Overexpression of Human α-Galactosidase A Results in Its Intracellular Aggregation, Crystallization in Lysosomes, and Selective Secretion

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Abstract. Human lysosomal α-galactosidase A (α-Gal A) was stably overexpressed in CHO cells and its biosynthesis and targeting were investigated. Clone AGA5.3-1000Mx, which was the highest enzyme overexpressor, produced intracellular α-Gal A levels of 20,900 U/mg (∼100 μg of enzyme/10^7 cells) and secreted ∼13,000 U (or 75 μg/10^7 cells) per day. Ultrastructural examination of these cells revealed numerous 0.25–1.5 μm crystalline structures in dilated *trans*-Golgi network (TGN) and in lysosomes which stained with immunogold particles using affinity-purified anti–human α-Gal A antibodies. Pulse-chase studies revealed that ∼65% of the total enzyme synthesized was secreted, while endogenous CHO lysosomal enzymes were not, indicating that the α-Gal A secretion was specific. The recombinant intracellular and secreted enzyme forms were normally processed and phosphorylated; the secreted enzyme had mannose-6-phosphate moieties and bound the immobilized 215-kD mannose-6-phosphate receptor (M6PR). Thus, the overexpressed enzyme's selective secretion did not result from oversaturation of the M6PR-mediated pathway or abnormal binding to the M6PR. Of note, the secreted α-Gal A was sulfated and the percent of enzyme sulfation decreased with increasing amplification, presumably due to the inaccessibility of the enzyme's tyrosine residues for the sulfotransferase in the TGN. Overexpression of human lysosomal α-*N*-acetylgalactosaminidase and acid sphingomyelinase in CHO cell lines also resulted in their respective selective secretion. In vitro studies revealed that purified secreted α-Gal A was precipitated as a function of enzyme concentration and pH, with 30% of the soluble enzyme being precipitated when 10 mg/ml of enzyme was incubated at pH 5.0. Thus, it is hypothesized that these overexpressed lysosomal enzymes are normally modified until they reach the TGN where the more acidic environment of this compartment causes the formation of soluble and particulate enzyme aggregates. A significant proportion of these enzyme aggregates are unable to bind the M6PR and are selectively secreted via the constitutive secretory pathway, while endogenous lysosomal enzymes bind the M6PRs and are transported to lysosomes.

Mammalian expression systems have proven invaluable for the stable, high-level production of human proteins which require various co- and posttranslational modifications for folding, stability, function and/or subcellular targeting (Wasley et al., 1987; Walls et al., 1989; Papkoff, 1989; Israel and Kaufman, 1989). Among available systems, SV-40–based vectors containing dominant selectable markers, such as dihydrofolate reductase, for gene amplification permit the stable integration and high-level expression of the selectable marker and gene of interest in mammalian cells (Kaufman, 1990*a*,*b*). Overexpression of human cDNAs in CHO cells results in the synthesis of recombinant proteins with posttranslational modifications similar to those of their native counterparts (Kaufman et al., 1988). These systems produce such large amounts of the recombinant protein that they facilitate efficient characterization of the protein's biosynthesis and targeting, as well as provide abundant protein for therapeutic evaluation. Examples of biologically functional human glycoproteins produced in CHO cells with amplifiable vectors include β-interferon (McCormick et al., 1984), granulocyte-macrophage–stimulating factor (Wong et al., 1985), tissue plasminogen activator (Kaufman et al., 1985), factor VIII (Kaufman et al., 1988), CD4 (Davis et al., 1990), and the glucocorticoid receptor (Alksnis et al., 1991).

Most of the human cDNAs overexpressed in CHO cells have encoded either secreted or membrane-associated proteins that were appropriately targeted. However, proteins trafficked to specific organelles have not been stably expressed, with the exception of the two resident endoplasmic reticulum (ER)[1] proteins, ERp72 and protein disulfide isomerase (Dorner et al., 1990). After stable, high-level overproduction, each of these glycoproteins, which normally are

1. *Abbreviations used in this paper*: α-Gal A, α-galactosidase; dFCS, dialyzed FCS; ER, endoplasmic reticulum; MEP, major excreted protein; M6PR, mannose-6-phosphate receptor; Mx, methotrexate; PCR, polymerase chain reaction; TGN, *trans*-Golgi network.

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retained in the ER by their KDEL signals, were in part targeted to the ER and selectively secreted. However, no mechanism for their selective secretion was identified. Thus, studies of the biosynthesis and targeting of overproduced lysosomal, ER, Golgi, or secretory pathway elements in CHO cells would determine if other proteins with specific intracellular trafficking signals are mislocalized and/or selectively secreted.

Among lysosomal enzymes, human α-galactosidase A (α-Gal A, α-D-galactosidase galactohydrolase, EC 3.2.1.22) provides a prototype for studies of the biosynthesis and targeting of overexpressed lysosomal hydrolases. Human α-Gal A is encoded by a single 14-kb housekeeping gene (Bishop et al., 1988*b*) localized to chromosomal region Xq21.33-Xq22 (Desnick et al., 1987). The processed 1.4-kb α-Gal A transcript, which encodes 429 amino acids including a signal peptide of 31 residues (Bishop et al., 1988*a*), is translated and co-translationally glycosylated in the ER into an ~50-kD precursor glycopeptide. After cleavage of the signal peptide, the glycopeptide undergoes modification of its *N*-linked oligosaccharide moieties in the Golgi apparatus, and then is transported to the lysosome via the mannose-6-phosphate receptor (M6PR)-mediated pathway (Lemansky et al., 1987). The mature lysosomal form of the enzyme is a soluble homodimeric glycoprotein with a native molecular mass of 101 kD (Bishop and Desnick, 1981). Mutations in the α-Gal A gene that result in deficient or absent enzymatic activity cause Fabry disease, a disorder characterized by progressive glycosphingolipid deposition in vascular lysosomes leading to early demise from renal, cardiac, or cerebral vascular disease (Desnick and Bishop, 1989).

In this communication, the stable, amplified overexpression of human lysosomal enzymes in mammalian cells is described. Amplified expression of human α-Gal A resulted in such high intracellular levels of the recombinant enzyme that crystalline structures were observed in membrane-limited structures presumed to be dilated *trans*-Golgi network (TGN) and in lysosomes. Of note, the majority of the overexpressed enzyme was selectively secreted into the culture medium. Among possible mechanisms for the selective secretion, several were eliminated: an altered cDNA construct, lack of the M6PR signal, and low affinity for the M6PR, suggesting that an alternative mechanism was responsible for the selective secretion of this lysosomal hydrolase.

Material and Methods

Construction of Plasmid p91-AGA for α-Gal A Overproduction

Construction of p91-AGA and demonstration of its transient expression have been previously described (Bishop et al., 1988). Briefly, the full-length human α-Gal A cDNA was digested with BamHI and PstI and the 1.45-kb insert fragment was purified and force-subcloned into plasmid pGEM-4 (Promega Biotec, Madison, WI) resulting in pGEM-AGA126. This plasmid was digested with HindIII, end-filled using Klenow polymerase and ligated to EcoRI linkers. After digestion with EcoRI, the 1.45-kb fragment was purified and cloned into the EcoRI site of the mammalian expression vector p91023(B) resulting in the construct, p91-AGA. The analogous expression constructs for human α-*N*-acetylgalactosaminidase (designated p91-AGB) and acid sphingomyelinase (designated p91-ASM) and the respective transient expression of each in COS-1 cells have been previously described (Wang et al., 1990; Schuchman et al., 1991).

Polymerase Chain Reaction Amplification, α-Gal A mRNA, and Gene Copy Number

Polymerase chain reaction (PCR) amplification of the integrated human α-Gal A cDNA sequences was performed using a DNA thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT). Primers designed to specifically amplify the α-Gal A coding sequence were used as previously described (Sakuraba et al., 1990). The PCR reactions were performed independently three times, and the products were ligated into pGEM-4Z for double-stranded sequencing (Sanger et al., 1977).

Total RNA from CHO cells was isolated and mRNA levels were estimated by dot-blot hybridization with ^{32}P-labeled α-Gal A cDNA to 10 μg of total RNA on nitrocellulose filters (Sambrook et al., 1990) using increasing amounts of the α-Gal A riboprobe for quantitation (Sakuraba et al., 1992). The filters were prehybridized overnight and then hybridized for 16 h with the ^{32}P-labeled α-Gal A cDNA probe. After washing, the filter was exposed to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY) for 6 h using two Cronex Lightening Plus (DuPont Instruments, Wilmington, DE) intensifying screens.

The α-Gal A gene copy number was estimated by Southern hybridization analysis (Sambrook et al., 1990). Briefly, genomic DNA was isolated from α-Gal A transfected clones AGA5.3-0Mx and AGA5.3-1000Mx and digested with EcoRI overnight. Aliquots (10 μg DNA) were electrophoresed in 0.7% agarose next to known amounts of human α-Gal A cDNA (1, 0.5, 0.25, and 0.125 ng). After Southern transfer, the filter was hybridized as above and the α-Gal A DNA bands were excised and quantitated by scintillography.

Cell Culture, Electrotransfection, and Gene Amplification

DG44 *dhfr*⁻ CHO cells were grown in DME with 0.05 mM hypoxanthine, 0.008 mM thymidine, 10% FCS, and antibiotics at 37°C in 5% CO_2 using standard techniques. After transfection, the recombinant *dhfr*⁻ DG44 CHO lines were grown in DME supplemented with 10% dialyzed FCS (dFCS) in the absence or presence of methotrexate (Mx).

