Biosynthesis, Processing, and Intracellular Transport of G<sub>M2</sub> Activator Protein in Human Epidermal Keratinocytes

THE LYSONSOMAL TARGETING OF THE G<sub>M2</sub> ACTIVATOR IS INDEPENDENT OF A MANNOSE-6-PHOSPHATE SIGNAL

(Received for publication, September 25, 1996, and in revised form, November 19, 1996)

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The processing, intracellular transport, and endocytosis of the G<sub>M2</sub> activator protein (G<sub>M2</sub>AP), an essential cofactor of β-hexosaminidase A for the degradation of ganglioside G<sub>M2</sub>, was investigated in human epidermal keratinocytes. The G<sub>M2</sub>AP precursor is synthesized as an 18-kDa peptide, which is singly glycosylated, resulting in 22-kDa high mannose and 24–27-kDa complex glycoforms. A small portion of the 22-kDa form bears phosphomannosyl residues. About 30% of the G<sub>M2</sub>AP precursor is secreted during 12 h after synthesis, consisting almost exclusively of complex glycoforms. In a post-Golgi compartment, the intracellular remainder is converted to a 20-kDa mature form within 24 h, bearing a heavily trimmed N-glycan on a 17-kDa backbone. Interestingly, even nonglycosylated G<sub>M2</sub>AP is delivered to the lysosome, as shown by tunicamycin treatment and subcellular fractionation. Also, its endocytosis is independent of carbohydrate-linked signals and is even more effective for nonglycosylated G<sub>M2</sub>AP. We conclude that a mannose-6-phosphate-independent pathway for the lysosomal delivery of G<sub>M2</sub>AP exists in cultured human keratinocytes.

The lysosomal degradation of cellular glycosphingolipids with short oligosaccharide chains requires the presence of non-enzymatic cofactors, the glycosphingolipid activator proteins (reviewed in Refs. 1 and 2). Five different activator proteins have been discovered to date. Four of them (sphingolipid activator proteins or saposins A–D) are proteolytically generated from a single precursor (3–5). The fifth activator, the GM2 activator protein (GM2AP), is the product of a separate gene (6). It is an essential cofactor of β-hexosaminidase (EC 3.2.1.52); M6P, mannose-6-phosphate; PNGase F, protein; BFA, brefeldin A; endo H, endoplasmic reticulum; hEK, human epidermal keratinocyte; It is an essential cofactor to peptide-hexosaminidase (EC 3.2.1.52); M6P, mannose-6-phosphate; PNGase F, protein; BFA, brefeldin A; endo H, endoplasmic reticulum; hEK, human epidermal keratinocyte.
short pulse times. Although no data were yet available on the biosynthesis and processing of lysosomal proteins in human epidermal keratinocytes (hEKS), we decided to choose this cell type for our study, since in hEKS, G$_{M3}$AP biosynthesis is 5-fold enhanced over human fibroblasts.²

With the present study, we established the biosynthesis and processing of G$_{M3}$AP in hEKS and we will show that an M6P-independent targeting pathway exists for G$_{M3}$AP in this cell type, which cannot be used by the precursors of cathepsins D and L. In this way, we also give first examples of lysosomal enzyme processing in hEKS. We will also show, that G$_{M3}$AP endocytosis is independent of known signals for N-glycan receptor-mediated endocytosis in hEKS.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The following reagents were purchased from commercial sources: MCDB 153 medium base, keratinocyte growth medium supplements, Dulbecco's modified Eagle's medium, N-acetylglucosaminyltamine (Biochrom, Berlin, Germany), cysteine- and methionine-free MCDB 153 medium base (Cytogen, Lohmar, Germany), bovine pituitary extract (Beckton Dickinson, Bedford, MA), porcine insulin, epidermal growth factor, brefeldin A, tunicamycin, yeast mannose, and protein G-Sepharose fast flow (Sigma), [³⁵S]cysteine and [³⁵P]orthophosphate (Amersham Corp.), [¹³¹I]albumin (ICN, Meckenheim, Germany), and β- and α-acetylgalactosaminidase (Endo H) and peptide N-glycanase F (PNGase F; New England Biolabs, Schwalbach, Germany). All other reagents were of the highest purity commercially available and obtained from Sigma, ICN, Merck (Darmstadt, Germany), and Serva (Heidelberg, Germany). Asialo-orosomucoid (desialylated α$_1$-acid glycoprotein, human) was a kind gift of Dr. G. Schwarzmann (Institut für Organische Chemie und Biochemie, Universität Bonn, Bonn, Germany).

**Cell Culture**—Epidermal keratinocytes from human foreskin were obtained according to the method of Rheinwald and Green (21). They were cultured at 0.1 mM Ca$^{2+}$ and 0.1% FBS and 0.1% antibiotics. At confluence, the cells were split in a 1:5 ratio resulting in approximately 70% confluency, the cell were split in a 1:5 ratio, resulting in seedin in densities of 2 x 10$^⁵$ cells/cm$²$. Passageing was repeated every 4–6 days. In the third or fourth passage, cells from a single donor were used for experiments.

