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

Yianni A. Ioannou, David F. Bishop, and Robert J. Desnick
Division of Medical and Molecular Genetics, Mount Sinai School of Medicine, New York 10029

Abstract. Human lysosomal α-galactosidase A (α-Gal A) was stably overexpressed in CHO cells and its biosynthesis and targeting were investigated. The 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, 1990a,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) proteins, ERP72 and protein disulfide isomerase (Dorner et al., 1990). After stable, high-level overproduction, each of these glycoproteins, which normally are

© The Rockefeller University Press, 0021-9525/92/12/1137/14 $2.00
The Journal of Cell Biology, Volume 119, Number 5, December 1992 1137-1150 1137

1. Abbreviations used in this paper: α-Gal A, α-galactosidase; dFCS, dia
lyzed FCS; ER, endoplasmic reticulum; MEP, major excreted protein; M6PR, mannose-6-phosphate receptor; Mx, methotrexate; PCR, polymerase chain reaction; TGN, trans-Golgi network.
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 32P-labeled α-Gal A cDNA to 10 μg of total RNA on nitrocellulose filters (Sumbrook 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 32P-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 (Sumbrook 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, Electrotansfection, and Gene Amplification

DG44 dfr CHO cells were grown in DME with 0.05 mM hypoxanthine, 0.008 mM thymidine, 10% FCS, and antibiotics at 37°C in 5% CO2 using standard techniques. After transfection, the recombinant dfr 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 MgCl2). Cells were then resuspended in phosphate buffered sucrose at ~665-1.0 x 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 days. After 2 wk of growth, ~1,100 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 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 to 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 CO2 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 (0.125% 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% OsO4 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 in Epon, sections were cut on a Porter-Blum OMU ultramicrotome (Lowick 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, the sections were fixed in 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% FCS and 2 mM methionine 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. Immune serum was collected 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-10 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 a 1.5-ml M6PR 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 Centricron-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 supernatant was discarded and the pellet was washed successively with (a) NPE 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) NPE 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. 5 μl of each precipitate were removed for analysis as described above.

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. To measure 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.

**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 visualized by using a fixed time of 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 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 increasing gradient of M6P as described by Dong and Saghian (1990). Fractions (1 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)-carboodimide, 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 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. 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 αα 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 1 M ethylendiamine. 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 sialidase 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 p9102303 (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 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 (Pallavici 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).

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

| Cell line | Mxt | α-Gal A specific activity |
|-----------|-----|-------------------------|
| DG44      |     | 175                     |
| AGA5.3    | 0.00| 360                     |
|           | 0.02| 417                     |
|           | 0.08| 687                     |
|           | 1.3 | 3,730                   |
|           | 2.0 | 4,450                   |
|           | 4.0 | 5,220                   |
|           | 250 | 9,540                   |
|           | 500 | 17,000                  |
|           | 1,000 | 20,900               |

*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.
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.
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 were metabolically labeled with [35S]methionine, the intracellular and secreted forms of α-Gal A were quantitatively immunoprecipitated, and their radioactivities were determined.

**Enzyme Secretion**

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 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, 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).

**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

---

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

| Cell line          | Butyrate Intracellular | Secreted | α-Gal A specific activity† |
|--------------------|------------------------|----------|---------------------------|
| AGA11              | −                       | 259      | 102                       |
|                    | +                       | 687 (2.7) | 675 (6.6)                 |
|                    | +, M6P‡                | 604 (2.3) | 700 (6.9)                 |
| AGA5.3-0 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 (~10⁷ 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.
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 32P-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 III. Intracellular and Secreted Levels of Various Lysosomal Enzymes from Untransfected DG44 and Stably Amplified AGA5.3-1000Mx Cells*

| Enzyme                  | DG44 Intracellular specific activity † | AGA5.3-1000Mx Intracellular specific activity † | DG44 Secreted specific activity ‡ | AGA5.3-1000Mx Secreted specific activity ‡ |
|-------------------------|----------------------------------------|-----------------------------------------------|----------------------------------|---------------------------------------------|
| α-Galactosidase A       | 176 ± 36                                | 15,500 ± 4,400                                | 98 ± 61                          | 12,100 ± 3,620                              |
| α-N-Acetylglactosaminidase | 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.