For electroporation, cells were trypsinized and centrifuged at 1000 *g* for 10 min at room temperature. The pellet was washed once with DME supplemented with 10% FCS and twice with ice-cold electroporation buffer (phosphate buffered sucrose; 272 mM sucrose, 7 mM sodium phosphate, pH 7.4, containing 1 mM $MgCl_2$). Cells were then resuspended in phosphate buffered sucrose at ~0.65-1.0 × 10^7 cells/ml. The cell suspension (0.8 ml) was placed in a 0.4-cm gap cuvette (Bio-Rad Laboratories, Richmond, CA) and 5-20 μg of plasmid DNA was added. After 10 min on ice, the cuvette was placed in the chamber of the "Gene Pulser" electroporation unit (Bio-Rad Laboratories) and was pulsed once at 400 V, 25 μF. The cuvette containing the pulsed cells was placed on ice for 10 min and then the cells were removed and placed in 100 mm tissue culture dishes containing 10 ml of DME supplemented with 10% FCS. For stable expression, the transfected DG44 cells were grown for 48 h, removed from the culture dish by trypsinization, and replated at a 1:15 ratio in DME supplemented with 10% dFCS. The media was replaced every 4 d. After 2 wk of growth, ~1,000 cell foci became visible, and 100 individual clones were isolated with cloning rings. The 15 clones that expressed the highest levels of α-Gal A activity were subjected to amplification en masse by step-wise growth in 0.02 and then 0.08 μM Mx. Of these, three clones, AGA5, AGA9, and AGA11, which had the highest enzymatic activities, were further amplified by sequential growth in 1.3, 20, 40, 80, 250, 500, and 1000 μM Mx.

Butyrate stimulation of the α-Gal A expressing CHO cells was performed as previously described (Dorner et al., 1989). Briefly, cells were plated in 100-mm dishes and allowed to grow in 10 ml of DME supplemented with 10% dFCS for 2 d. The media was removed and replaced with 10 ml of DME supplemented with 10% dFCS containing 5 mM sodium butyrate. Cells were incubated for 16 h at 37°C in a CO_2 incubator, and then cells and culture media were harvested and the α-Gal A activity was determined.

Ultrastructural and Immunolabeling Studies

AGA5.3-1000Mx cells were grown to confluency in 100-mm dishes. After trypsinization (0.25% trypsin, EDTA), they were washed twice in PBS and pelleted at 1,500 *g* for 5 min at room temperature. Cells were then fixed for 1 h with 3% glutaraldehyde in PBS, followed by fixation in PBS-buffered 1% OsO_4 for 30 min at room temperature. Samples were then dehydrated with graded steps of ethanol, infiltrated with propylene oxide, and embed-

ded in Embed 812 (Electron Microscopy Sciences, Fort Washington, PA). 1-μm sections were cut from representative areas. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate, and then were viewed with an electron microscope (JEM 100CX, Jeol USA, Peabody, MA).

For immunodetection, sections were prepared as above, and after embedding, they were mounted on Formvar-coated nickel grids (Formvar Scientific, Marietta, OH), incubated with goat serum in PBS for 30 min at 37°C to block nonspecific binding, washed six times with PBS, and then incubated with affinity-purified rabbit anti-α-Gal A antibodies for 1 h. The sections were washed extensively as above and then incubated with 10-nm gold particles conjugated to protein A (Amersham Corp., Arlington, IL) for 1 h at 37°C. After washing with PBS, sections were fixed with 3% glutaraldehyde in PBS for 15 min at room temperature, washed again with PBS, and then examined under the electron microscope.

Metabolic Labeling Experiments

Confluent cultures of the indicated subclones in 100-mm dishes were washed once with 5 ml of methionine-free DME and then incubated for 30 min in 5 ml of methionine-free DME in a 37°C incubator. The media was replaced with 1 ml of methionine-free DME containing 10% dFCS and 50–100 μCi of [^{35}S]methionine. After incubation at 37°C for 3–5 min, the radioactive media was removed and the cells were washed twice with DME containing 10% FCS. The cells were chased for the indicated times in 5 ml of DME containing 10% FCS and 2 mM methionine. For overnight labeling, cultures received 5 ml of methionine-free DME supplemented with dFCS, glutamine, antibiotics, and 200 μCi of [^{35}S]methionine.

For ^{32}P-labeling, the cells were washed with phosphate-free DME supplemented with 10% dFCS and incubated with 1 mCi of [^{32}P]orthophosphate for 24 h at 37°C with 5% CO_2. For [^{35}S]sulfate labeling, confluent cultures in 100-mm dishes were switched to sulfate-free DME supplemented with 10% dFCS. After addition of [^{35}S]sulfate (50–100 μCi), the cultures were incubated in a 37°C-CO_2 incubator for 24 h. The cells and media were harvested for analysis as described above.

Antibody Production and Immunoprecipitation Studies

Monospecific α-Gal A antibodies were raised in New Zealand white rabbits initially immunized with 250 μg of purified recombinant α-Gal A in Freund's complete adjuvant. The rabbits were boosted with 100 μg of α-Gal A in Freund's incomplete adjuvant 6 wk later and serum was collected 8 and 12 d later. Subsequent boosts (100 μg) were given every 2 mo followed by bleeding 10 d later.

To affinity purify the rabbit anti-human-α-Gal A antibodies, purified recombinant α-Gal A was coupled to 10 ml of Affigel-15 at a concentration of 1 mg protein per milliliter gel, according to the manufacturer's instructions (Bio-Rad Laboratories). Immune serum (10 ml) was chromatographed over the 10-ml Affigel-α-Gal A column, washed with 200 ml of 10 mM Tris buffer, pH 7.5, followed by 200 ml of 10 mM Tris buffer, pH 7.5, containing 500 mM NaCl. The antibodies were eluted with 30 ml of 100 mM glycine, pH 2.5, into a tube containing 5 ml of 1 M Tris buffer, pH 8.0. The antibodies were concentrated and resuspended in 10 mM Tris buffer, pH 7.5, with a Centricon-30 concentrator (Amicon Corp., Beverly, MA).

For immunoprecipitation studies, cells were lysed by the following procedure. Cells grown in 100-mm culture dishes were washed twice with 5 ml of PBS and 1 ml of lysis buffer (50 mM sodium phosphate, pH 6.9, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 0.2 mM PMSF) was added to each dish. After incubation at 4°C for 10 min, the lysates were transferred to 1.5-ml microcentrifuge tubes. Cell debris was removed by centrifugation (8,000 *g* for 15 min at 4°C).

Immunoprecipitation was carried out as previously described (Sambrook et al., 1990). Briefly, 0.5 ml of cell lysate or culture media was placed in a 1.5-ml microcentrifuge tube and 50 μl of preimmune rabbit serum was added. The mixture was incubated at 4°C for 1 h with gentle agitation. 50 μl of Pansorbin (Calbiochem Corp., La Jolla, CA) was added and incubation was continued for 30 min. The mixture was clarified by centrifugation at 8,000 *g* for 5 min, 10 μl of monospecific affinity-purified rabbit anti-human-α-Gal A antibody was added, and the mixture was then incubated for an additional 1 h at 4°C with gentle rocking. Pansorbin (100 μl) was added and the incubation was continued for 30 min as above. The tertiary *Staphylococcus aureus* cell-antibody-antigen complex was collected by centrifugation as above. The supernatant was discarded and the pellet was washed successively with (*a*) NEP buffer (50 mM sodium phosphate, pH 6.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% gelatin) supplemented with 0.5 M NaCl) (*b*) NEP buffer with 0.1% SDS, and (*c*) 10 mM Tris buffer, pH 7.5, containing 0.1% NP-40. The immunoprecipitated protein was denatured by heating at 100°C for 5 min in the presence of 2% SDS and 100 mM dithiothreitol. *S. aureus* cells were removed by centrifugation at 8,000 *g* for 5 min at room temperature.

For quantitative immunoprecipitation, the total α-Gal A activity in the cell lysate and 10 ml of media was determined. To aliquots of the cell lysate and media, each containing 100 U of α-Gal A, a fourfold excess of rabbit anti-human α-Gal A antibodies was added, and then the volume of each was adjusted to 0.5 ml with lysis buffer. Immunoprecipitation was carried out as above. This procedure reproducibly immunoprecipitated > 99% of human α-Gal A in the cell lysate or medium. The polyclonal antibody weakly cross-reacted with the CHO α-Gal A. Similarly, the rabbit anti-human β-hexosaminidase β chain antibodies quantitatively immunoprecipitated human or CHO β-hexosaminidase A and B isozymes. The standard incubation mixture described above contained 1 μl of the antibody and the quantitative immunoprecipitation incubations were performed as described above for α-Gal A.

To quantitate the percent secretion of human α-Gal A and endogenous CHO β-hexosaminidases A and B, ~10^7 cells were labeled with 50 μCi of [^{35}S]methionine for 2 h as above, and then chased with 5 ml of DME containing 10% FCS and 2 mM methionine for 16 h. After immunoprecipitation of the intracellular and secreted α-Gal A or CHO β-hexosaminidases, the radioactivity of the immunoprecipitated enzyme was determined in 10 ml of liquid scintillation fluid (Aquasol; New England Nuclear, Boston, MA) using an LKB 1219 RackBeta scintillation counter. The total radiolabeled α-Gal A or total CHO β-hexosaminidases in the cells and media was calculated based on the cpm of the immunoprecipitated 100 U of either enzyme from each source.

SDS-PAGE and Autoradiography

PAGE was carried out under reducing conditions as described by Laemmli (1970) in 1.5-mm thick slab gels containing 10% acrylamide. The gel was fixed in 10% acetic acid and 20% methanol for 30 min and then soaked in Amplify (Amersham Corp.) for 30 min with agitation. Gels were vacuum dried for 90 min (Hoeffer Scientific Instruments, San Francisco, CA) and then autoradiographed with Kodak X Omat AR film (Eastman Kodak Co.) for 4–24 h.

M6PR Affinity Chromatography

The 215-kD M6PR coupled to Affigel-10 (Bio-Rad Laboratories) at a concentration of 0.4 mg/ml of packed gel was a generous gift of Dr. Stuart Kornfeld (Washington University, St. Louis, MO). Media containing the secreted enzyme(s) was mixed 1:1 with binding buffer (50 mM imidazole, pH 7.0, 150 mM NaCl, 0.05% Triton X-100, 5 mM sodium-β-glycerolphosphate, 0.02% sodium azide) and applied to the 1.5 × 0.8-cm M6PR column at a flow rate of 0.3 ml/min. After sample application (5 ml), the column was washed with 10 ml of binding buffer and then eluted with an exponentially increasing gradient of M6P as described by Dong and Sahagian (1990). Fractions (1.0 ml) were collected and assayed for enzymatic activity using the appropriate 4-methylumbelliferyl-substrate as described below.