**Antibodies**—A goat antiserum raised against recombinant G$_{M3}$AP (23) was used for all experiments. Sheep anticalphaglobin L or D were commercially available (BioAss, Schwalbach, Germany); goat anti-β-hexosaminidase β-chain was a kind gift of Dr. R. Proia (NIDDK, National Institutes of Health, Bethesda, MD).

**Metabolic Labeling**—Epidermal keratinocytes grown in 60-mm dishes were kept in 1.5 ml of medium lacking methionine and cysteine for 2 h. When inhibitors were added, they were already present during this starvation period. All inhibitors were added from 1000-fold concentrated stock solutions in ME SO. Half of the medium was then removed, and the cells were labeled with [³⁵S]cysteine (5.55 MBq; specific activity, >37 TBq/nmol) for 60 min. After the pulse period, the cells were washed twice with phosphate-buffered saline, and the chase was initiated by addition of 1.8 ml of complete medium. The media were saved, and the cells were extracted. Phosphate labeling was performed analogously, except that the labeling medium was phosphate deficient. 18.5 MBq of [³²P]orthophosphate (specific activity, >111 TBq/nmol) were used in a pulse of 5 h.

**Endocytosis Experiments**—Epidermal keratinocytes grown in 75-cm² flasks were stained for 2 h in the presence of 10 μM NTI CI and labeled with 22.2 MBq of [³⁵S]cysteine in 4 ml of medium containing 10 μM NTI CI for 8 h. Nonglycosylated G$_{M3}$AP was obtained by labeling of hEKS in the simultaneous presence of 10 μM NTI CI and 5 μg/ml tunicamycin. The secretions were dialyzed overnight against two 500-ml changes of MCDB 153 medium base in dialysis tubing with a 10-kDa cutoff to remove the inhibitors and were added to three 60-mm dishes of unlabelled cells in a total volume of 2 ml/dish. Endocytosis was allowed to proceed for 24 h. Afterward, the cells were extracted and immunoprecipitated as described below. Of the endocytosis media, 200 μl were filled up to 700 μl with cell extraction buffer (see below) and immunoprecipitated.

**Preparation of Cell Extracts and Immunoprecipitation**—After the pulse-chase experiment, the cells were washed twice with ice-cold phosphate-buffered saline and scraped off with 0.7 ml of ice-cold lysis buffer (phosphate-buffered saline containing 5 mM EDTA, 0.5% bovine serum albumin, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each leupeptin and pepstatin A). After the removal of insoluble matter by a 20-min centrifugation at 17,500 x g and 4 °C, 10 μl of the extracts were saved for acid precipitation. Immunoprecipitation was carried out as described (24), except that 3.5 μl of goat anti-G$_{M3}$AP serum/ml of sample and 10 μl of protein G-Sepharose/μl of serum were used. Finally, the immune complexes were solubilized by boiling in 0.5% SDS/1% β-mercaptoethanol.

Deglycosylation of the precipitates with endo H or PNGase F was carried out according to the manufacturer's instructions. The samples were subjected to reducing SDS gel electrophoresis in the Tris-Tricine buffer system of Schägger and von Jagow (25) using 12.5% polyacrylamide in the separating gel. Afterward, the gels were soaked in Amplify (Amersham), dried, exposed overnight to a Fuji BAS 1000 imaging system screen, and recorded with a Fuji BAS 1000 system. For a permanent record, the gels were exposed to Kodak BioMax MR x-ray film.

**Magnetic Isolation of Lysosomes**—The preparation of ferromagnetic dextran and the magnetic isolation of lysosomes were carried out essentially as described by Rodriguez-Paris et al. (26) with the following modifications.³ The cells were loaded with the iron/dextran probe for 24 h and chased for at least 10 h. They were harvested by trypsinization and lysed in homogenization buffer (27) supplied with 0.5 mM 4-aminophenylmethanesulfonyl fluoride (Calbiochem) and 1 μg/ml each leupeptin and pepstatin A by 20 passages through a 24-gauge needle. Nuclei and insoluble components were removed by centrifugation (750 x g, 5 min, 4 °C). The supernatant was passed over a steel wool column placed in a magnetic cell-sorting device (Milenyi Biotec, Bergisch-Gladbach, Germany). The flow-through was collected. After washing with homogenization buffer (5 x 1 ml), the column was removed from the magnet for elution of lysosomes. Nondestructive elution was achieved with 5 x 1 ml of homogenization buffer. Destructive elution was performed with 5 x 1 ml of water. Thereafter, each fraction was assayed for the following marker enzymes as described (28): alkaline phosphatase (plasma membrane), lactate dehydrogenase (cytoplasm), NADPH-dependent cytochrome-C reductase (endoplasmic reticulum), galactosyl-transferase (Golgi apparatus), and succinate dehydrogenase (mitochondria). β-Hexosaminidase (lysosomes) was assessed as described (29) using (4-nitrophenyl)-β-D-N-acetylglucosaminide as a substrate. For hEKS, it was found that 60–80% of the preloaded lysosomes were lost into the 750 x g pellet. However, losses of 40–60% were also observed with unlabelled cells. More than 90% of total protein and all nonlysosomal marker enzyme activities were recovered from the flow-through. Only 6 ± 2% of total hexosaminidase activity could be recovered from the nondestructive eluate and were contaminated by mitochondria and some plasma membrane, but sufficiently enriched lysosomal material was eluted destructively. 36 ± 10% of hexosaminidase was found in this fraction, being ± 9-fold enriched (mean of six experiments in all cases) and was contaminated only by trace amounts of mitochondrial and plasma membrane marker activities.

