitates led to the complete loss of hAC-specific radiolabel. This was taken as evidence that phosphate-containing residues of both the hAC precursor and β-subunit correspond to mannose 6-phosphate residues. Phosphorylation of the polypeptide backbone was not detected.

In order to investigate the influence of the individual glyco-
sylation sites on the processing and trafficking of hAC, we substituted glutamine for normal asparagine in the six potential N-glycosylation consensus sequences (Asn-X-Thr/Ser) by site-directed mutagenesis. The resulting cDNA constructs were designated GS1–6, respectively.

Each construct was inserted into the eukaryotic expression vector pSV-Sport-1 and transiently expressed in COS-1 cells to analyze the effects of these mutations on the corresponding hAC polypeptide. Duplicate sets of transfectants using different batches of purified plasmid DNA were metabolically-pulse labeled with [35S]methionine/cysteine for 30 min, and the hAC polypeptides were immunoprecipitated (Fig. 7). The major hAC wild-type precursor migrated as the hAC precursor from fibroblasts at an apparent molecular mass of 53 kDa. The hAC precursors derived form ΔGS 1–5 migrated at slightly reduced molecular mass values of 51–52 kDa (with the mass of ΔGS1 being between wild type and mutants ΔGS2–5), suggesting that these sites are presumably N-glycosylated under normal conditions. The ΔGS6 precursor is of the same size as the wild-type polypeptide (53 kDa).

Downstream processing of recombinant wild-type and ΔGS6 hAC resulted in the same heterodimeric mature enzyme complex as observed in normal fibroblasts (40-kDa β-subunit and 13-kDa α-subunit) (Fig. 8). The removal of glycosylation sites 2 and 4 led to mature hAC forms with a regular 13-kDa α-subunit and β-subunit with slightly reduced molecular mass (38 kDa) compared with the wild-type β-subunit. The mutants ΔGS1, -3, and -5 showed no proteolytic maturation of the hAC precursor over the whole chase period (Figs. 8 and 9).

Again, a significant secretion of recombinant wild-type hAC or any mutagenized hAC was not observed, although transiently transfected COS cell usually release considerable amounts of lysosomal proteins into the medium due to high expression levels. Comparable with the fibroblast studies, only poor amounts of hAC precursor of 47 kDa were detected in the culture medium >8 h after biosynthesis (not shown).

To demonstrate that the altered electrophoretic mobilities of the mutant hAC polypeptides shown in Figs. 8 and 9 were due to the elimination of individual oligosaccharide chains, transfected COS-1 cells were pulse-labeled (30 min) and chased (12 h), and subsequently the hAC immunoprecipitates were deglycosylated with either PNGase F or Endo H, respectively (Fig. 9). Deglycosylation with PNGase F gave rise to a 13-kDa band and a 28-kDa band for the wild-type hAC and the mutants ΔGS2, -4, and -6. Treatment of the wild type, ΔGS2, ΔGS4, and ΔGS6 immunoprecipitates with Endo H led to a 29–30-kDa band for the β-subunit. The mutant hACs ΔGS1, -3, and -5, as mentioned above, were not processed to the mature heterodimer, suggesting that these glycosylation sites are essential for the proper routing of the hAC precursor to the acidic organelles.


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**TABLE I**
Relative hAC activities in the cell homogenates of COS-1 cells transfected with various hAC cDNA constructs

| Construct | Relative activity (nmol substrate/h/mg protein) |
|-----------|-----------------------------------------------|
| Wild type | 3.51 ± 0.12                                   |
| Mock      | 0.81 ± 0.40                                   |
| ΔGS1      | 0.87 ± 0.12                                   |
| ΔGS2      | 3.11 ± 0.03                                   |
| ΔGS3      | 0.81 ± 0.19                                   |
| ΔGS4      | 3.39 ± 0.03                                   |
| ΔGS5      | 0.81 ± 0.34                                   |
| ΔGS6      | 3.17 ± 0.03                                   |

**DISCUSSION**

Preliminary results on the molecular properties of purified hAC from human spleen and processing studies in transfected COS cells indicated that hAC is a heterodimeric glycoprotein derived from a single precursor protein (21). Analysis of the early steps in hAC biosynthesis revealed the occurrence of a pair of precursor proteins of 55 and 55 kDa, respectively. Since these pulse labeling studies are inappropriate to show whether both forms are derived from alternatively spliced transcripts or by co- or posttranslational processing events, we also performed short pulse/chase experiments. Evidence for an early processing event comes from the studies depicted in Fig. 4a (left panel), where the 55-kDa precursor band increases over the whole chase period to the account of the 55-kDa form. Endoproteolytic processing of the hAC precursor into the mature heterodimeric hAC obviously takes place in the acidic organelle such as endosomes and lysosomes, since treatment of fibroblasts with NH4Cl (Fig. 4a) or brefeldin A (not shown), a fungal metabolite that blocks the vesicular transport from the endoplasmic reticulum to the Golgi, prevents the formation of the mature enzyme. Strong evidence for lysosomal hAC processing is further provided by the analysis of I-cell disease fibroblasts. I-cell disease fibroblasts are characterized by an inherited deficiency in UDP-N-acetylgalactosaminyl-β-galactosylceramide N-acetylgalactosaminyl-1-phosphotransferase activity (33), the enzyme responsible for the first step in the formation of mannos-6-phosphate (M6P) residues on N-linked carbohydrate moieties. Under normal conditions, M6P residues bind to the cation-independent M6PR in the Golgi, from where they are shuttled to the acidic compartiments of the cell. Improper formation of M6P residues prevents the correct targeting of lysosomal proteins. As a result, significantly increased amounts of lysosomal proteins are secreted into the cell culture medium instead. This could also be demonstrated in the cell culture medium of metabolically labeled I-cell disease fibroblasts in which markedly increased levels of hAC precursor were detected. On the other hand, intracellular hAC precursor was depleted within few hours after biosynthesis. Similar results were obtained with NH4Cl-treated normal fibroblasts. Under these conditions, loss of radiolabeled intracellular hAC precursor and secretion of hAC precursor into the extracellular space was observed. Secretory hAC from NH4Cl-treated fibroblasts corresponds in size to the hAC precursor detected in the cell culture medium of normal, untreated fibroblast cultures but differs in the molecular mass when compared with hAC precursor detected in the medium of I-cell disease fibroblasts. Deglycosylation of secreted hAC forms indicated that the difference in the apparent molecular mass is due to altered N-glycosylation, since both precursor forms consist of a polypeptide backbone of the same size. The molecular reason for this difference in carbohydrate processing is not yet clear, but it may possibly be secondary to the genetic defect in I-cell disease fibroblasts.