To assess the amount of purified, secreted recombinant α-Gal A that bound the M6PR as a function of enzyme concentration, increasing amounts of enzyme (200–10,000,000 U) were applied in a constant volume of 100 μl of binding buffer to the 1-ml M6PR column at pH 6.0. The receptor column was washed with five column volumes of binding buffer and then eluted with five column volumes of binding buffer containing 5 mM M6P. The enzymatic activity bound and eluted under these conditions was determined as above. In addition, 300,000 U of highly concentrated recombinant α-Gal A (30 mg/ml) was intra- and intermolecularly cross-linked with 1-ethyl-3-(3-dimethylaminopropyl-cardodimide), and used for the receptor binding assay as described above.

To evaluate the affinity of the bound enzyme for the M6PR column, increasing concentrations of purified, secreted α-Gal A were applied as above. After washing the column with binding buffer to elute unbound enzyme, the bound α-Gal A was eluted in two fractions: one with five column volumes of binding buffer containing 0.005 mM M6P to release enzyme bound with low affinity, followed by a second with five column volumes of binding buffer containing 5 mM M6P to elute the enzyme bound with high affinity. The relative affinity of the bound enzyme for the receptor was expressed as one over the percent of total enzyme eluted at 0.005 mM M6P.

In Vitro Studies of α-Gal A Aggregation

The possible formation of insoluble α-Gal A aggregates at varying enzyme and hydrogen ion concentrations was investigated. Using a stock solution of purified, secreted α-Gal A (16 mg/ml in 10 mM Tris buffer, pH 7.0), appropriate aliquots were placed in glass borosilicate tubes and the volumes were brought to 200 μl with distilled water so that with the addition of 100 μl of the appropriate buffer (0.5 M 2-(*N*-morpholino)ethanesulfonic acid, at pH 5.0, 5.5, 6.0, 6.5, or 7.0), the final α-Gal A concentrations (0.1-10 mg/ml) would be achieved at specific pH values. After incubation for 10 min at room temperature, the turbidity of each solution was determined by measuring the OD at 650 nm in a spectrophotometer (Spectronic 1201, Milton Roy Co., Rochester, NY) using a 1-cm path cuvette. As a control, 1 mg/ml of purified, secreted α-Gal A was mixed with increasing BSA concentrations (0.1-10 mg/ml), and the turbidity of each solution was determined. Similar experiments were performed with solutions of α-Gal A (10 mg/ml) and BSA (2 mg/ml) at decreasing pH (from pH 7.0 to 5.0). After incubation and centrifugation as above, the supernatants and pellets were subjected to SDS-PAGE.

Enzyme and Protein Assays

The α-Gal A activities in the cell lysates and media were determined using 5 mM 4-methylumbelliferyl-α-D-galactopyranoside (4MU-α-Gal) (Genzyme Corp., Cambridge, MA) as previously described (Bishop and Desnick, 1981). Briefly, a stock solution of 5 mM 4MU-α-Gal was prepared in 0.1 M citrate/0.2 M phosphate buffer, pH 4.6, in an ultrasonic bath. The reaction mixture, containing 10-50 μl of cell extract and 150 μl of the stock substrate solution, was incubated at 37°C for 10-30 min. The reaction was terminated with the addition of 2.3 ml of 0.1 M ethylenediamine. The fluorescence was determined using a Ratio-2 System Fluorometer (Optical Technology Devices, Elmsford, NY). 1 U of activity is the amount of enzyme that hydrolyzed 1 nmole of substrate per hour. The activities of α-mannosidase, β-galactosidase, β-hexosaminidase, β-glucuronidase, acid phosphatase, and α-*N*-acetylgalactosaminidase were measured using the appropriate 4-methylumbelliferyl substrate. The activity of acid sphingomyelinase was determined according to Gal et al. (1975). Protein concentrations were quantitated by the fluorescamine method (Bohlen et al., 1973) as modified by Bishop et al. (1978).

Results

Transfection and Amplification of p91-AGA in dhfr⁻ CHO Cells

Recombinant clones stably expressing human α-Gal A were obtained by electrotransfection of *dhfr⁻* DG44 CHO cells with the expression vector p91023(B) (Wong et al., 1985) containing the murine dihydrofolate reductase cDNA as the selectable marker and the full-length human α-Gal A cDNA (designated p91-AGA). Initial growth of transfected cells in medium lacking nucleosides resulted in over 1,000 positive clones, from which 100 were isolated and found to express α-Gal A at levels ranging from 200-1,800 U/mg protein (data not shown). The three subclones with the highest α-Gal A activities (designated AGA5, AGA9, and AGA11) were grown in the presence of 0.02 and then 0.08 μM Mx to sequentially amplify the integrated p91-AGA sequences. Two AGA5 subclones (AGA5.3 and DG5.9) had the highest intracellular α-Gal A levels (4,900 and 4,200 U/mg protein, respectively). Therefore, subclone AGA5.3 was used for subsequent step-wise amplification with Mx. Table I shows the α-Gal A activities in the sequentially amplified AGA5.3 subclones which were assayed after growth in the absence of Mx for 3 wk to determine the levels of stably expressed enzyme following removal of the selection pressure (Pallavicini et al., 1990). When grown at the highest Mx concentration (1,000 μM), the AGA5.3 subclone (designated AGA5.3-1000 Mx) expressed 20,900 U/mg of α-Gal A activity, an intracellular level ~120-fold greater than that in the untransfected parental DG44 cells. The human α-Gal A cDNA copy number in AGA5.3-0Mx and AGA5.3-1000Mx cells was estimated by Southern hybridization analyses to be 4 and 25, respectively, whereas the amount of α-Gal A transcript in AGA5.3-1000Mx cells was at least 10-fold greater than in the transfected, unamplified AGA5.3-0Mx cells (data not shown).

*Table I. Intracellular and Secreted Levels of Recombinant α-Gal A from Sequentially Amplified AGA5.3 Subclones**

Cell line	Mx‡	α-Gal A specific activity§	
		Intracellular	Secreted
DG44		175	100
AGA5.3	0.00	360	135
	0.02	417	185
	0.08	687	280
	1.3	3,730	1,630
	20	4,450	1,920
	40	5,220	2,280
	250	9,540	4,250
	500	17,000	8,470
	1,000	20,900	13,100

* Activities determined 3 wk after Mx removed from the culture media. Values are means of triplicate determinations.
‡ Mx concentration (μM) used to amplify the expression of p91-AGA.
§ Cellular and secreted specific activities are expressed as units per milligram of cell protein.

Overexpression Results in Crystalline Structures Containing Human α-Gal A in Membrane-Limited Vesicles

Ultrastructural examination of the stably amplified AGA5.3-1000Mx cells revealed numerous 0.25-1.5 μm crystalline bodies which had ordered triangular lattices in membrane-limited vesicles throughout the cytoplasm (Fig. 1 *A* and *B*). The repeat within these crystalline structures was ~20 nm. These structures were particularly abundant in lysosomes (Fig. 1 *A*) and in vesicles which appeared to be dilated TGN (Fig. 1 *B*) (Hand and Oliver, 1984; Griffiths and Simons, 1986; McCracken, 1991). Of note, normal Golgi structures were not observed in these cells, whereas Golgi complexes were readily identified in the parental DG44 cells (Fig. 1 *E*, *inset*). When osmium-glutaraldehyde-fixed sections of the AGA5.3-1000Mx cells were incubated with affinity-purified rabbit anti-human α-Gal A antibodies and then with Protein A-conjugated gold, these crystalline structures were specifically stained by the gold particles (Fig. 1 *C* and *D*). That the crystalline structures were immunogold labeled, even though the sections were fixed in osmium-glutaraldehyde, suggested that these structures were primarily, if not solely, composed of the human enzyme.

To determine whether the crystalline structures were present in clones expressing lower levels of α-Gal A, clones AGA5.3-0Mx, -1.3Mx, -250Mx, and -1000Mx were grown to confluency and examined by electron microscopy. Although the TGN was increasingly dilated with increasing α-Gal A expression, only the AGA5.3-1000Mx clone contained crystalline arrays in lysosomes. Similarly, clone