**RESULTS**

**Biosynthesis and Processing of G$_{M3}$AP in hEKS**—hEKS were pulse-labeled with [³⁵S]cysteine for 1 h and chased for 2, 6, 24, and 48 h. G$_{M3}$AP was immunoprecipitated from the medium (Fig. 1A, left panel) and from cell extracts (Fig. 1A, right panel). G$_{M3}$AP was not yet detectable in the medium after the pulse period, whereas an intense single band of 22 kDa was precipitated from the cell extract. After a 2-h chase, an additional band of 24 kDa appeared in the medium as well as in the cells. In the medium, the intensity of this band increased within the next 10 h but then remained constant during a chase time of 48 h. This secreted form of G$_{M3}$AP was accompanied by at least three additional weaker bands of 25, 26, and 27 kDa and by traces of the intracellular 22-kDa form and an 18-kDa form. In the cells, however, the initial band doublet of 22 and 24 kDa persisted for at least 48 h without being further processed.

² G. J. Glombitza, and K. Sandhoff, unpublished observations.

³ E. Becker, G. J. Glombitza, and K. Sandhoff, unpublished procedures.
Removal of the GM2AP-N-glycan by endo H or PNGase F (Fig. 1B) revealed that most of the apparent molecular mass difference between individual GM2AP forms was due to carbohydrate processing events. The early 22-kDa band and the 24–27-kDa forms shared the same 18-kDa peptide backbone, but the 22-kDa GM2AP bore an endo H-sensitive carbohydrate, whereas the N-glycan of the 24–27-kDa GM2AP was endo H-resistant. Experiments with more closely spaced chase times (not shown) demonstrated that about 70% of the 22-kDa GM2AP was converted to the 24–27-kDa forms during the first 6 h of chase. At 6 h of chase, conversion of the 18-kDa peptide to a 17-kDa product was already detectable, indicating the onset of proteolytic processing on the peptide chain of GM2AP. Processing was complete after 24 h, and no endo H-sensitive glycoforms could be detected at this time any more.

For comparison, cathepsin L was subsequently immunoprecipitated from the cells and media (not shown). It was chosen as a representative of soluble lysosomal enzymes not involved into glycosphingolipid metabolism and because it has a unique N-glycosylation site (30), just like GM2AP. After 24 h of chase, a 42-kDa precursor was found in the medium, and two mature forms of 29 and 24.5 kDa (31) were detected intracellularly. The oligosaccharides side chains of all forms were entirely endo H-sensitive.

Fig. 2 summarizes the results for GM2AP schematically. It anticipates some of the observations given in detail later, but it is intended as a guide to the large number of GM2AP forms and their intracellular localization. In hEKS, GM2AP is synthesized as a precursor with a backbone size of 18 kDa after translocation into the ER and removal of the signal peptide. The precursor is cotranslationally glycosylated at its unique N-glycosylation site, yielding a 22-kDa protein with a high mannose type carbohydrate chain (precursor, high mannose (PHM)). Passing through the Golgi apparatus, a significant amount of this early precursor is converted to an endo H-resistant glycoform of 24 kDa, which most likely bears a complex type oligosaccharide (precursor, complex (PC)) and is partially secreted. Glycoforms of 25–27 kDa are generated from a minor fraction of the 24-kDa precursor immediately before its secretion. They presumably bear multiantennary glycan structures (precursor, multiantennary (PMA)), and only trace amounts of them are found inside the cell. A small portion (~5%) of the secreted material consists of 22-kDa GM2AP. The secretion ceases between 6 and 12 h after synthesis, when about one-third of the total amount of precursor present after a 1-h pulse is found outside the cell.

