Two Mechanisms for the Recapture of Extracellular G_{M2} Activator Protein: Evidence for a Major Secretory Form of the Protein†

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ABSTRACT: The G_{M2} activator protein is a small monomeric protein containing a single site for Asn-linked glycosylation. Its only proven *in vivo* function is to act as a substrate specific cofactor for the hydrolysis of G_{M2} ganglioside by lysosomal β-hexosaminidase A. However, we and others have shown it can act as a general glycolipid transporter at neutral pH *in vitro*. Any other possible *in vivo* functions would require that some of the newly synthesized activator molecules not be targeted to the lysosome. The lysosomal targeting mechanism for the activator has not been conclusively identified. While earlier reports suggested that it is likely through the mannose-6-phosphate receptor, another more recent report demonstrated that deficient human cells could recapture nonglycosylated, bacterially produced activator, suggesting its use of an alternate targeting pathway. Here, we demonstrate that the mannose-6-phosphate pathway is likely the major intracellular, biosynthetic route to the lysosome, as well as a high affinity recapture pathway for the endocytosis of activator protein from extracellular fluids. Additionally, we show that there exists a second lower affinity recapture pathway that requires its native protein structure, is carbohydrate independent, and likely does not involve its ability to bind glycosphingolipids in the plasma membrane. Finally, we document that the pool of newly synthesized precursor activator protein contains a majority of molecules with a complex-type oligosaccharide, which cannot contain a functional mannose-6-phosphate targeting signal. These molecules makeup the secreted forms of the protein in normal human fibroblasts.

Hydrolysis of G_{M2} ganglioside (G_{M2})[1] to G_{M3} ganglioside involves the hydrolysis of the terminal nonreducing β-linked GalNAc residue. This reaction requires the participation of three separate gene products. Two of these are the α and β subunits of the lysosomal heterodimeric β-hexosaminidase A (Hex A). The third is the small, monomeric globular protein called G_{M2} activator protein (activator), which severs as a substrate specific cofactor for Hex A. The critical *in vivo* role that is played by the activator protein is demonstrated by the occurrence of the AB variant form of G_{M2} gangliosidosis, a severe lysosomal storage disease cause by the lack of a functional activator protein [reviewed in Gravel et al. (1995)].

The major intracellular transport pathway for most lysosomal proteins which do not contain a transmembrane domain, *e.g.*, Hex, is via the mannose-6-phosphate receptor (MPR). Like all lysosomal proteins, the activator is synthesized in the rough endoplasmic reticulum (RER). In the ER, Asn-linked glycosylation also occurs at selected Asn-X-Ser/Thr sites (Kornfeld & Kornfeld, 1985). Glycosylation may be followed by the addition of phosphate markers to one or more high mannose-type oligosaccharides in order to specifically target the protein to the lysosome (Bach et al., 1979; Kornfeld, 1986). Targeting is accomplished through interactions with one of two, cation-dependent and/or cation-independent MPRs (CD-MPR and CI-MPR) in the trans-Golgi (Griffiths et al., 1988) (biosynthetic route). However, there is usually a small percentage of each mannose-6-phosphate (M6P)-containing lysosomal protein that fails to interact with the MPR and is secreted. This percentage becomes large in the case of cells transfected with a cDNA encoding a lysosomal enzyme, presumably because its overexpression saturates the MPRs (Anson et al., 1992). The larger (270 kDa) CI-MPR also serves as the insulin-like growth factor II (IGF-II) receptor on the plasma membrane (Goda, 1988). This additional cellular localization allows the CI-MPR to recapture M6P-containing proteins from extracellular fluids and transport them to the lysosome (endocytotic route).

The importance of the MPR system is evident from the occurrence of two related inherited autosomal recessive diseases known as I-cell disease and pseudo-hurler polydystrophy. These diseases are caused by defects in the UDP-*N*-acetylglucosamine-1-phosphotransferase: lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase, the lysosomal-targeting enzyme (Nolan & Sly, 1989). Although the MPR system is vital, its involvement with targeting

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[1] Abbreviations: activator, G_{M2} activator protein; Hex A, hexosaminidase A; G_{M2} ganglioside, GalNAcβ(1−4)-[NANAα(2−3)]-Galβ-(1−4)-Glc-ceramide; G_{M3} ganglioside, NANAα(2−3)-Galβ(1−4)-Glc-ceramide; MPR, mannose-6-phosphate receptor; M6P, mannose-6-phosphate; ER, endoplasmic reticulum; SDS−PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; CHO, Chinese Hamster Ovary; CRM, cross-reacting material; MEM, minimum essential media; FCS, fetal calf serum.