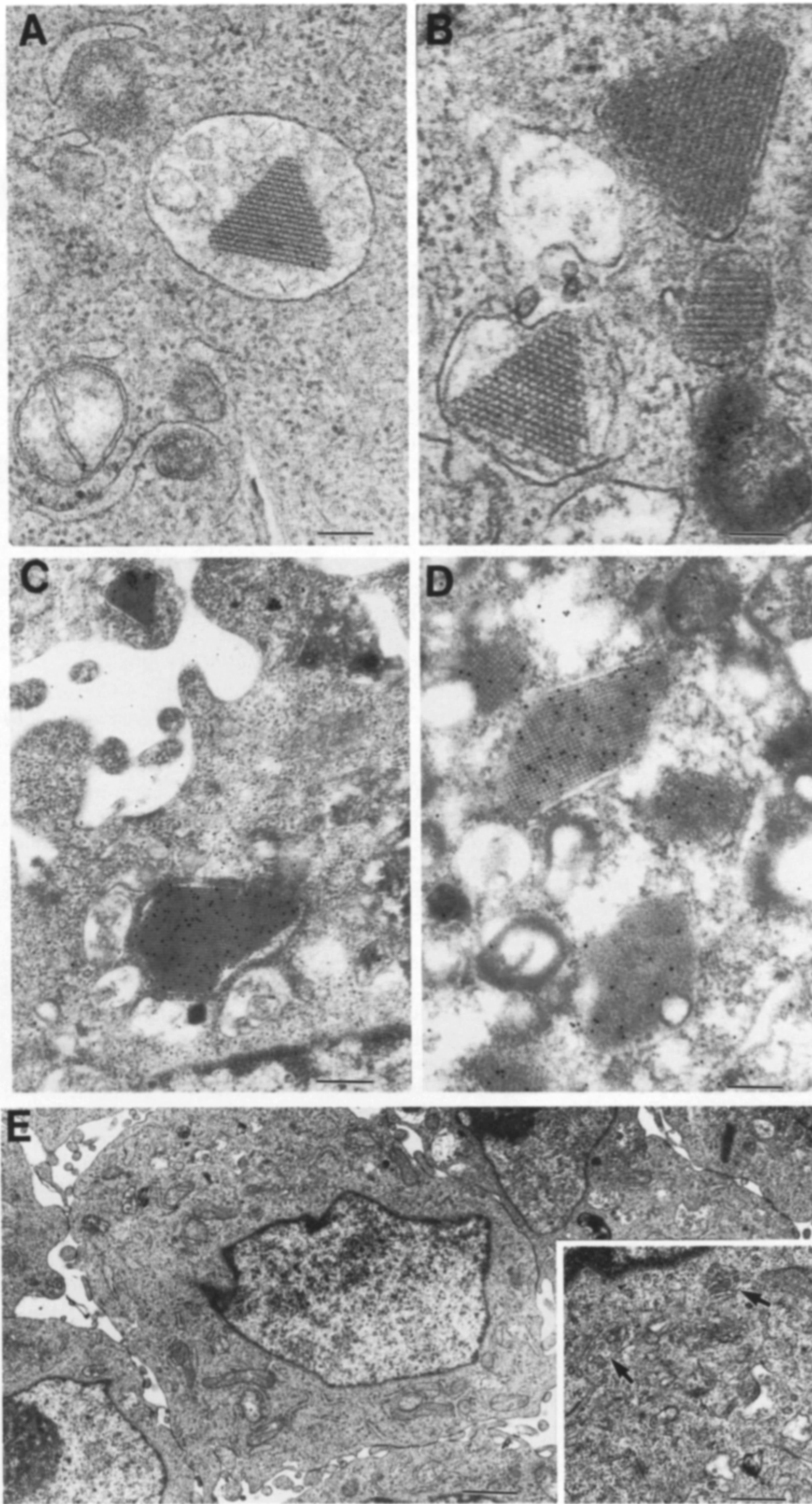

Figure 1. AGA5.3-1000Mx CHO cells contain crystalline structures of overexpressed human α-Gal A. Electron micrographs of AGA5.3-1000Mx cells showing crystalline structures in single membrane-limited vacuoles (*A*) and in vesicles, presumably in the dilated *trans*-Golgi (*B*). (*C* and *D*) Immunoelectron microscopic localization of human α-Gal A with 10-nm colloidal gold particles. (*E*) Electron micrograph of parental *dfhr*⁻ DG44 cells; inset showing Golgi complex (*arrows*) in *dfhr*⁻ DG44 cells. Bars: (*A*) 0.15 μm; (*B*) 0.10 μm; (*C*) 0.31 μm; (*D*) 0.19 μm; (*E*) 1.11 μm, (*inset*) 0.5 μm.

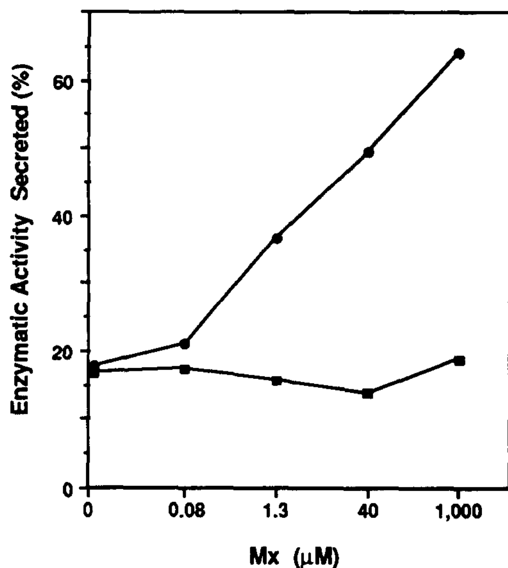

Figure 2. Pulse-chase studies demonstrate increasing secretion of overexpressed α-Gal A in the AGA5.3 amplification series while CHO β-hexosaminidase secretion remains unchanged. AGA5.3 cells growing at the indicated concentrations of Mx were labeled with 50 μCi of [^{35}S]methionine for 2 h and then chased with 2 mM methionine for 16 h. Human α-Gal A (●) and CHO β-hexosaminidases (■) were individually immunoprecipitated from the cells and media, and the percent of each newly synthesized enzyme that was secreted was determined as described in Materials and Methods.

AGA5.3-1.3Mx was stimulated with 5 mM sodium butyrate for 18 h to increase transcription of the integrated vector containing the α-Gal A cDNA (see below) and then examined ultrastructurally. Compared to the untreated clone, butyrate treatment resulted in the presence of dilated organelles including many membrane-bound structures containing dense material (not shown). These results indicated that crystal formation was α-Gal A concentration dependent. Furthermore, to assess whether crystal formation was specific to α-Gal A, clone AGB14.8-1000Mx, which overexpresses α-*N*-acetylgalactosaminidase, was examined. No crystalline arrays were observed, but numerous dilated structures were seen (not shown) similar to those in the AGA5.3-1.3Mx clone, suggesting that expression of the recombinant α-*N*-acetylgalactosaminidase had not reached the critical level necessary for crystal formation.

High Expression Clones Secrete Human α-Gal A

Interestingly, the media from the sequentially amplified AGA5.3 subclones also had increasing concentrations of α-Gal A activity (Table I). Notably, the AGA5.3-1000Mx subclone secreted recombinant α-Gal A at a level of 13,100 U/mg cell protein per 24-h period (∼18,000 U/ml media), or over 130-fold that in the medium of untransfected *dhfr*⁻ DG44 cells. To determine the percentage of newly synthesized α-Gal A secreted by the amplified AGA5.3 clones, cells selected at sequentially increasing Mx concentrations were metabolically labeled with [^{35}S]methionine, the intracellular and secreted forms of α-Gal A were quantitatively immunoprecipitated, and their radioactivities were determined.

*Table II. Effect of Butyrate on Intracellular and Secreted Levels of Human α-Gal A from AGA11 and AGA5 Subclones**

Cell line	Butyrate	α-Gal A specific activity‡	
		Intracellular	Secreted
AGA11	−	259	102
	+	687 (2.7)‖	675 (6.6)
	+, M6P§	604 (2.3)	700 (6.9)
AGA5.3-O Mx	−	485	89
	+	1,460 (4.7)	947 (10.6)
AGA5.3-1000Mx	−	12,700	3,830
	+	14,700 (1.2)	9,510 (2.5)

* Each cell line (∼10^7 cells) was grown for 12 h in the presence or absence of 5 mM butyrate, then the cells and media were harvested and assayed for α-Gal A activity.
‡ α-Gal A specific activity expressed as units per milligram of cell protein.
§ 5 mM M6P added to the medium to block reuptake of secreted enzyme.
‖ Fold increases resulting from butyrate treatment shown in parentheses.

Of the total radiolabeled α-Gal A immunoprecipitated by the transfected, but unamplified, AGA5.3-0Mx clone, about 15% was secreted. With increasing amplification, the percent of synthesized enzyme secreted increased such that the AGA5.3-1000Mx subclone secreted ∼65% (Fig. 2).

Increased α-Gal A mRNA Results in Increased Enzyme Secretion

To assess whether the secretion of recombinant α-Gal A by the CHO cells was dependent on the amount of the p91-AGA transcript, AGA11 (which did not secrete recombinant α-Gal A), AGA5.3-0Mx, and AGA5.3-1000Mx cells were grown in the presence of 5 mM butyrate, which was previously shown to specifically increase transcription of stably integrated p91023(B) constructs (Andrews and Adamson, 1987; Dorner et al., 1989). As shown in Table II, growth of the AGA11 cells in butyrate resulted in a 2.7-fold increase in the intracellular α-Gal A specific activity, while the amount of secreted enzyme increased over 6.6-fold. When 5 mM M6P was added to the growth medium, the intracellular and secreted activities reflected the small expected changes due to blocking the reuptake of the secreted enzyme. Of interest, the butyrate stimulation effect was inversely proportional to the degree of p91-AGA amplification; for example, the secretion of α-Gal A by the transfected, but unamplified AGA5.3-0Mx clone increased 10.6-fold, whereas the secretion by the most highly amplified AGA5.3-1000Mx clone was increased only 2.5-fold. Consistent with these results, butyrate increased the p91-AGA mRNA concentrations, as shown for the human α-Gal A transcript by RNA/DNA hybridization analyses of the unamplified, but transfected, AGA5.3-0Mx and the AGA5.3-1000Mx clones (Fig. 3). Thus, the level of α-Gal A secretion was proportional to the α-Gal A transcript concentration.

Secretion of Human α-Gal A Is Specific

To assess whether the secretion of the overexpressed α-Gal A was due to saturation of the M6PR-mediated pathway for lysosomal targeting, the culture medium from clone AGA5.3-1000Mx was assayed for the presence of increased levels of other lysosomal enzymes which would also be

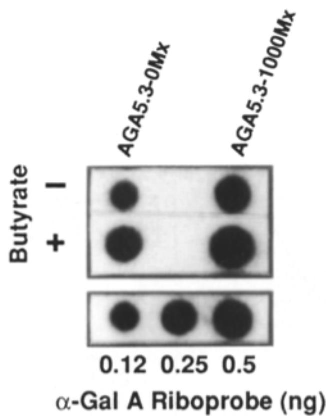

Figure 3. The concentration of human α-Gal A mRNA in AGA5.3 cells is markedly increased by sodium butyrate. Total RNAs (10 μg) from AGA5.3-0Mx and AGA5.3-1000Mx cells were applied to a nitrocellulose filter and hybridized to ^{32}P-labeled α-Gal A cDNA. RNA was isolated before (*minus*) and after (*plus*) addition of 5 mM sodium butyrate to the growth media for 16 h. *α-Gal A riboprobe* indicates the amounts of the human α-Gal A riboprobe applied as standards for quantitation (Sakuraba et al., 1992). Note that butyrate increased the mRNA concentration even in unamplified AGA5.3 cells.