Simultaneously, late processing events occur on the protein backbone as well as on the oligosaccharide of the intracellular forms, suggesting that the bulk of GM2AP is then entering the lysosome. The intermediates of this late processing cover the entire molecular mass range between 20 and 24 kDa. After 24 h, processing has yielded a 20-kDa mature form (M) with a 17-kDa protein backbone. Its carbohydrate side chain is completely endo H-resistant and probably heavily trimmed, since the mass shift between the precursor glycoforms and M is 2 or 4 kDa, but proteolytic processing accounts for only 1 kDa of this apparent loss (compare with Fig. 1).

The half-life of mature GM2AP must be well beyond the chosen chase times, since no significant decrease in overall signal intensity was observed even after 72 h of chase (not shown). Varying amounts (2–4%) of the total secreted material consisted of 18-kDa dP, suggesting that a part of GM2AP had escaped N-glycosylation intracellularly, since a trace of dP was also observed within the cells.

Carbohydrate Phosphorylation of GM2AP—in hEKS were pulse-labeled with [33P]phosphate to assess which glycoform of GM2AP

**FIG. 1.** Processing of GM2AP in hEKS. hEKS were pulse-labeled with [35S]cysteine for 1 h and chased for the times indicated. Media and cell lysates were immunoprecipitated for GM2AP, and the precipitates were separated by SDS-PAGE. A, glycosylated GM2AP; B, GM2AP after glycosidase treatment. The apparent molecular masses and glycosylation states are indicated at the right.

**FIG. 2.** Schematic representation of GM2AP processing in hEKS. The localization of individual glyco and protein forms of GM2AP during the course of transport and processing as determined by metabolic labeling and immunoprecipitation is shown. Thick arrows, major pathways; thin arrows, minor pathways. Approximate transit or conversion times are indicated for each step. Blocking agents are given for transitions that could be inhibited. BafA1, bafilomycin A1.
A 1-h pulse at 37 °C, hEKs were chased at 14 °C for 6 h, which should trap early GM2AP forms by freeing vesicle transport between the ER and the Golgi apparatus (32). Only a trace of Pc was detected in the medium (Fig. 4, lane 1). In the cells, most of the total signal corresponded to P1IM (Fig. 4, lanes 2–4), and no mature form appeared after this chase time. This is consistent with the view that the Golgi type modifications in the oligosaccharide chain of GM2AP are caused by a temperature shift and brefeldin A (BFA) treatment.

After a 1-h pulse at 37 °C, hEKs were chased at 14 °C for 6 h, which should trap early GM2AP forms by freeing vesicle transport between the ER and the Golgi apparatus (32). Only a trace of Pc was detected in the medium (Fig. 4, lane 1). In the cells, most of the total signal corresponded to P1IM (Fig. 4, lanes 2–4), and no mature form appeared after this chase time. This is consistent with the view that the conversion of P1IM to Pc is a carbohydrate processing event caused by Golgi type modifications in the oligosaccharide chain of GM2AP. The small amounts of Pc present at this point of chase were processed to a mature form, which gave a clear indication that GM2AP matures in a compartment distal to the Golgi apparatus. Two additional hitherto unobserved products appeared after the temperature shift, one of 19 kDa and one of 16.5 kDa (Fig. 4, lane 2). Their origin remained obscure, and they disappeared after glycosidase treatment (Fig. 4, lanes 3 and 4), thus revealing that they did not represent M and dM. Release of the temperature block after 24 h fully restored the usual processing pattern (not shown).

An unexpected result was obtained when the cells were exposed to BFA (5 μg/ml), a fungal macrolide antibiotic, which blocks anterograde vesicular transport from the ER but does not interfere with retrograde membrane flow between Golgi and the ER (33). In this respect, BFA would have been predicted to exert essentially the same effects on GM2AP transport and maturation as the 14 °C shift. Fig. 4, lanes 5–14, shows that this was not quite the case. If the cells were treated with BFA during the starvation period, a 1-h pulse, and a 6-h chase, the secretion of GM2AP was completely prevented (Fig. 4, lane 5). Within the cells, however, a band triplet of 22, 23, and 24 kDa was found (Fig. 4, lane 8) instead of the expected PIM. These forms proved to be largely endo H-resistant (Fig. 4, lane 9). After 24 h of chase in the presence of BFA, the triplet had been converted to P1 (Fig. 4, lanes 10 and 11) with the usual dp backbone (Fig. 4, lane 12). This result also supported the view that the proteolytic processing events leading to the generation of the 17-kDa mature peptide must take place in a post-Golgi compartment, presumably in endosomes and/or lysosomes. No alteration of the N-glycan was observed for cathepsin L, the precursor still being in its high mannose form even after 24 h of chase in the presence of BFA (Fig. 4, lanes 17 and 18).