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soluble lysosomal proteins is neither exclusive nor universal. There are at least four major types of exceptions (1) some enzymes which lack a transmembrane domain are nonetheless fully transported to the lysosomes of I-cell fibroblasts, *e.g.*, glucocerebrosidase, which is not phosphorylated in normal cells (Aerts et al., 1988; Miller et al., 1981). (2) Some proteins, despite containing the proper mannose-6-phosphate tag, are still primarily secreted by certain cell types. The best studied of this type of protein is cathepsin L (Lazzarino & Gabel, 1990; Sahagian & Gottesman, 1982). (3) While some tissues (*e.g.*, skin fibroblasts) from I-cell patients are deficient in most soluble lysosomal enzymes others (*e.g.*, lymphoblasts, liver, and kidney) contain near normal levels (Nolan & Sly, 1989). (4) Even in tissues where these enzymes are deficient, they are not totally absent (Nolan & Sly, 1989).

One possible MPR-independent sorting mechanism has been suggested from the observation that the single precursor polypeptide of the four other sphingolipid activator proteins (SAP A-D, produced by proteolysis of the precursor in the endosome/lysosome), pro-saposin (not structurally related to the G_{M2} activator), forms a membrane-associated complex with cathepsin D and glucocerebrosidase, which facilitates their transport to the lysosome (Rijnboutt et al., 1991; Zhu & Conner, 1994). Previous data on the types of carbohydrate moieties present in the mature (lysosomal) saposins indicated the presence of both complex and high mannose forms (Ito et al., 1993; Yamashita et al., 1990). Recently, it has been reported that prosaposin contains M6P. However, the report concludes that its biosynthetic route to the lysosome is only partially dependent on this signal. Additionally, the authors conclude that its endocytotic route is through a totally carbohydrate-independent mechanism. They also suggest that the signal for endocytosis is protein-based and not associated with the protein's ability to bind glycosphingolipids on the plasma membrane (Vielhaber et al., 1996).

The two major Hex isozymes have been well studied in both normal and I-cell fibroblasts, and they behave in the manner typical of most MPR-transported lysosomal enzymes (Miller et al., 1993; Van Elsen & Leroy, 1979; Vladutiu, 1984; Vladutiu & Rattazzi, 1981). However, similar studies of the activator have not been reported. Two previous reports have suggested that the activator is transported to the lysosome via a MPR. First, there was a 2.5-fold increase in the amount of activator in the serum of a single I-cell patient, and secondly, there was a similar 2.5-fold increase in the secretion of the activator from normal fibroblasts (although the intracellular level was reported to be unaffected) grown in the presence of NH_4Cl (Banerjee et al., 1984). NH_4Cl specifically causes the secretion of proteins normally targeted to the lysosome through the MPR pathway by preventing endosomal acidification and the uncoupling/recycling of the MPRs, as well as other receptors (Burg et al., 1985). However, these data are far from conclusive as the secretion of other MPR-targeted enzymes is increased 10−50-fold with a concomitant loss of intracellular enzyme in both circumstances (Creek et al., 1983; Wiesmann et al., 1971). Recently it was demonstrated that *Escherichia coli* expressed activator (nonglycosylated) could be recaptured by AB-variant fibroblasts (Klima et al., 1993), suggesting that either the activator is not normally transported via the MPR or it is able to enter the cell through a second endocytotic pathway. Additionally, the activator protein like cathepsin L (described above) contains only a single site for Asn-linked oligosaccharide attachment (Fürst et al., 1990; Xie et al., 1991). Interestingly, while the location of the oligosaccharide, which is the primary site of phosphorylation in the human α and β subunits of Hex (Sonderfeld-Fresko & Proia, 1989; Weitz & Proia, 1992), is conserved in the aligned Hex sequences from mouse, cat, and boar (unpublished observation), the position of the single oligosaccharide sites in human and mouse activator do not align (Bellachioma et al., 1993). Because phosphorylation is dependent on a certain protein conformation and the location of a nearby oligosaccharide (Baranski et al., 1992; Cantor et al., 1992; Cantor & Kornfeld, 1992), this lack of conservation suggests that the activator site may not be efficiently phosphorylated.

In this report, we identify the MPR pathway as the likely, major biosynthetic route for the incorporation of the activator in lysosomes. We also demonstrate that a large percentage of the newly synthesized activator does not contain the M6P tag and is normally secreted. Finally, we confirm the presence of a secondary, lower affinity mechanism for the recapture of the activator from the extracellular medium and characterize some of its requirements. This mechanism is likely the primary endocytotic route for the activator.