*Table IV. Intracellular and Secreted Levels of Recombinant α-Gal A from Sequentially Amplified AGA9 Subclones**

Mx‡	α-Gal A specific activity§ Intracellular	Secreted
0.00	377	200
0.02	254	120
0.08	678	424
1.3	1,565	2,090
10	2,610	1,970
20	9,480	7,980

* Average of three independent determinations.
‡ Mx concentration in μM.
§ Cellular and secreted specific activity expressed as units per milligram of cell protein.

secreted by default. As shown in Table III, the activities of seven lysosomal enzymes in the medium of AGA5.3-1000Mx cells were essentially the same or lower than those in the medium of the *dhfr⁻* DG44 parental cell line. These findings indicated that the secretion of α-Gal A was specific and was not due to saturation of the M6PR-mediated pathway. In addition, the intracellular activities of these enzymes in the AGA5.3-1000Mx cells were essentially unchanged or only slightly lower compared to those in the parental *dhfr⁻* DG44 cells (Table III), further indicating that the M6PR-mediated pathway was not saturated and that the secretion of α-Gal A was specific.

Efforts were then directed to determine if the secretion of α-Gal A was (*a*) the result of a serendipitous mutation introduced into the α-Gal A cDNA during the construction, introduction, or amplification of the expression plasmid in the CHO cells (Calos et al., 1983) or (*b*) due to an alteration of the protein-sorting machinery unique to the AGA5.3 subclones. PCR amplification and sequencing of 10 independent subclones containing the α-Gal A cDNA insert from the amplified AGA5.3-1.3Mx cells and the highly amplified AGA5.3-1000Mx cells revealed that the sequence was completely intact (data not shown). To demonstrate that the selective-secretion phenotype was not specific to the AGA5.3 clones, an independent clone, AGA9, which was secreting little, if any, recombinant α-Gal A following removal of the selective pressure (i.e., α-Gal A activity in the media ~200 U/mg cell protein), was grown in sequentially increasing Mx concentrations. After selection in 20 μM Mx, clone AGA9-20Mx had intracellular and secreted levels of α-Gal A activity of 9,400 U/mg and 7,900 U/mg, respectively (Table IV), indicating that the secretion phenotype was not unique to the AGA5.3 subclones.

To further characterize the selective secretion of the overproduced α-Gal A, the biosynthesis and targeting of human α-Gal A and human β-hexosaminidases A and B were determined in the series of sequentially amplified AGA5.3 subclones. The intracellular and secreted forms of [^{35}S]methionine-labeled α-Gal A and CHO β-hexosaminidases A and B were immunoprecipitated from the cells and media, and the percent of each total enzyme that was secreted by the sequentially amplified AGA5.3 cell lines was determined. As the secretion of recombinant α-Gal A increased dramatically, the amounts of endogenous CHO β-hexosaminidases A and B secreted by these cells remained essentially unchanged or decreased slightly (Fig. 2), consistent with the selective secretion of recombinant α-Gal A.

Finally, the possibility that the secretion of human α-Gal A was due to the absence of functional M6P moieties on the recombinant enzyme was assessed. Parental *dhfr⁻* DG44 and AGA5.3-1000Mx cells were metabolically labeled with

*Table III. Intracellular and Secreted Levels of Various Lysosomal Enzymes from Untransfected DG44 and Stably Amplified AGA5.3-1000Mx Cells**

Enzyme	Intracellular specific activity‡ DG44	AGA5.3-1000Mx§	Secreted specific activity‡ DG44	AGA5.3-1000Mx
α-Galactosidase A	176 ± 36	15,500 ± 4,400	98 ± 61	12,100 ± 3,620
α-*N*-Acetylgalactosaminidase	11 ± 3.2	9.5 ± 1.1	50 ± 4.5	43 ± 0.6
β-Galactosidase	4.0 ± 0.2	2.3 ± 0.25	12.9 ± 5.0	16.9 ± 4.4
β-Glucuronidase	266 ± 88	136 ± 30	109 ± 16	73 ± 29
α-*L*-Fucosidase	245 ± 34	190 ± 27	1,000 ± 198	695 ± 101
β-Hexosaminidase	705 ± 161	754 ± 340	2,240 ± 405	1,810 ± 197
α-Mannosidase	5.3 ± 3.4	7.7 ± 3.7	135 ± 50	137 ± 24

* Average of three independent determinations.
‡ Cellular and secreted specific activity expressed as units per milligram of cell protein ± 1 SD.
§ Cells selected at 1,000 μM Mx.

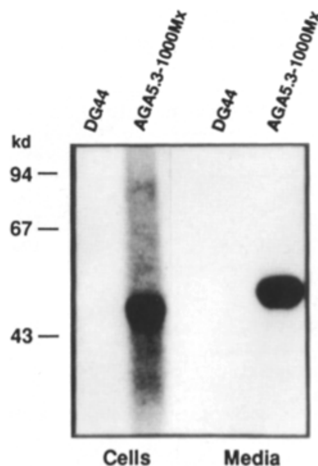

Figure 4. Intracellular and secreted forms of overexpressed human α-Gal A are phosphorylated. Parental *dhfr⁻* DG44 cells and AGA5.3 cells were each labeled with 1 mCi of [32P]orthophosphate for 24 h. The cells and media were immunoprecipitated with affinity-purified anti-human α-Gal A polyclonal antibodies and the immunoprecipitates were analyzed on SDS-PAGE. Note that the secreted enzyme had a slightly higher molecular weight than the intracellular form due to differential post-translational modifications.

[32P]orthophosphate for 12 h and then the cell extracts and media were immunoprecipitated and subjected to SDS-PAGE. As shown in Fig. 4, both the intracellular and secreted forms of α-Gal A were phosphorylated. To demonstrate that the phosphate residues were in M6P moieties with normal M6PR-binding affinities, the AGA5.3-1000Mx cells and human fibroblasts were grown in the presence of [35S]methionine and 10 mM NH_4Cl for 16 h; then, their secretions were individually chromatographed on a 1-ml column of immobilized 215-kD M6PR (Varki and Kornfeld, 1983) and eluted with an exponential gradient of M6P as described by Dong and Sahagian (1990). As shown in Fig. 5, the peaks of secreted α-Gal A from human fibroblasts (Fig. 5 *A*) and AGA5.3-1000Mx cells (Fig. 5 *B*) were both eluted at about 1 mM M6P. Similarly, the radiolabeled peaks for the other M6P-containing proteins secreted in the media from each cell line were also eluted at essentially the same M6P concentrations. These results indicated that the native and recombinant secreted forms of α-Gal A had normal affinities for the M6PR.

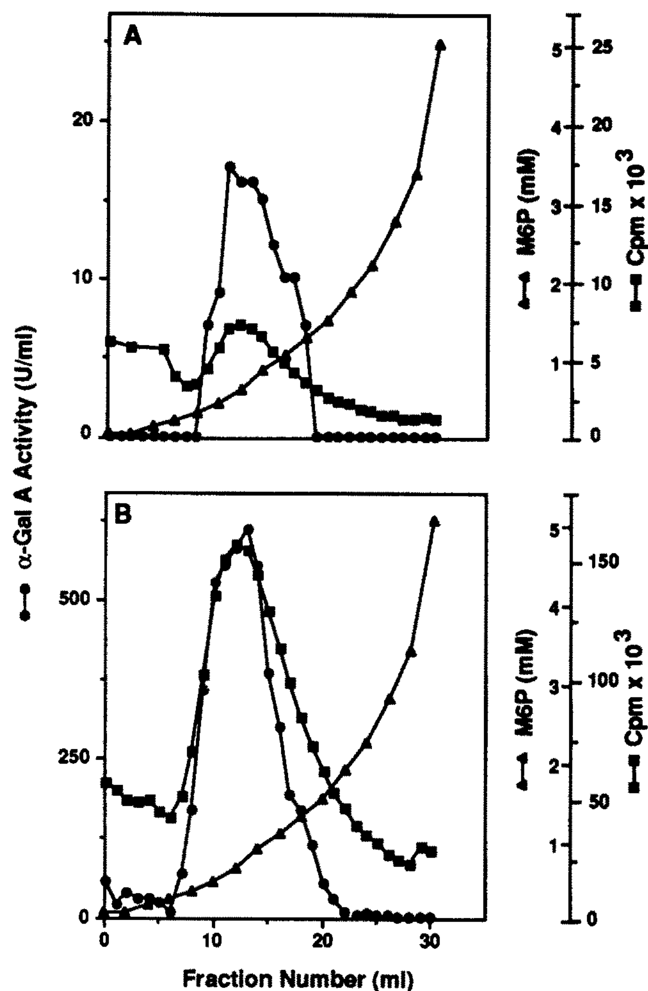

Figure 5. Recombinant secreted human α-Gal A has normal affinity for the M6PR. Media (1 ml) from 10^7 normal human fibroblasts and AGA5.3-1000Mx cells grown in the presence of [35S]methionine and 10 mM NH_4Cl for 16 h, were separately chromatographed on a 1-ml column of immobilized 215-kD M6PR and eluted with an exponential gradient of M6P (▲) according to the method of Dong and Sahagian (1990). The elution profiles of the secreted α-Gal A (●) from human fibroblasts (*A*) and from the ADA5.3-1000Mx cells (*B*) were essentially the same, the peak activities eluted at about 1 mM M6P. In addition, the elution profiles for the radiolabeled M6P-containing proteins (■) were similar, indicating that neither the native or recombinant secreted forms of α-Gal A had decreased affinities for the M6PR.

Other Lysosomal Enzymes Overexpressed by CHO Cells Are Selectively Secreted

To determine if the overexpression of other lysosomal enzymes would result in their selective secretion, the full-length human cDNAs encoding α-*N*-acetylgalactosaminidase and acid sphingomyelinase were subcloned into the p91023(B) vector. The respective constructs (designated p91-AGB and p91-ASM) were individually introduced by electroporation into *dhfr⁻* DG44 CHO cells, and the expression construct in each transfected cell line was amplified as above. The stable overexpression of each lysosomal cDNA resulted in high levels of intracellular enzymatic activity as well as the enzyme's selective secretion. For example, following step-wise amplification of p91-AGB, the sequentially amplified clones expressed increasing intracellular levels of active α-*N*-acetylgalactosaminidase and secreted significant amounts of this lysosomal glycosidase (Table V). Similarly, when the p91-ASM construct was amplified, high levels of active recombinant enzyme were detected in the cells and medium (data not shown). Both secreted recombinant enzymes had phosphomannosyl signals for receptor-mediated binding, as demonstrated by their respective normal binding and elution profiles when chromatographed on the 215-kD M6PR column and eluted with M6P (data not shown).