To assess whether the modified glycoevolution pattern had any effect on GM2AP maturation, the cells were chased in the presence of BFA for 24 h from either 24 h in the absence of the drug. In this case, secretion was reconstituted but rose to 50% (Fig. 4, lane 7). In the cells, P1 was processed to mature GM2AP (Fig. 4, lanes 13 and 14), indicating that the complex glycoform of the GM2AP precursor is also competent for lysosomal transport. Checking for the phosphorylation state of the BFA-induced glycoforms, we treated hEKs with BFA during a 6-h pulse with 33P phosphate and a 24-h chase. Only P1IM was phosphorylated.
amounts of P C were secreted into the chase media. After 6 h of chase, GM2AP bears phosphomannosyl residues. This again argued for the view that only a minor subfraction of the GM2AP with ammonium chloride or protease inhibitors. Ammonium chloride induces hypersecretion of lysosomal enzyme precursors by increasing the intraorganellar pH of acidic compartments (34). The elevated pH probably prevents dissociation of the proteolytic step. Treating the cells with pepstatin A (0.1 mM) had no effect on GM2AP maturation.

Processing of GM2AP in the presence of NH4Cl (Fig. 5, left panel). High amounts of P C, M1, and Pm were secreted into the chase media. After 6 h of chase, both P H1M and P C were immunoprecipitated from the cells. Only 10% of total GM2AP remained in the cells after 24 h of chase, which were in the P C form and not processed to mature GM2AP. Similar results were obtained (not shown) when hEKs were exposed to bafilomycin A1, an inhibitor of the vesicular proton pump (38), or to monensin, a Na+ and K+ ionophore, which disturbs transport at a late Golgi stage (39).

In contrast, 60% of the cathepsin L precursor had been secreted after 24 h of chase in the presence of NH4Cl, but the remaining 40% had been converted to the 29-kDa form instead of the usual 21-kDa form. These compounds are potent inhibitors of Golgi mannosidases prematurely terminating the trimming of N-glycans at high mannosic stages (reviewed in Ref. 41). The maturation of GM2AP was not affected by these drugs, but both inhibitors led to the generation of entirely endo H-sensitive glycoforms, including even secreted and mature GM2AP. In conjunction with the results of the BFA treatment presented in Fig. 4, it therefore seemed questionable whether the carbohydrate of GM2AP has any significance for its lysosomal delivery at all. Consequently, we suppressed the cotranslational attachment of its N-glycan by tunicamycin treatment of hEKs. Tunicamycin interferes with the biosynthesis of the dolichol-phosphate precursor of N-linked carbohydrate side chains, thereby globally preventing N-glycosylation (41, 42).

hEKs were treated with tunicamycin (5 μg/ml) during the starvation period and a 1-h pulse with [35S]cysteine. They were chased for 0, 6, and 24 h in the absence of the drug and immunoprecipitated for GM2AP (Fig. 6). Qualitatively, the same GM2AP band pattern was obtained as for deglycosylated samples from pulse-chase experiments conducted without tunicamycin (compare with Fig. 1B). dP was precipitated from the media. The intensity of which increased over 24 h of chase. In the cells, dP appeared after the pulse, showing first signs of proteolytic processing after 6 h of chase and having been completely converted to dM after 24 h. Tunicamycin treatment seemed to have no adverse effect on GM2AP synthesis and stability. Overall protein synthesis dropped to 70% as judged by trichloroacetic acid precipitation of the lysates, but the immunoprecipitable proportion of GM2AP remained constant under these conditions. The combined intensity of dP and dM did not significantly decrease over the chase period. The kinetics of GM2AP transport also seemed to be unaffected compared with the normal situation. However, two additional bands (16 and 14 kDa) appeared, which had not been observed in the absence of the inhibitor and which were not subjected to further proteolytic processing. Again, the secretion of the GM2AP precursor rose to 50% instead of the normal 30%, which had also been observed after release of the BFA block.
Quite another situation was met with cathepsin L (Fig. 6, right four lanes). Here, a nonglycosylated precursor of 38 kDa was synthesized and completely excreted into the medium. Similar results were obtained for cathepsin D (not shown).

This experiment indicated that an alternative pathway for lysosomal targeting exists in hEKs, which allows effective delivery of G32AP to the lysosome even when no N-glycan is attached to its protein backbone. This mechanism obviously does not operate on the precursors of cathepsins L and D.

In an attempt to determine whether the transport of G32AP or cathepsin L is a membrane-associated process, we tried to recover their precursors from the cellular scaffold of hEKs differentially permeabilized with saponin according to the method of Rijnboutt et al. (19). However, G32AP as well as cathepsin L could only be detected in the saponin supernatant, not in the membrane pellet (not shown).

**Lysosomal Localization of Mature G32AP by Magnetic Isolation of hEK Lysosomes**—To prove the lysosomal localization of processed G32AP, hEK lysosomes were isolated by magnetic fractionation.