MATERIALS AND METHODS

Generation of a CHO Cell Line Permanently Transfected with pEF-NEO-ACT. A 5′-*Bam*HI-*Bam*HI-3′ fragment containing the complete coding sequence of the activator was obtained by the polymerase chain reaction (PCR). Purified plasmid pEF-NEO was provided by Dr. Don Anson (Anson et al., 1992), Department of Chemical Pathology, Adelaide Children's Hospital, Adelaide, SA, Australia. The vector pAct1 (Xie et al., 1991) was used as a template for PCR. One 83 nucleotide 5′-primer and one 26 nucleotide 3′-primer were synthesized. The sequence of the 5′ primer was 5′-CGGGATCCCCCAGCTACAGTCGGAAACCA-TCAGCAAGCAGGTCATTGTTCCAACATGGC-GTCCCTGATGCAGGCTCCCCTCCTG-3′, where the first 53 bp consists of a *Bam*HI linker sequence, underlined, and a 5′ noncoding region (enhancer), which may increase the mRNA translational efficiency (Anson et al., 1992; Potter et al., 1984). Nucleotides 54−83 correspond to positions 1−30 of the G_{M2} activator cDNA. The sequence of the 3′ primer was 5′-GGATCCTGGGAGTTTGGCCTTGGCAA-3′, which corresponds to positions 646−665 and a *Bam*HI linker sequence. The reaction mixture for PCR contained 10 mM each of the four deoxynucleotide triphosphates (dNTP), 0.5 μg of each of the primers, 20 ng of template DNA, and 1.5 units of Taq polymerase (Bio/Can Scientific Inc.) in 50 μL of Taq buffer. PCR was performed in 30 cycles each of 30 s at 94 °C, 30s at 58 °C and 90s at 72 °C, using the conditions recommended by Perkin-Elmer Cetus Corporation. The PCR-amplified product was electrophoresed in 0.8% (w/v) agarose gel, visualized by ethidium bromide staining, and extracted by gene clean (Bio/Can Scientific Inc.). The PCR product was then incubated with 10 units of T4 polynucleotide kinase (Pharmacia) in 1× T4 polynucleotide kinase buffer containing 10 mM of ATP at 37 °C for 1 h, then 6 units of Klenow fragment of polymerase I (Gibco BRL) were added and 0.5 mM each of four dNTP followed by incubation at room temperature for another 2 h. The PCR product (complete coding region of the activator with enhancer) was cloned into pEF-NEO at a preinserted *Bam*H1 site. Colonies containing recombinant plasmid were selected and sequenced by the dideoxy chain-

termination method (Sanger et al., 1977) to confirm that they contained the correct nucleotide sequence.

Transfection of pEF-NEO-ACT in CHO Cells and the Isolation of a Clone Producing High Levels of Activator Protein. pEF-NEO-ACT (above) was transfected into CHO cells by the "Calcium Phosphate Transfection System" as per the manufacturer's recommendations (BRL Life Technologies, Inc). About 2.0 μg of supercoiled pEF-NEO-ACT was used to transfect a 100 mm plate of CHO cells at ~60% confluency. The cells were maintained in selection media containing 0.8 mg/mL (total, 400 μg/mL active) of G418 (Sigma). Nontransfected CHO cells were used as selection controls. The control cells died after ~2 weeks in the selecting media. The cells remaining in the plate that were transfected were split 1 in 20 into selecting media. Individual clones appeared after 1 week. Colonies were picked with cloning rings and grown in 24-well dishes (Flow Laboratories, Inc.). Western blot analysis was performed as previously described (Xie et al., 1992) to identify the clone for large-scale expression that secreted the largest amount of activator into its medium.

Purification of the Activator Secreted by Transfected CHO Cells. The medium from transfected CHO cells was passed through an octyl-sepharose column. The activator was then eluted using 1% octylglucoside. Final purification was achieved by molecular sieve chromatography on Sephacryl S-200. The various buffers and conditions used have been previously described for the purification of activator from human kidney (Novak & Lowden, 1994).

Production of the His_6-G_{M2} Activator Fusion Protein from E. coli. With minor modifications, we used the same basic methods for the synthesis by *E. coli*, purification, and refolding of a His_6-factor X-G_{M2} activator fusion protein (His_6-activator) as reported by Klima et al. (Klima et al., 1993). These modifications have been previously reported (Smiljanic-Georgijev et al., 1997).