Sulfation of Secreted α-Gal A Decreases with Overexpression

To determine if the intracellular and secreted forms of recombinant α-Gal A were posttranslationally sulfated, the amplification series of AGA5.3 subclones were each grown in medium containing [35S]sulfate for 16 h, and the recombinant human α-Gal A and endogenous CHO β-hexosaminidases were immunoprecipitated from the cells and the media. As shown in Fig. 6, the secreted form of α-Gal A was sulfated, whereas the cellular form was not. The radiolabeled sulfate moiety on the recombinant secreted enzyme

Table V. Intracellular and Secreted Levels of Recombinant α-GalNAc from Sequentially Amplified AGB14.8 Subclones*

Cell line	Mx‡	α-GalNAc specific activity§	
		Intracellular	Secreted
DG44		11	50
AGB14.8			
	0.00	140	66
	0.02	226	ND
	0.08	390	ND
	10	1,130	385
	20	2,270	920
	80	1,910	1,650
	250	2,580	2,100
	1,000	3,730	3,460

* Average of three independent determinations.
‡ Mx concentration in μM.
§ Cellular and secreted specific activity expressed as units per milligram of cell protein.

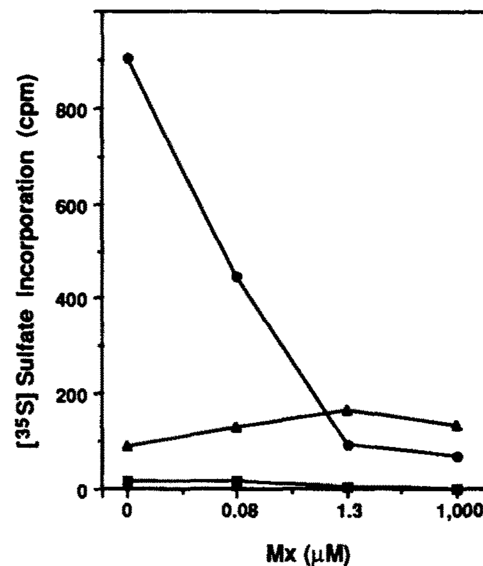

Figure 6. Increasing overexpression of human α-Gal A results in decreasing sulfation of the secreted enzyme. Sequentially amplified AGA5.3 cells (10^7) were labeled with 50 μCi of [^{35}S]sulfate in sulfate-free media for 24 h. Recombinant α-Gal A and CHO β-hexosaminidase activities (100 U) were immunoprecipitated from the cells and media and the amount of radioactive sulfate incorporation was determined. Note that the incorporation of radioactive sulfate in secreted human α-Gal A (●) decreased with increasing amplification (0.08, 1.3, and 1,000 μM Mx) and secretion, while the sulfate incorporation into endogenous CHO β-hexosaminidases was essentially unchanged (▲). No sulfate incorporation was observed in intracellular α-Gal A (■).

was resistant to alkaline hydrolysis, indicating that the linkage was a tyrosine-*O*-sulfate rather than sulfate bound to carbohydrate (Hüttner and Baeuerle, 1988). Of particular interest was the finding that the level of radiolabeled sulfate incorporated into the secreted α-Gal A decreased as the amount of secreted enzyme increased, while the level of radiolabeled sulfate incorporated into the secreted CHO β-hexosaminidases remained essentially unchanged (Fig. 6).

α-Gal A and α-N-Acetylgalactosaminidase Aggregate at High Concentration and Low pH

Since it was conceivable that the overexpression of α-Gal A resulted in the formation of soluble and particulate aggregates that did not bind to, or were inefficiently bound by, the M6PR and/or the sulfotransferase in the TGN, the possible aggregation of α-Gal A was assessed in vitro at varying enzyme and hydrogen ion concentrations. As shown in Fig. 7 *A*, the amount of α-Gal A precipitated from a 10-mg/ml solution increased as the pH was lowered; at pH 7.0, less than 3% (< 3 × 10^5 U) was precipitated, compared to ∼30% (> 2 × 10^6 U) at pH 5.0. At pH 6.0, the estimated pH of the TGN (Griffiths and Simons, 1986), about 12% of the enzyme-formed particulate aggregates that could be pelleted by centrifugation at 15,000 *g*. Figure 7 *B* shows that the turbidity, as a measure of aggregation (Halper and Stere, 1977), of solutions containing 0.1–10 mg/ml of α-Gal A at either pH 5.0 or 7.0 increased as a function of enzyme concentration. Moreover, the turbidity of a 1-mg/ml α-Gal A solution was essentially unaffected by the presence of increasing albumin concentrations from 0.1–10 mg/ml at pH 5.0 (Fig. 7 *B*; control). Finally, electrophoresis of the supernatant and pellet fractions from solutions containing α-Gal A (10 mg/ml) and BSA (2 mg/ml) incubated at varying hydrogen ion concentrations revealed that the increasing precipitation of α-Gal A with decreasing pH was enzyme specific, as the BSA did not precipitate over this pH range. Of note, purified α-*N*-acetylgalactosaminidase also formed aggregates when solutions containing > 500,000 U/ml were incubated at pH 6.0 (Y. A. Ioannou, and R. J. Desnick, unpublished results).

To determine the effects of α-Gal A aggregation at lower pH on its binding to the M6PR, increased concentrations of α-Gal A were applied to a column of immobilized M6PR at pH 6.0. The binding of the purified, secreted enzyme to the receptor became saturated when ∼2,000 U were bound (Fig. 8 *A*, *inset*). However, as the concentration of the applied enzyme was markedly increased, the binding capacity of the receptor column was considerably increased (Fig. 8 *A*). For example, when 1,000,000 U/100 μl were applied, 40,000 U were bound. The affinity of the bound enzyme on the M6PR column was determined by applying increasing concentrations of enzyme to the column, followed by sequential elution with 0.005 mM M6P to release enzyme bound with low affinity and then with 5 mM M6P to release the remaining bound enzyme. As shown in Fig. 8 *B*, the relative affinity of α-Gal A for the M6PR column decreased when increasing enzyme concentrations were applied, suggesting that increasingly concentrated enzyme (i.e., aggregates) bound the receptor, albeit with decreasing affinity.

To determine whether the increased capacity of the receptor column was compatible with the application and binding of enzyme aggregates in the concentrated enzyme solution, purified α-Gal A (30 mg/ml) was cross-linked with EDC at pH 5.0 and then adjusted to pH 6.0. When 300,000 U of the cross-linked enzyme solution was applied to the M6PR column, more than 100,000 U of cross-linked enzyme were bound and eluted with 5 mM M6P. In contrast, when the same amount and concentration of uncross-linked enzyme was applied, only 3,000 U were bound and eluted (Fig. 8 *A*).

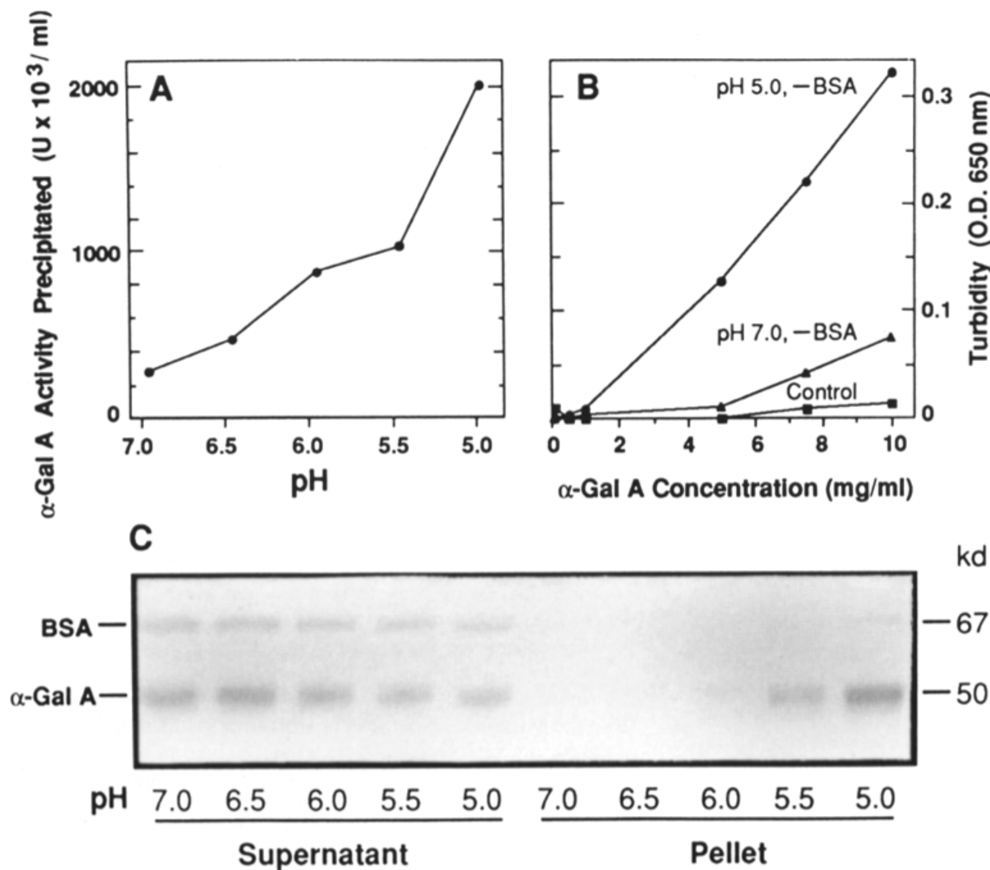

Figure 7. Aggregation of purified secreted α-Gal A is enzyme concentration and pH dependent. (*A*) Precipitation of α-Gal A (10 mg/ml) with decreasing pH. Note that ∼12% of incubated enzyme was precipitated at pH 6.0, the estimated pH of the TGN. (*B*) Turbidity of increasing concentrations of secreted α-Gal A at pH 5.0 (●) and pH 7.0 (▲) in the absence of BSA. As a control for nonspecific precipitation with increasing protein concentration, secreted α-Gal A (1 mg/ml) was mixed with increasing BSA concentrations (0.1-10 mg/ml) at pH 5.0 (■). (*C*) SDS-PAGE of the supernatant and pellet fractions from mixtures of purified secreted α-Gal A (10 mg/ml) and BSA (2 mg/ml) incubated at decreasing pH values. Note that α-Gal A was precipitated with decreasing pH, whereas the concentrations of soluble and precipitated BSA were essentially unchanged.