For cold experiments, hEKs were preloaded for 24 h with a probe of colloidal iron coupled to dextran, which was ingested by nonspecific endocytosis. After a chase of at least 10 h, the cells were lysed, a 750 × g supernatant was subjected to magnetic separation of lysosomes as described under “Experimental Procedures.” The combined flow-through and wash fractions (flow-through) as well as the destructively eluted fractions (eluate) were immunoprecipitated for G32AP, and the samples were separated by SDS-PAGE. The positions of individual G32AP forms are given at the right, with designations according to Fig. 1. *, sample carryover from neighboring lanes, which have been removed. G32AP distribution and enrichment are indicated below the panel and were calculated from the immunoprecipitable proportion of G32AP relative to total protein.

![Diagram](image)

**Fig. 7. Lysosomal localization of G32AP.** hEKs were pulse-labeled with [35S]cysteine for 5 h in the presence or absence of 5 μg of tunicamycin/ml of medium as indicated. The cysteine label was chased by loading the cells with s.c. in complete medium for 14 h. Afterward both the magnetic probe and the cysteine label were chased for another 10 h. The cells were subjected to magnetic separation of lysosomes as described under “Experimental Procedures.” The combined flow-through and wash fractions (flow-through) as well as the destructively eluted fractions (eluate) were immunoprecipitated for G32AP, and the samples were separated by SDS-PAGE. The positions of individual G32AP forms are given at the right, with designations according to Fig. 1. *, sample carryover from neighboring lanes, which have been removed. G32AP distribution and enrichment are indicated below the panel and were calculated from the immunoprecipitable proportion of G32AP relative to total protein.

For the localization of nonsglycosylated G32AP, hEKs were pulse-labeled with [35S]cysteine for 5 h in the presence or absence of tunicamycin. They were chased for 14 h in the presence of the magnetic probe and for another 10 h in its absence. Fig. 7 shows that mature G32AP could be immunoprecipitated from the flow-through and, more significantly, from the lysosomal fraction of the magnetic separation, regardless of whether it was glycosylated or not. G32AP was 2-fold depleted from the flow-through and about 45-fold enriched in the destructive eluates from these separations, which essentially parallels the hexosaminidase enrichment given above.

In this manner, we showed that in hEKs, G32AP is properly targeted to the lysosome even if no N-glycan is cotranslationally attached to its unique N-glycosylation site.

**Endocytosis of G32AP**—We examined whether the carbohydrate would be of any relevance for the uptake of exogenously added G32AP by offering the [35S]cysteine-labeled secretions of ammonium chloride-treated hEKs to unlabeled cells for a 24-h period in the presence of M6P or glucose-6-phosphate (G6P), 1 mg/ml yeast mannan, 1 mg/ml asialo-orosomucoid (ASOM), or a combination of G6P and the latter two at the above concentrations, respectively (indicated at the bottom). Afterward, the cell lysate and 10% of the medium were immunoprecipitated for G32AP (left panel). The untreated controls and M6P-treated cells were also immunoprecipitated for cathepsin L and hexosaminidase β-chain (right panel), and the samples were separated by SDS-PAGE. The positions of individual protein forms are indicated on both sides of each panel. Hexosaminidase bands were assigned according to Ref. 61. α, traces of hexosaminidase α-chain coprecipitating with β-chains from heterodimeric hexosaminidase A; ββ(2), singly glycosylated form of ββ peptide (62), s, molecular mass standard (14.3, 21.3, and 30 kDa are visible in the left panel; 14.3, 21.3, 30, 46, and 69 kDa are visible in the right panel).

Three additional G32AP bands appeared in the medium besides PC and PMA, which were assigned to PHM, dP, and an unknown component according to their molecular masses. The endocytosis of G32AP was not significantly affected by any of the inhibitors (Fig. 8, *left panel*). Under M6P, glucose-6-phosphate, mannan, and asialo-orosomucoid alone, the uptake was 95–120% of the untreated control. It dropped to 75% in the presence of the inhibitor mixture, which we believe to be a nonspecific effect due to overloading of the serum-free medium with unusual additives, since none of the inhibitors had an adverse effect when applied alone. The endocytosis of cathepsin L was only slightly affected by M6P, whereas the uptake of hexosaminidase β-chain was almost completely inhibited in the presence of M6P (Fig. 8, *right panel*). The relative molar concentrations offered in the media were calculated to be 4.4:1:1.1 for G32AP, cathepsin L, and hexosaminidase β-chain, respec-
GM2AP precursor is secreted, and more than 90% of these to a complex type oligosaccharide. About one-third of the hexosaminidase kDa, it is very likely that the signal peptide of GM2AP is independent of the known N-ments) of the offered GM2AP was recovered from the cells after 24 h in this case.

Both findings indicate that endocytosis of GM2AP is largely independent of the known N-glycan-linked signals for receptor-mediated endocytosis in hEKs.