Recapture of the Activator from the Media by Fibroblasts from an AB-Variant Patient. A fibroblast cell line from a 16 month old black female patient with the AB-variant form of G_{M2} gangliosidosis, homozygous for a mutation that causes a Cys^{138} to Arg substitution in the G_{M2} activator protein (GM01675) (Schröder et al., 1991; Xie et al., 1992), was obtained from the Human Genetic Mutant Cell Line Repository, Camden, NJ. This substitution mutation renders the cells CRM negative for the mature activator (Xie et al., 1992). Normal fibroblasts, line 3858, were provided by the Department of Genetics Cell Culture Service, The Hospital For Sick Children, Toronto, Ontario. All cell lines were maintained at 37 °C in 5% CO_2 in α-MEM (minimum essential medium, Gibco-BRL), supplemented with antibiotics and 10% FCS (fetal calf serum, v/v). Varying amounts of purified activator from transfected CHO cell media were added to 10 mL of media with and without 10 mM M6P in which fibroblasts from the AB-variant patient were growing to produce a final concentration between 90 and 460 nM. The cells were grown in this media for 2 days, harvested, washed, lysed by freeze−thawing, and then analyzed by Western blot (Xie et al., 1992). A similar procedure (without added M6P) was carried out using the nonglycosylated, refolded, functional wild-type activator MRGS$(H)_6$GSIEGR-S^{32}-I^{193} (Ni^{2+} binding and vector sequences encode the first 16 residues, residues S^{32}-I^{193} correspond to the mature activator) and a nonglycosylated, refolded, nonfunctional, mutant, truncated form MRGS$(H)_6$GSIEGR-S^{32}-L^{157}-WSCPVGSPPGTTA (the last 13 residues result from a frame-shift mutation in the activator cDNA) produced in bacteria (Smiljanic-Georgijev et al., 1997) except that the final concentration of the activator in the media was 500−600 nM. Finally, 5 nmol of the nonglycosylated wild-type activator was preincubated with two amounts of G_{M2} ganglioside, 10 or 100 nmol, in 500 μL of α-MEM without FCS. The G_{M2} ganglioside was first dissolved in methanol−chloroform (3/1), the necessary volume for the preincubation was dried under nitrogen, redissolved in α-MEM, and sonicated for 20 s before the addition of the activator. After 2 h at 37 °C, each mixture was added to separate wells of AB-variant fibroblasts cultured in 5 mL of α-MEM supplemented with antibiotics and 10% FCS.

Analysis of the Distribution of Activator Molecules from Normal Human Fibroblasts with a High Mannose or a Complex-Type Oligosaccharide Moiety: (A) Isolation of a Semipurified Sample of Newly Synthesized Activator from Normal Human Fibroblasts. Normal human fibroblasts were grown (24× P150 dishes) in α-MEM containing 10% FCS and antibiotics until they reached confluency. Then, 10 mM NH_4Cl was added to the medium. After 2 days, the medium was replaced by CHO SFM II (serum free medium, Gibco-BRL) medium plus 10 mM NH_4Cl. The cells were grown, and the medium was collected and replaced at days 7 and 12 of culture. The pooled medium (~1 L) was cleared (centrifuged) and applied to a 4.5 mL octyl-sepharose (Pharmacia) column. The column was washed with 200 mL of 10 mM sodium phosphate buffer, pH 6, containing 0.1 M NaCl and eluted in the same buffer containing 0.75% octylglucoside (1 column volume was passed through and column elution stopped for at least 4 hours, then the elution was continued). The pooled fractions containing semipurified activator protein (OD_{280}, total yield 1.3 mg) were concentrated to 200 μL in a Centricon-10 (Amicon). This procedure was repeated without the addition of NH_4Cl to the cellular medium.

(B) Glycosidase Digestion. The effects on the apparent M_r of the semipurified activator from the medium of cells grown in NH_4Cl after treatment with each of three glycosidases dissolved in 10 mM sodium phosphate buffer, pH 5, were determined. The enzymes used were (a) *N*-Glycosidase F, (b) Endoglycosidase F (*N*-Glycosidase F-Free), or (c) Endoglycosidase H. Each digestion was conducted in duplicate as follows. First, the denaturation of 2.5 μL (16 μg) of semipurified activator (above) was accomplished by addition of 5 μL of denaturing buffer [1.2% SDS (w/v), 3% β-mercaptoethanol (v/v), 12% octyl-β-glucoside (w/v)]. Depending on the enzyme used (points a−c above), the denaturing buffer was either (a) not diluted, (b) diluted 1:2, or (c) diluted 1:4 with 10 mM sodium phosphate buffer, pH 5. Then the samples were heated at 60 °C for 20 min and 8 μL of more phosphate buffer was added. Second, the appropriate amount of each glycosidase (a) 0.4 unit in 12 μL, (b) 0.2 unit in 10 μL, or (c) 0.004 unit in 10 μL was added to the cooled samples. Third, leupeptin, 1 μg/μL of reaction mix, was added to one of each duplicate. Finally, all samples were incubated overnight at 37 °C. These digestions were repeated for the enriched activator from the medium of cells not grown in NH_4Cl; however, no duplicate samples with leupeptin added were made. Each digestion was then analyzed by Western blot (Xie et al., 1992). Ribonuclease B and carboxypeptides Y (yeast) were used as positive controls for endoglycosidase H digestion of high

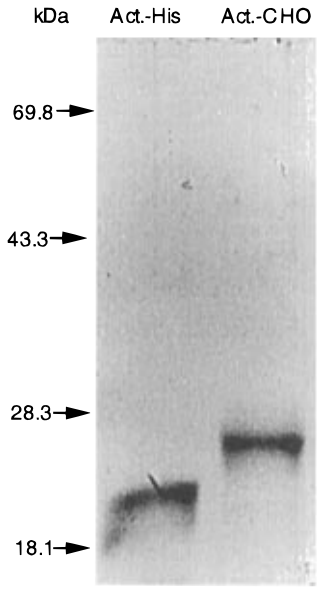

FIGURE 1: SDS−PAGE analysis of purified activator protein from transformed bacteria, Act.-His, and the medium of transfected CHO cells, Act.-CHO. The gel was stained with Coomassie Blue. The positions of the various M_r markers used are indicated on the left.

mannose oligosaccharides (Boehringer Mannheim, Inc.). In this case, the section of the gel containing the separated, digested, and undigested controls was cut out prior to blotting and stained with Coomassie blue.