Discussion

Stable Overexpression of Human α-Gal A

Mx-induced amplification of the bicistronic human α-Gal A/DHFR expression construct in CHO cells resulted in the stable, high-level synthesis of active α-Gal A, which was targeted to lysosomes as well as selectively secreted. After amplification in 1 mM Mx, ∼25 copies of the stably integrated vector transcribed sufficient α-Gal A mRNA to produce intracellular levels of the soluble lysosomal glycosidase that were over 120 times the endogenous activity in nontransfected CHO cells. The recombinant enzyme was synthesized in such large amounts that ordered crystalline structures were observed in markedly dilated TGN and in numerous, enlarged lysosomes (Fig. 1 *A* and *B*). That the crystalline structures were composed of human α-Gal A was demonstrated by immunogold labeling with affinity-purified anti-human α-Gal A antibodies (Fig. 1 *C* and *D*). Such hypertrophy and dilation of the TGN has been observed in secretory cells that were stimulated to secrete (Hand and Oliver, 1984), or in response to pathologic processes (Hand and Oliver, 1983).

Notably, ∼65% of the synthesized enzyme was selectively secreted, attaining a level of α-Gal A activity in the media that was 130 times greater than that of the secreted CHO enzyme. Evidence for this being a selective process, was the finding that the trafficking of the endogenous lysosomal enzymes was not impaired, as their activity levels in the cells and culture medium were either unchanged or slightly decreased. These findings indicated that the M6PR-mediated pathway was not saturated, and that the selective secretion of the recombinant enzyme was due to an alternative mechanism.

Potential Mechanisms for Selective Secretion of α-Gal A

Possible mechanisms for the selective secretion of the overexpressed lysosomal enzyme included: (*a*)) a critical mutation in the α-Gal A cDNA expression construct such as the elimination of an essential glycosylation site; (*b*) an alteration in the lysosomal protein transport and/or sorting machinery unique to the AGA5.3 clones such that the majority of the highly expressed enzyme was secreted; (*c*) improper synthesis or modification of the M6P moieties on the enzyme due to the high level of its synthesis; and/or (*d*) an unusually low affinity of the recombinant enzyme for the M6PR. Each of these mechanisms was excluded experimentally.

To rule out a possible mutation in the α-Gal A cDNA introduced during construction and integration of the vector, the integrated human α-Gal A cDNAs from AGA5.3-1.3Mx and AGA5.3-1000Mx cells were PCR-amplified. 10 subclones of each were completely sequenced in both orientations, and no mutations were identified. The possibility that a unique alteration in the lysosomal or secretory trafficking machinery occurred in the AGA5.3 cells was eliminated by the amplification of another transfected clone (AGA9) which then selectively secreted the recombinant enzyme. That the M6P moiety was intact on the secreted recombinant enzyme, was demonstrated by M6PR-binding and elution studies (Fig. 5 *A*). In addition, the secreted enzyme was purified to

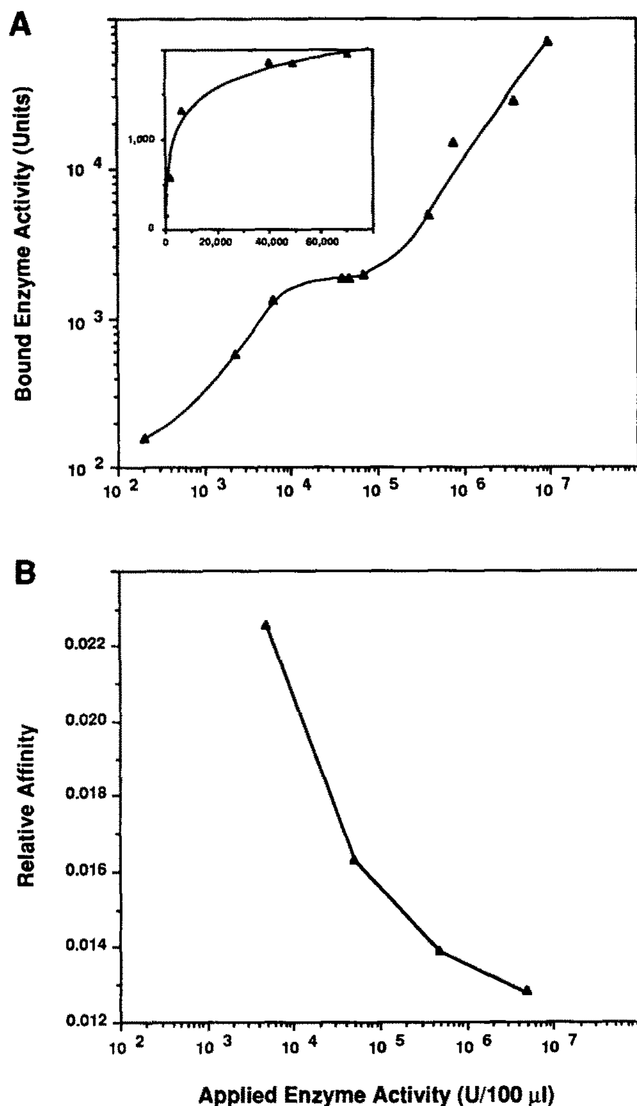

Figure 8. Purified α-Gal A aggregates can bind the M6PR. (*A*) Increasing concentrations of purified secreted α-Gal A (200–10,000,000 U/100 μl) were applied to the M6PR column at pH 6.0 in a constant volume of 100 μl. When dilute concentrations of α-Gal A were applied, the column was saturated at about 2,000 U (*inset*). Application of increasing α-Gal A concentrations resulted in a marked increase in enzyme binding to the column; i.e., when 1,000,000 and 10,000,000 U were applied, greater than 20,000 and 50,000 U were bound, respectively. (*B*) The same concentrations of recombinant α-Gal A were applied to the column as in (*A*). After washing of the column with binding buffer at pH 6.0, the column was eluted with five column volumes of washing buffer containing 0.005 mM M6P to elute enzyme bound with low affinity and then, with the same buffer containing 5 mM M6P to release the remaining enzyme. The relative affinity of binding is expressed as one over the percent of enzyme eluted at 0.005 mM M6P.

homogeneity and its physical and kinetic properties as well as its NH_2-terminal sequence were the same as those of the native lysosomal enzyme and the purified intracellular recombinant enzyme (Y. A. Ioannou and R. J. Desnick, unpublished results). The only notable differences between the recombinant intracellular and secreted enzymes were in their carbohydrate structures and their sulfation. Whereas the intracellular enzyme had its carbohydrate moieties trimmed in the lysosome and was not sulfated, the secreted enzyme was glycosylated and sulfated, consistent with its transport through the secretory pathway. Furthermore, to prove that the secretion of human α-Gal A was not unique to this protein, the cDNAs encoding two other human lysosomal hydrolases, α-*N*-acetylgalactosaminidase and acid sphingomyelinase, also were inserted into the p91023(B) expression construct and amplified in transfected CHO cells (Table IV). Cells that were high expressors of each enzyme also selectively secreted the respective phosphorylated recombinant enzyme into the medium.

Overexpression Results in the Selective Secretion of Other Eukaryotic "Lysosomal" Enzymes

In yeast, the overexpression of vacuolar carboxypeptidase Y resulted in the selective secretion of over 50% of the synthesized enzyme, as the normally glycosylated precursor form (Stevens et al., 1986). Similarly, overexpression of the yeast vacuolar proteinase A gene resulted in the selective secretion of its enzyme precursor (Rothman et al., 1986). It has been proposed that these yeast glycoproteins have subcellular targeting signals located in their peptide sequences that recognize a specific sorting receptor. However, the fact that these overproduced proteins were secreted, whereas other vacuolar proteins were not, precluded the existence of a specific receptor that became saturated. It is notable that the levels of secretion of these yeast vacuolar proteins were proportional to their gene dosage, mRNA levels, and degree of expression.

In transformed murine fibroblasts (NIH 3T3 cells), the major excreted protein (MEP) is cathepsin L (Sahagian et al., 1982; Troen et al., 1987), a lysosomal cysteine protease with functional M6P moieties. The synthesis and selective secretion of MEP are markedly increased in response to viral transformation, certain growth factors, and tumor promoters (Dong et al., 1989; Dong and Sahagian, 1990). For example, transformation of NIH 3T3 cells with Kirsten virus resulted in a 25-fold increase in the synthesis of MEP, causing the enzyme to be selectively secreted. The mechanism for the selective secretion of MEP in the Kirsten virus–transformed cells apparently results from the low affinity of MEP for the M6PR (Dong et al., 1989; Lazzarino and Gabel, 1990). Of note, the amplified expression of murine or human cathepsin L in NIH 3T3 cells resulted in levels of secretion comparable to those in NIH 3T3 cells transformed by the Kirsten virus (Kane et al., 1988). In addition, human cathepsin D has been stably transfected into BHK cells, but the intracellular and secreted levels of recombinant enzyme were only several times higher than in control cells (Horst and Hasilik, 1991); immunoprecipitation of the intracellular and secreted forms of cathepsin D focused on their differential glycosylation; the mechanism of secretion was not investigated. These examples illustrate mechanisms that might account for the selective secretion of their respective proteins. However, these mechanisms have been eliminated for the selective secretion of overexpressed human α-Gal A, since the M6PR-mediated pathway was not saturated, nor was the affinity of the α-Gal A M6P moieties for the receptor decreased.