DISCUSSION

In this article, we have clearly established that in hEKs, GM2AP is synthesized as a 22-kDa precursor (P21M) bearing a single high mannose N-linked oligosaccharide chain on a peptide backbone of 18 kDa. In the Golgi apparatus, at least 70% of P21M is converted to 24-kDa PC by remodeling of the N-glycan to a complex type oligosaccharide. About one-third of the GM2AP precursor is secreted, and more than 90% of these secretory forms consist of PC. The intracellular remainder is segregated from the secretory pathway and processed in a post-Golgi compartment to yield a 20-kDa mature form (M), which bears an endo H-resistant N-glycan on a 17-kDa peptide (summarized in Fig. 2).

Thus, the early and late processing of GM2AP shows many features typical of soluble lysosomal enzymes (reviewed in Ref. 44). It is synthesized as a precursor protein, the N-glycan of which receives Golgi type modifications. It is partially secreted into the medium, and it becomes subject to proteolytic and glycolytic processing events in acidic compartments after segregation. The molecular mass of the major secreted form (PC, 24 kDa) was in agreement with earlier findings (10). Regarding the increased half-width of the mature GM2AP band, one may assume that M is microheterogeneous in its N-glycan and/or its protein backbone. It should be noted in this context that two protein species were also present in M purified from human kidney: one major form with Phe34 and a minor one with Ser32 as the amino terminus (45). The molecular mass found for dM (17 kDa by SDS-PAGE) is close to its calculated molecular mass from cDNA and protein sequencing data (17,369 Da starting from Phe34). Since dP has an apparent molecular mass of 18 kDa, it is very likely that the signal peptide of GM2AP is removed on entry into the ER. The algorithm of von Heijne (46) predicts signal peptidase cleavage sites at Gin22 and His24, leading to precursors of 18,561 or 18,362 Da, respectively.

Very recently, a report by Wu et al. (47) proposed that an alternatively spliced form of GM2AP (Met1-Glu142 plus Val-Ser32 as the amino terminus) may exist in human placenta and fibroblasts, which in vitro has an activating effect on cestrialidase but not on hexosaminidase. Taking into account the expected decrease in molecular mass of such a splice variant (~5 kDa for all forms), we re-screened our fluorographs for the presence of GM2AP (PA). At least for hEKs, we were not able to detect GM2AP-related products in the relevant molecular mass window. Shortened GM2AP forms appeared only when inhibitors of early transport (see Fig. 4, lane 2) or tunicamycin (see Fig. 6) were used. However, they were not processed in the same manner as full-size GM2AP, and we assume that they represent prematurely and aberrantly processed forms arising from the presence of the inhibitors.

P21C first appears in the medium 60–90 min after synthesis, a value that is typical for other lysosomal enzymes too (44). In hEKs, however, its secretion does not cease before 12 h after synthesis, and its final maturation takes more than 12 h. Similar kinetics were observed for cathepsin L. It seems that segregation and transport of at least these two lysosomal proteins is somewhat delayed between late Golgi and lysosomal compartments. To our knowledge, this is the first study dealing with the processing of lysosomal proteins in hEKs. Since the maturation of lysosomal protein precursors occurs in ranges from minutes to days depending on cell type and species (44), we attribute these slowed-down kinetics to a yet uncharacterized effect specific to hEK.

Furthermore, we have shown that GM2AP is targeted to the lysosomes by a M6P-independent pathway in hEKs. After Klima et al. (12) had found that recombinant, nonglycosylated GM2AP is effectively endocytosed by human fibroblasts, the question arose whether its intracellular transport would also be at least partially independent of M6P or other carbohydrate-linked signals. Several different lines of evidence demonstrated that such a pathway must exist in hEKs.

First, we have shown that only a minor fraction of GM2AP, possibly 10%, bears phosphomannosyl residues, and that this tag is confined to P21M. Specifically, at least 70% of P21M are converted to P21S and since 70% of GM2AP precursors are retained intracellularly, a significant proportion of M should be derived from P21C. The endo H resistance of M also suggests that it may be derived mainly from a precursor with complex type oligosaccharides. It has to be kept in mind, however, that extensive trimming of mannose-rich N-glycans may abolish recognition by endo H (44). In addition, we could trap P21C exclusively by BFA treatment and observed that it is correctly processed to M on detoxification of the cells (Fig. 4, lanes 13 and 14). Under BFA, the oligosaccharide of P21M was remodeled almost instantaneously by the action of Golgi glycosidases and glycosyltransferases, which retain at least part of their activity in the fused ER-Golgi compartment (49). It may well be that the underphosphorylated N-glycan of GM2AP is a preferred substrate for such modifications, since M6P serves as a protecting group against Golgi glycosidase trimming (44, 48, 50).

Indeed, the efficiently phosphorylated carbohydrate of cathepsin L would be under no circumstances converted to a complex form in hEKs.

Second, only P21C is hypersorbed on NH4Cl treatment. For enzymes sorted via M6P receptors, NH4Cl interferes with sorting in a post-Golgi compartment, and the secretions are highly enriched in phosphorylated precursor glycoforms (35). Therefore, it is likely that P21C passes a similar base-sensitive compartment, and the high selectivity by which NH4Cl induces its hypersorption and not that of P21M suggests that it is mainly P21C that moves through this compartment. P21C would then be the dominant precursor to M, implying that the M6P receptor system possibly plays only a minor role in the segregation of GM2AP, since P21C does not bear phosphomannosyl residues.