RESULTS

The media (800 mL) from 20 P-150 culture plates containing the transfected CHO cell clone expressing the human activator yielded about 1 mg of purified activator (Figure 1). Functional assays with purified Hex A and labeled G_{M2} ganglioside demonstrated that its functionality is 1.9-fold higher than that of the activator synthesized in bacteria, purified (Figure 1), and refolded (Smiljanic-Georgijev et al., 1997).

At low concentrations of the purified CHO cell-produced activator (90 nM), the recapture of the protein by AB-variant fibroblasts was nearly totally blocked by the presence of 10 mM M6P (Figure 2, lanes 5 and 6). However, when the concentration of the activator was increased ~5-fold, significant amounts of the activator were recaptured by the cells, even in the presence of 10 mM M6P (Figure 2, lanes 1 and 2). Whereas the recapture of the activator in the presence of 10 mM M6P appeared to be increasing in a dose-dependent manner, recapture without M6P appeared to be nearly saturated, even at the lowest concentrations (Figure 2). Thus, there are two mechanisms for the recapture of activator functioning in human fibroblasts, a high affinity mechanism, which is inhibited by M6P, and a lower affinity mechanism, which is not.

Since each activator contains only one potential site for N-linked glycosylation (Xie et al., 1991), it was possible to determine the percentage of molecules containing a high mannose versus a complex-type oligosaccharide by incubating samples with various endo-glycosidases. Activator was isolated from the media of human fibroblasts grown in the presence or absence of NH_4Cl and semipurified. The samples were then incubated with either glycopeptidase F as a control for the location of the deglycosylated products [*i.e.*, cleaves all types of Asn-linked oligosaccharides, endoglycosidase F free of any glycopeptidase F (cleaves high mannose and hybrid, and at a slower rate, biantennary, but not tri- or tetraantennary complex-type oligosaccharides)] or endoglycosidase H (cleaves high mannose and hybrid, but not complex-type oligosaccharides) (see the information sheets from Boehringer Mannheim, Inc. http://biochem.boehringer-mannheim.com/ for specificities). In the case of the activator pool from NH_4Cl-containing medium, endoglycosidase F and endoglycosidase H treatments removed some, but not all of the oligosaccharide from the activator molecules present. Thus, the majority of these activator molecules contain a complex rather than a high mannose-type oligosaccharide (Figure 3A). A small amount of unglycosylated activator could also be seen in the untreated lane (Figure 3A). The pool of activator molecules from the medium lacking NH_4Cl differed in two ways. There was an apparent increase in the proportion of nonglycosylated activator present and there was no detectable increase in the amount of the nonglycosylated form after endo-H treatment (Figure 3B). In this experiment, positive controls were included to confirm that the endo-H was functional (Figure 3C).

In order to determine the requirements for the low affinity recapture system, we used nonglycosylated, refolded wild-type activator produced in bacterial (Figure 4, Act.-His) and a nonfunctional, truncated form of the activator produced by the same system (Smiljanic-Georgijev et al., 1997) as a control (Figure 4, Act.-T). The two forms of the activator were compared at concentrations similar to the upper limits evaluated for the glycosylated CHO cell protein (Figure 2). After 2 days in media containing ~500 nM of functional activator, an amount nearly equivalent to that contained in normal fibroblasts was found inside the AB-variant cells (Figure 4, Act.-His and N-Fibro). However, at a concentration of ~600 nM, no nonfunctional activator was detected in the AB-variant cells (Figure 4, Act.-T and AB-Var). Thus, the lower affinity recapture mechanism requires the functionality and/or native structure of the activator protein, but not an oligosaccharide. These data also confirm that a nonspecific, bulk fluid-phase endocytotic pathway (Wilson et al., 1993, Xie & Mahuran, 1994) is not responsible for the recapture of the activator. To determine if the recapture system was dependent on the ability of the activator to bind glycosphingolipids, we first incubated 5 nmol of wild-type bacterial activator with 10 or 100 nmol of G_{M2} ganglioside in 0.5 mL of buffer, before adding each mixture to 5 mL of FCS-containing culture medium. The recapture of the activator was not inhibited by either concentration of the ganglioside and may even have been slightly enhanced (Figure 5). Taken together, these data indicate that the mechanism of recapture by the cell is through the recognition of a folded protein domain in the wild-type activator, not present, or misfolded in the truncated form of the activator.