Of interest, the selective secretion of the yeast vacuolar proteins, murine and human cathepsins L, human cathepsin

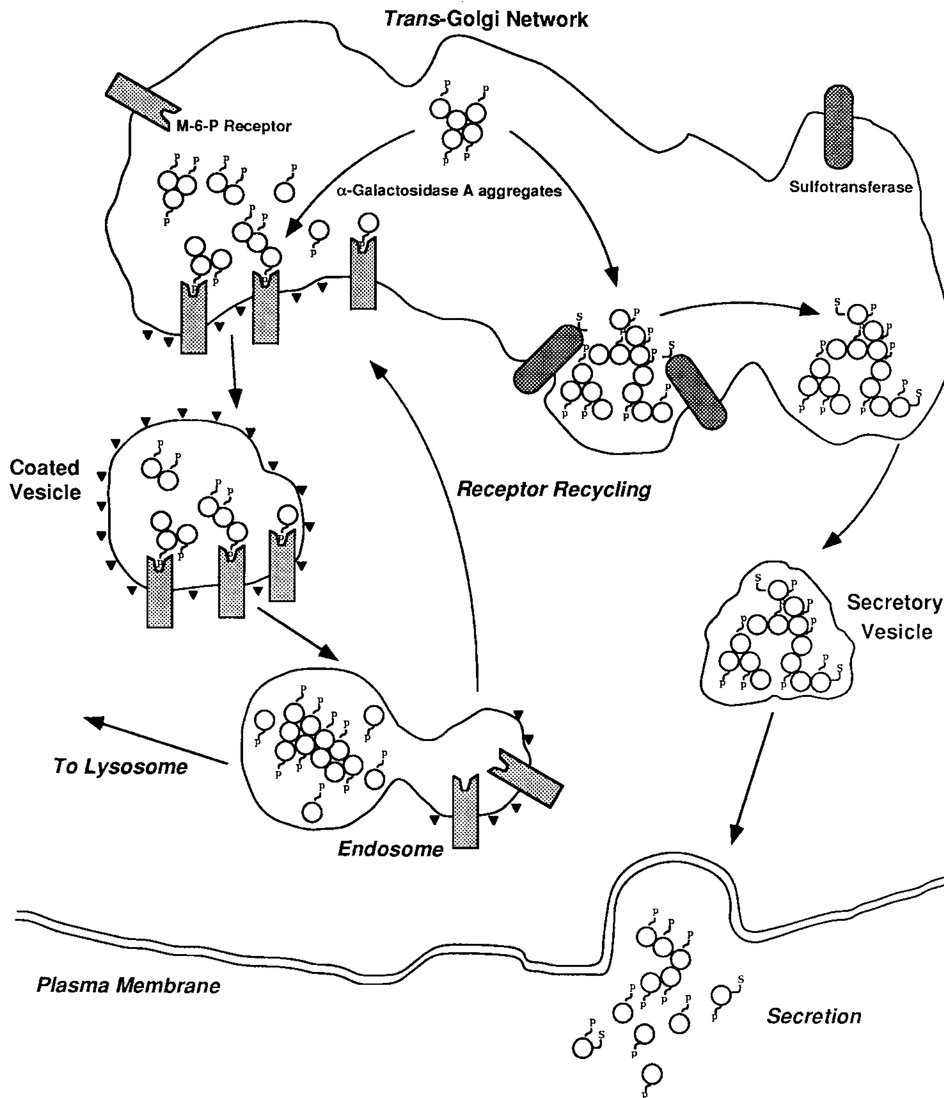

Figure 9. Aggregation-secretion model for selective secretion of human α-Gal A overexpressed in CHO cells. High-level overexpression in CHO cells of human α-Gal A or other lysosomal enzymes that normally are targeted to the lysosome results in their selective secretion due to their aggregation and the resultant inaccessibility of their M6PR signals. The enzyme undergoes normal posttranslational processing until it arrives in the TGN, where the overexpressed enzyme undergoes protein-protein interactions and forms smaller soluble and larger particulate aggregates (Fig. 1, *A–D*), due to lower pH of the TGN. The TGN becomes dilated with the overexpressed enzyme (see Fig. 1, *B* and *D*). Some aggregates and soluble enzyme with exposed M6P signals are trafficked to lysosomes, while the majority of aggregates whose M6P are not accessible are exocytosed by default via the constitutive secretory pathway. In addition, decreased sulfation may occur as the tyrosines in the enzyme aggregates destined for secretion are unavailable to the sulfotransferase. This model may explain the selective secretion of other overexpressed proteins that normally are targeted to specific organelles.

D in mammary tumors (Capony et al., 1989; Rochefort et al., 1989, 1990), recombinant secreted human α-Gal A, and α-*N*-acetylgalactosaminidase all share the common feature of overexpression. In fact, the butyrate stimulation experiments emphasized that even a transient increase in the α-Gal A mRNA levels in transfected cells could result in the increased secretion of α-Gal A. The increase in secretion was proportional to the increase in α-Gal A mRNA levels (Fig. 3). Consistent with this finding, the transient expression of various lysosomal enzymes in COS-1 cells results in their respective secretion into the medium (Y. Ioannou and R. J. Desnick, unpublished results). Analogously, the overexpression of two ER resident proteins, ERp72 and protein disulfide isomerase was characterized by their accumulation in the ER and their selective secretions (Dorner et al., 1990). The overexpression of ERp72 did not cause secretion of endogenous protein disulfide isomerase and vice versa, indicating a selective-secretion mechanism, albeit unknown, unique to the overexpressed ER proteins.

The Aggregation-Secretion Hypothesis: Overexpression Leads to Aggregation, Precipitation, and Rerouting Via the Constitutive Secretion Pathway

An "aggregation-secretion" model is proposed to account for the rerouting of human α-Gal A as a prototype for overproduced lysosomal enzymes and other targeted proteins. As depicted in Fig. 9, the overproduced enzyme is normally synthesized and processed until it reaches the TGN. In this structure, the overproduced enzyme is accumulated and subjected to a markedly more acidic environment, pH ~6.0 (Griffiths and Simons, 1986), which causes increased protein-protein interactions that generate soluble and particulate α-Gal A aggregates. As a result of such aggregation, the enzyme's M6P moieties become inaccessible or less accessible for binding to the M6PR. The aggregates with inaccessible M6P moieties, by default, are rerouted via the constitutive secretory pathway (Helms et al., 1990). A certain

amount of the synthesized enzyme would remain soluble or form soluble and particulate aggregates which can interact with the M6PR and would be transported to lysosomes, accounting for the high-level intracellular accumulation of α-Gal A. In addition, a portion of the secreted enzyme would become soluble and would be internalized and targeted to lysosomes after binding to the M6PR on the cell surface. An alternative hypothesis for the delivery of enzyme aggregates to lysosomes could involve autophagy of the aggregate-containing portions of the TGN.

Three lines of experimental evidence support the aggregation-secretion model. First, purified secreted α-Gal A formed precipitates in vitro with increasing enzyme concentration and decreasing pH (Fig. 7). In fact, ∼12% of the purified enzyme was precipitated at a concentration of 10 mg/ml and a pH of 6.0, consistent with the estimated hydrogen ion concentration of the TGN, and the fact that crystalline structures were abundant in dilated TGN (Fig. 1 *B* and *D*). It should be noted that the protein concentration in cells and their organelles is very high (e.g., > 200 mg/ml in liver; Srere, 1987), and that the α-Gal A concentration of 10 mg/ml may be much less than that in the TGN of the AGA5.3-1000Mx cell line. Second, the secreted form of human α-Gal A was sulfated as expected, since secreted molecules are usually sulfated in the TGN as they proceed through the constitutive secretory pathway (Huttner and Baeuerle, 1988). The human α-Gal A sequence contains two tyrosine residues (Y55 and Y57) within an amino acid context that conforms to four of the five guidelines for tyrosine sulfation (Huttner and Baeuerle, 1988). Of note, the percent of sulfated secreted enzyme decreased with increasing enzyme secretion, whereas the sulfation levels of the endogenous CHO β-hexosaminidase isozymes were essentially unchanged (Fig. 6 *A* and *B*). This finding suggested that the sulfotransferase in the TGN was unable to sulfate all of the secreted α-Gal A, presumably due to the formation of enzyme aggregates which hindered access of the tyrosine moieties to the sulfotransferase. Furthermore, it should be noted that analysis of the oligosaccharide structures on the secreted enzyme revealed phosphorylated high mannose, complex, and hybrid chains (Y. A. Ioannou, and R. J. Desnick, unpublished results), indicating that the enzyme was soluble and appropriately modified in the Golgi complex. This finding is consistent with the sulfation data and supports the occurrence of enzyme aggregation in the TGN. However, it should also be noted that other possible mechanisms may be responsible for the selective secretion of the overexpressed enzyme, including some as yet unknown receptor, binding protein, or other component of protein sorting. Third, when high concentrations of purified, secreted α-Gal A at pH 6.0 were applied to the M6PR column, the amount of enzyme bound was increased markedly (Fig. 8), consistent with the formation and binding of enzyme aggregates. The affinity of enzyme binding decreased when higher concentrations were applied to the column, suggesting that the larger aggregates were less tightly bound due to their mass or steric configuration. A cross-linked enzyme concentrate similarly bound to the receptor column, indicating that cross-linked aggregates had available M6P signals for receptor binding. Thus, these in vitro studies indicated that high concentrations of the recombinant enzyme aggregated, and that some of these aggregates bound to the M6PR, albeit with decreased binding affinity. Analogously, enzyme aggregates with exposed M6P signals that formed in the AGA5.3-1000Mx cells would be targeted to the lysosome, while aggregates in which critical M6P moieties were masked, presumably would be secreted by default via the secretory pathway, consistent with the "aggregation-secretion" hypothesis.

Thus, the cellular response to the overproduction of lysosome-targeted proteins is to transport those containing available M6P residues to the lysosome and to reroute the majority of the overproduced (and presumably aggregated) proteins through the constitutive secretion pathway. Clearly, the amplification series of overexpressing α-Gal A CHO cells provides an unique experimental mammalian system to efficiently characterize the biosynthesis, posttranslational modifications, and mechanisms responsible for the lysosomal targeting and selective secretion of this prototype lysosomal enzyme, thereby providing further insight into the nature of protein transport and sorting in mammalian cells. In addition, the fact that large amounts of recombinant human α-Gal A are secreted by CHO cells permits the scaled-up production and purification of the recombinant enzyme for crystallography and for trials of enzyme replacement therapy in patients with Fabry disease. Thus, the overexpression of lysosomal and perhaps other targeted proteins in CHO cells provides a convenient approach to study protein biosynthesis and sorting as well as produce large amounts of the protein for structural analyses and/or therapeutic applications.

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