Finally, and most strikingly, we found that GM2AP was correctly targeted at an apparently normal rate even when its glycosylation had been suppressed by tunicamycin treatment (Figs. 6 and 7), suggesting that the lysosomal targeting signal resides on its protein backbone rather than on its carbohydrate. Interestingly, the secretion of the GM2AP precursor rose from 30 to 50% under tunicamycin. This difference is close to the 10% value estimated to be the mannose-6-phosphorylated fraction of GM2AP. Usually, tunicamycin treatment strongly enhances the secretion of lysosomal enzymes transported in re-
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...response to the M6P system (51–53), which then are shed from the cells as unprocessed precursors (52, 53). In this respect, the nonglycosylated precursors of cathepsin L and D behaved as expected.

Thus, the intracellular transport of GM2AP adds to an increasing number of reports about correct targeting of lysosomal enzymes independent of phosphomannosyl residues. Such phenomena have been described for cathepsin D in HepG2 cells (14, 19), in breast cancer cells (15), and in ICD lymphoblasts (16), as well as for β-asparaginylglucosaminidase (17) and α-glucosidase (18) in transfected COS1 cells. In the cases of cathepsin D (14, 15, 19, 54), α-glucosidase (18), glucocerebrosidase and sulfatide activator protein (19), and cathepsin L (55), a M6P-independent membrane association of the precursors was observed. We could not demonstrate a membrane association of the GM2AP precursor by saponin permeabilization, but we cannot exclude a weak interaction, which may be disrupted by this treatment. For α-glucosidase, the membrane association is mediated by an uncleaved signal peptide (18). However, our data indicate that the signal peptide of GM2AP is instantaneously removed on entry into the ER.

Since the function of GM2AP is to serve as a lipid-binding protein (1), and since GM2AP is able to transfer gangliosides from donor to acceptor liposomes in vitro (56), an intriguing option would be that it is transported and segregated in direct association to the lipid bilayer. Such an interaction has been proposed for glucocerebrosidase, which does not bear M6P residues (57) and is possibly bound to acidic phospholipids (58).

Comparing the transport of GM2AP and cathepsin L in HEKs, it seems that two different targeting mechanisms operate on these proteins. The efficient segregation of cathepsin L is crucially dependent on the presence of an N-glycan, which is not the case for GM2AP. On the other hand, acidification of the transporting machinery is essential for the delivery of GM2AP, whereas it is less substantial to the targeting of cathepsin L. In this respect, each of both proteins displays one feature typical of M6P-dependent and one typical of M6P-independent transport. Obviously, the unknown recognition system responsible for GM2AP segregation shows a similar pH dependence as the M6P-receptor system. The transport of cathepsin L might proceed in a manner analogous to myelocytes and macrophages, in which base-sensitive and base-insensitive packaging mechanisms are known to exist in parallel (reviewed in Ref. 44).

The endocytosis of GM2AP is also independent of the known signals for carbohydrate-mediated internalization (Fig. 8). We had expected that M6P would not affect its uptake, since more than 90% of the offered secretions are made up of PC and the P3MA, which are not phosphorylated. However, its endocytosis remained equally unaffected by inhibitors of mannose and asialoglycoprotein receptors. Nonglycosylated GM2AP is also internalized, as had already been shown earlier for human fibroblasts (12), but we were surprised to find that its uptake seemed almost twice as effective as for the glycosylated form. A similar observation has been made for the sphingolipid activator protein precursor in fibroblasts (24), and it has been suggested that the carbohydrate might partially mask an epitope involved in recognition between this protein and a yet unknown receptor system on the cell surface. It may again be speculated that GM2AP reaches the interior of the cell by binding to lipid components of the cell surface.

In the granular layer of the epidermis, keratinocytes start to form acidic lamellar granules, which contain precursors of specialized stratum corneum lipids, lysosomal hydrolases, and a vesicular ATP-dependent proton pump (reviewed in Refs. 59 and 60). The contents of these storage bodies is eventually shed into the uppermost, cornified layer of the epithelium. The present work was performed with basal keratinocytes, in which the basal characteristics were maintained by culture at low calcium concentrations (0.1 mM), suppressing the onset of terminal differentiation. Further experiments, however, may address the question of whether GM2AP is also targeted to the lamellar granules in differentiated keratinocytes and whether this targeting involves the same lysosomal type delivery mechanism as observed in basal keratinocytes, or whether a separate routing strategy is used. Additionally, future work may reveal the nature of the mechanisms responsible for the M6P-independent intracellular targeting and uptake of GM2AP and whether both of these processes are mediated by the same or different targeting systems.
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