DISCUSSION

A signal for lysosomal incorporation is one or more M6P residues linked to a high-mannose type oligosaccharide (Burg et al., 1985; Kornfeld, 1990); secretory proteins normally contain complex-types of oligosaccharides (Kornfeld & Kornfeld, 1985). Normal cells can be induced to specifically secrete their newly synthesized lysosomal proteins containing this tag (along with their normally secreted proteins) when grown in the presence of NH_4Cl. Previous reports have demonstrated that lysosomal proteins containing the M6P signal in normal cells are also tagged when expressed in transfected CHO cells. Because of the large amount of the

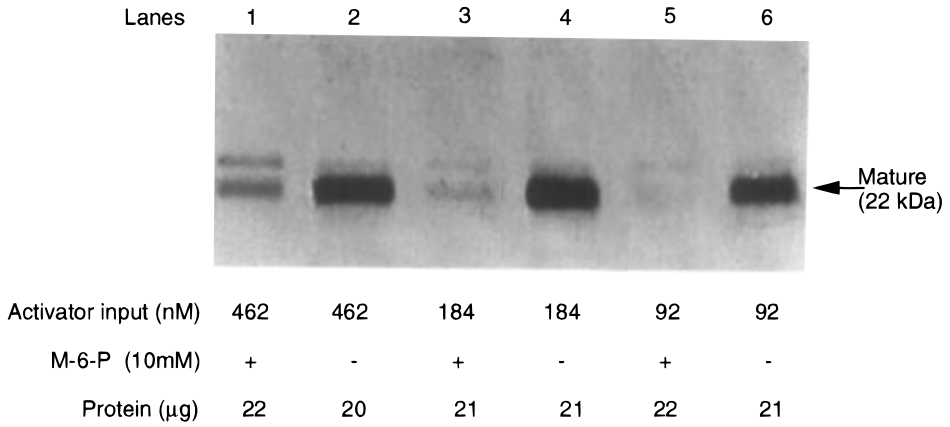

FIGURE 2: Western blot of fibroblast-lysate from a patient with the AB-variant form of G_{M2} gangliosidosis after growth in media containing various concentrations of functional, glycosylated activator, purified from transfected CHO cell medium [Activator input (nM)]. Duplicate samples at each activator concentration are shown, either with (+) or without (−) the addition of 10 mM mannose-6-phosphate (M6P). Total cellular protein loaded in each lane is also shown [Protein (μg)].

tagged protein expressed by selected cloned cells, much of it escapes the MPRs and is secreted. Thus, with these cells, the addition of NH_4Cl to the media does not substantially increase secretion (Anson et al., 1992; Bielicki et al., 1993). We used this method to produce milligram quantities of the human precursor activator (~26 kDa), which we could purify from a protein-free CHO cell media by a two-step column procedure (Figure 1).

Fibroblasts from a patient with the AB-variant form of G_{M2} gangliosidosis (Xie et al., 1992) were tested for their ability to recapture purified CHO cell activator precursor in the presence and absence of 10 mM M6P (Figure 2). The data clearly indicated that at concentrations of activator <100 nM the recapture of the protein is largely blocked by the presence of M6P in the medium (Figure 2, lanes 5 and 6). However, at higher concentration of activator (still >10000-fold less than that of M6P), a second MPR-independent pathway appears to be functioning (Figure 2, lanes 1 and 2). The delivery of the activator to the lysosome was confirmed by its decrease in M_r to that of the mature form (22 000) (Burg et al., 1985). However, a M_r form of the activator intermediate between the precursor and mature forms was apparently selectively excluded from the MPR-dependent pathway (Figure 2). Multiple bands have previously been reported for the purified activator protein and attributed to oligosaccharide heterogeneity (Kuwana et al., 1995; Novak & Lowden, 1994). However, since these studies dealt with the intracellular protein, it was unclear if the heterogeneity was the result of differential processing of the activator's single oligosaccharide in the Golgi, producing molecules with different types of oligosaccharides, or if it was the result of the same type of oligosaccharide in different stages of degradation in the lysosome [such as seen with human Hex (O'Dowd et al., 1988; Sonderfeld-Fresko & Proia, 1989)]. Nonetheless, complex oligosaccharides (which do not contain M6P) have been shown to produce a higher apparent M_r on SDS−PAGE than do high mannose type oligosaccharides (Segrent & Jackson, 1972).

The question of oligosaccharide heterogeneity at the prelysosomal stage was examined by growing normal (untransfected) human fibroblasts in protein-free media either containing or lacking NH_4Cl. The former population (+NH_4Cl) should contain all the newly synthesized activator molecules, while the latter should contain only the population that is normally secreted and not readily recaptured by the cell. The relative percentage of each pool which retained their single high mannose type oligosaccharide as they pass through the Golgi, *i.e.*, were protected from further processing by their mannose-6-phosphate tag (Hasilik & von Figura, 1981), was determined by incubating samples with various endoglycosidases. These data indicate, that while the majority of oligosaccharides present in the NH_4Cl pool are complex, some do contain an endo-H cleavable oligosaccharide (Figure 3A). Interestingly, a small amount of unglycosylated activator was also be seen in the untreated lane (Figure 3A), which was increased significantly in the NH_4Cl-free pool (Figure 3B), indicating that transport out of ER does not strictly require glycosylation. In the activator pool secreted from, but not recaptured by cells grown in normal medium, there was no detectable high mannose oligosaccharides (Figure 3B). Thus, it is likely that the proportion of the NH_4Cl pool that did, ~30%, is not normally secreted, suggesting that the biosynthetic pathway is primarily MPR-dependent.