The enzymatic activity of hAC in the cell lysate and in the supernatant/blood plasma of I-cell disease lymphocytes and fibroblasts has been formerly determined by Ben-Yoseph et al. (34). They report that intracellular hAC activity is reduced by ~60%, whereas secreted hAC activity is increased 4-fold, suggesting that the hAC precursor may have intrinsic activity. However, in an attempt to express recombinant hAC in a baculovirus/insect cell system, we produced huge amounts of secretory nonprocessed, enzymatically active hAC precursor without any enzyme activity.2 In consideration of our hAC processing data in I-cell disease fibroblasts, we would rather assume the hAC activity measured from the I-cell medium to originate from mature hAC. Since I-cells are known to secrete various lysosomal hydrolases, it is tempting to speculate whether processing of hAC may also occur extracellularly by yet unspecified proteases and thus lead to increased levels of medium hAC activity.

The considerable half-life of hAC (>20 h; data not shown) suggests that it has a significant stability toward the proteolytic lysosomal environment. Especially, the prolonged half-life of the hAC precursor differs in its stability when compared with other lysosomal hydrolases. This observation is further supported by previous reports of a significant resistance of hAC activity toward the treatment with the protease trypsin (38). Similar results were also obtained from the incubation of recombinant hAC precursor (see above) with enriched lysosomal extracts from human placenta. In these experiments, recombinant hAC precursor exhibited a remarkable stability toward degradation despite the aggressive hydrolytic environment.2

In contrast to most other soluble lysosomal hydrolases investigated so far, secretion of hAC precursor from untreated normal fibroblasts is almost negligible. After a chase period of as long as 8 h, only traces of hAC precursor, even in the high level expression COS cell system, were found in the cell culture medium, indicative of a highly effective intracellular sorting mechanism for hAC. In contrast, secretion of several other lysosomal sphingolipid hydrolases (i.e. acid sphingomyelinase, arylsulfatase A, or β-hexosaminidases) were detected as early as 2 h after metabolic labeling. A potential membrane affinity of the highly hydrophobic hAC may be the reason for this. Since the nonglycosylated α-subunit as well as the N terminus of the β-subunit exhibit extremely hydrophobic properties (Fig. 10), it is plausible to assume a hydrophobic interaction between the membrane lipid phase and the hAC. The lysosomal hydrolysis of ceramide by hAC additionally requires the assistance of

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2 K. Ferlinz, G. Kopal, K. Bernardo, T. Linke, B. Breiden, U. Neumann, F. Lang, E. H. Schuchman, and K. Sandhoff, unpublished results.
sphingolipid activator proteins SAP-C or SAP-D (6). Several different hypotheses exist as to how these small, nonenzymatic proteins stimulate ceramide hydrolysis in vivo. They could possibly act as weak physiological detergents, which render ceramide more water-soluble. They might also act as so-called “lifetases,” elevating ceramide above the lipid bilayer toward the active center of hAC. In another model, SAPs are thought to disturb the packing of the lipid bilayer and making ceramide more accessible to hAC or may directly mediate the interaction between ceramide and hAC by generating a ternary protein-lipid complex (6, 39).

The major endocytic route for the hAC precursor is the M6PR-dependent pathway. Normal fibroblasts, which were treated with mannose 6-phosphate in order to block M6PR (predominantly cation-dependent M6PR) located at the plasma membrane completely failed to endocytose radiolabeled hAC precursor. A sorting mechanism via M6PR is further supported by the identification of phosphate label on N-linked carbohydrate structures. Consequently, knock-out mouse fibroblasts deficient in M6P receptors (35, 36) are not able to internalize detectable amounts of hAC precursor.

Glycosylation of a protein may control its folding and stability and regulate its activity (40). In this report, we further investigated the influence of N-glycosylation sites (Asn-X-Ser/Thr) (41) on targeting/processing and enzyme activity of hAC. In another model, SAPs are thought to possibly act as weak physiological detergents, which render proteins stimulate ceramide hydrolysis.

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Fig. 10. Hydrophobicity profile of hAC.
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