The recapture experiments were repeated with functional and nonfunctional (truncated) forms of the activator produced in bacteria (Smiljanic-Georgijev et al., 1997) at a concentration similar to the upper range we used with the CHO cell protein. For this study, 10 mM leupeptin was also added to the media to prevent any differential degradation of the nonfunctional protein in the lysosome (Brown & Mahuran, 1991, Brown et al., 1989). Without leupeptin, a decrease in M_r (~1000) was observed on SDS−PAGE for the recaptured wild-type activator, indicating lysosomal incorporation (data not shown). However, with leupeptin added, no change in M_r was observed (Figure 4). Our data demonstrated that the MPR-independent pathway readily endocytosed only the functional, bacterially produced activator. Thus, this pathway is not the bulk endocytotic pathway that we have previously demonstrated to be present in these AB-variant fibroblasts (Xie & Mahuran, 1994). This pathway nonspecifically internalizes fluid-phase components from the medium (Wilson et al., 1993). In our earlier study, we needed to use a very sensitive enzyme assay (normally used for ELISAs) in order to detect the bulk endocytosis of horseradish peroxidase, added at a level of 2.5 mg/mL of serum-free medium (Xie & Mahuran, 1994). In the present study, the maximum amount of activator added was 0.1 mg/mL (600 nM), and at this level no intracellular truncated activator could be detected (Figure 4). The presence of this MPR-independent recapture

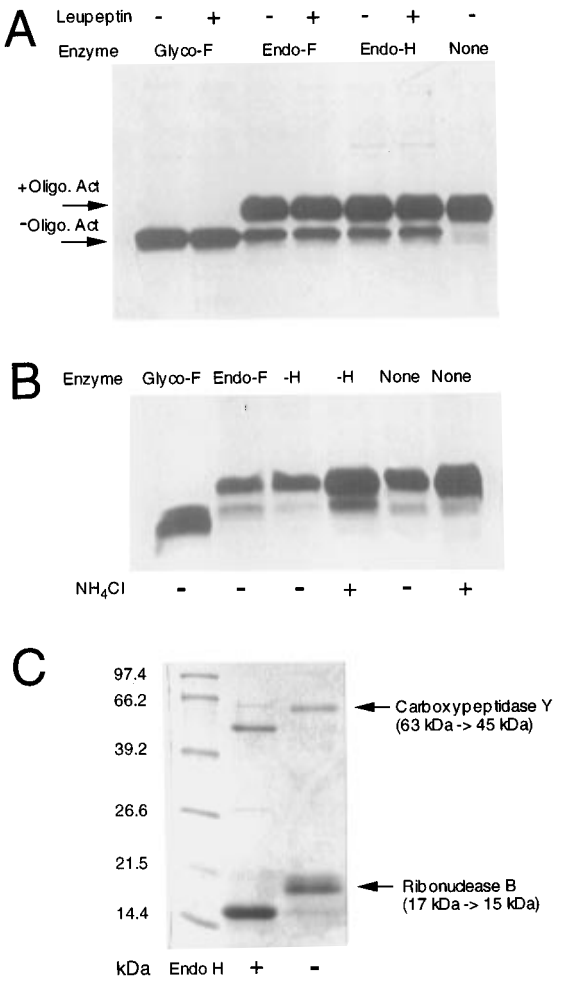

FIGURE 3: (A) Western blot of semipurified activator protein from NH_4Cl-containing medium in which normal human fibroblasts were grown. Samples were treated with either (a) *N*-glycosidase F, *i.e.*, glycopeptidase F (Glyco-F); (b) endoglycosidase F, *N*-glycosidase F-free (Endo-F); or (c) endoglycosidase H (Endo-H). Two samples were incubated with each glycosidase, one contained leupeptin (+) and the other did not (−). An untreated sample is also shown (None) along with the position of the glycosylated (+Oligo. Act) and unglycosylated (−Oligo. Act) forms of the activator. (B) A similar experiment is shown for a pool of semipurified activator from the medium of normal fibroblasts that did not contain NH_4Cl (NH_4Cl). Two samples from panel A, which were grown in NH_4Cl (+) containing medium, were also included as controls. (C) A positive control for the function of Endo-H. This is a section of the gel shown in panel B which was exsized and stained with Coomassie B blue. It contains a mixture of 50 μg of yeast carboxypeptidase Y and ribonucleease B.

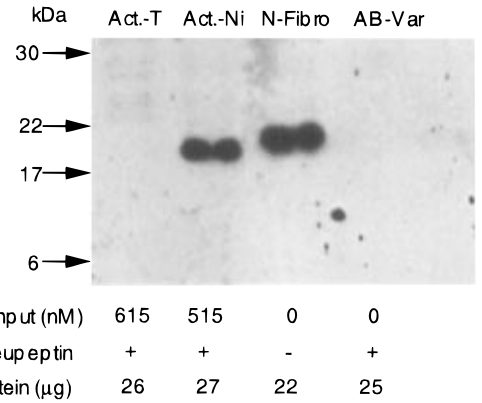

FIGURE 4: Western blot analyses of normal fibroblast lysate, N-Fibro, and fibroblasts from a patient with the AB-variant form of G_{M2} gangliosidosis, AB-Var (detecting endogenous activator protein). The AB-variant fibroblasts were further analyzed after growth in media containing either 515 nM of functional, nonglycosylated wild-type activator (Act.-Ni) or 615 nM of nonfunctional, nonglycosylated truncated activator (Act.-T), purified from transformed bacteria and 10 mM Leupeptin (+). Total cellular protein loaded in each lane is shown [Protein (μg)]. The positions of M_r markers are shown on the left.

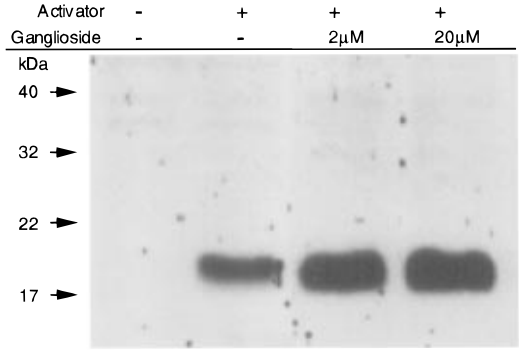

FIGURE 5: Western blot of fibroblast-lysate from a patient with the AB-variant form of G_{M2} gangliosidosis before (left lane) and after (second lane) growth in media containing 1 μM of unglycosylated wild-type activator. For the two right lanes, the activator was mixed and preincubated with either a 2- or a 20-fold molar excess of G_{M2} ganglioside before each mixture was added to the cell medium.

pathway could explain the previous findings of only a 2.5-fold increase in the level of activator in the serum of an I-cell patient and in the medium of normal cells grown in NH_4Cl (Banerjee et al., 1984).

The recapture system was also not inhibited by preincubating the activator with a 2- or a 20-fold excess of G_{M2} ganglioside (Figure 5). Thus, binding of the activator to plasma membrane lipids does not appear to be the mechanism responsible for its recapture from the extracellular media, and it appears that a domain within the folded polypeptide is required.

The above conclusion is similar to the one made after the study of extracellular recapture of pro-saposin (Vielhaber et al., 1996). In this study, the authors did not detect a M6P inhibitable recapture mechanism. However, the concentration of pro-saposin used in the study was not determined and thus, a second higher affinity system could have been missed.

The data contained in this report indicate that a large fraction of the activator is secreted by normal cells and can then be recaptured, with or without a bound glycolipid. When this conclusion is combined with the demonstrated ability of the activator to bind and transport a wide variety of glycolipids at neutral pH (Smiljanic-Georgijev et al., 1997), it suggests that the activator could serve as a glycolipid and ganglioside transport protein, accelerating their endocytosis and lysosomal degradation. Given the number of cellular functions linked to plasma membrane gangliosides, [*e.g.*, (Ferrari et al. (1995), Nagai, (1995), Nakamura et al. (1994), Rabin and Mocchetti (1995), Saito et al. (1995), Tettamanti and Riboni (1993), Yates et al. (1995), and Zeller and Marchase (1992)], controlling the amounts and types of glycolipids present on the cells' surfaces would be an important *in vivo* function.

Another published manuscript on the biosynthesis and processing of the activator in human epidermal keratinocytes (Glombitza et al., 1997) appeared prior to the final revisions made to this report. These authors also found that the majority of the carbohydrate structures in the pool of

activator molecules are complex and that the complex forms had a higher apparent M_r on SDS−PAGE. As well, nonglycosylated forms of the activator were detected, and leupeptin was found to inhibit maturation of the activator. However, some of their other conclusions differed from our. They estimated that only 10% of the activator is phosphorylated and that 70% is retained intracellularly. Thus, they concluded that there must be a major MPR-independent biosynthetic pathway. They also found that the recapture by other keratinocytes of labeled activator from the medium of cells grown in NH_4Cl was not inhibited by M6P. However, in their study the contribution of the endocytotic pathway to the intracellular forms of the activator was not considered, and the amount of activator added to the media for the recapture experiments was not calculated. Alternatively, just as cathepsin D is transported by a MPR-dependent pathway in human fibroblasts, but by a MPR-independent pathway in I-cell lymphoblasts (Glickman & Kornfeld, 1993), these differences may be cell-specific.

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