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

| Mx (μM) | Intracellular α-Gal A specific activity † | Secreted α-Gal A specific activity ‡ |
|---------|------------------------------------------|-------------------------------------|
| 0.00    | 377                                      | 200                                 |
| 0.02    | 254                                      | 120                                 |
| 0.08    | 678                                      | 424                                 |
| 1.0     | 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.
Intracellular and secreted forms of overexpressed human α-Gal A are phosphorylated. Parenteral dhfr- DG44 cells and AGA5.3 cells were each labeled with 1 mCi of \[^{32}P\]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.

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 \[^{32}P\]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.

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 \[^{35}S\]methionine and 10 mM NH_4Cl for 16 h, were separately chromatographed on a 1-ml column of immobilized 215-kDa M6PR (Varki and Komfeld, 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. 5A) and AGA5.3-1000Mx cells (Fig. 5B) 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.

Profiles when chromatographed on the 215-kDa 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 \[^{35}S\]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

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-kDa M6PR column and eluted with M6P (data not shown).
was resistant to alkaline hydrolysis, indicating that the linkage was a tyrosine-O-sulfate rather than sulfate bound to carbohydrate (Hüttnner 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 × 10⁶ U) was precipitated, compared to ~30% (> 2 × 10⁶ 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).
Discussion

Stable Overexpression of Human \( \alpha \)-Gal A

MX-induced amplification of the bicistronic human \( \alpha \)-Gal A/DHFR expression construct in CHO cells resulted in the stable, high-level synthesis of active \( \alpha \)-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 \( \alpha \)-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 \( \alpha \)-Gal A was demonstrated by immunogold labeling with affinity-purified anti-human \( \alpha \)-Gal A antibodies (Fig. 1 C and D). Such hypertrophy and dilatation 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 \( \alpha \)-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 \( \alpha \)-Gal A

Possible mechanisms for the selective secretion of the overexpressed lysosomal enzyme included: (a) a critical mutation in the \( \alpha \)-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 \( \alpha \)-Gal A cDNA introduced during construction and integration of the vector, the integrated human \( \alpha \)-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
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.

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 diluted TGN (Fig. 1B 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; Sreer, 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. 6A 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. Ioannou, and R. J. Desnick, unpublished results), indicating that the enzyme was sulfated 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.

The authors thank Dr. Ronald Gordon (Mount Sinai School of Medicine, New York, NY) for ultrastructural studies and immunodetection microscopy, Dr. Lawrence Chassin (Columbia University, New York, NY) for the gift of the dlyfr DO44 CHO cell line, Dr. Randal Kaufman (Genetics Institute, Inc., Cambridge, MA) for the gift of the p91023(B) vector, Dr. Stuart Kornfeld (Washington University School of Medicine, St. Louis, MO) for the gift of the immobilized 215-kD M6PR, and Dr. Mario Rattazzi (Mount Sinai School of Medicine, New York, NY) for the rabbit anti-human β-hexosaminidase β-chain antibodies and for helpful discussions and review of the manuscript.

These studies were supported in part by a grant (RO1 DK34045) from the National Institutes of Health, a grant (I-518) from the March of Dimes Birth Defects Foundation, a grant (RR-00071) for the Mount Sinai General Clinical Research Center from the National Center for Research Resources, National Institutes of Health, and a grant (1 P30 HD27786) for the Child Health Research Center at Mount Sinai from the National Institutes of Health. Y. Ioannou was the recipient of a National Institutes of Health postdoctoral fellowship (5 T32 HD07105).

Received for publication 11 March 1992 and in revised form 7 August 1992.

References

Aalknis, M., T. Barkhem, P.-E. Strömstedt, H. Ahola, E. Kutoh, J.-A. Gustafsson, L. Poellinger, and S. Nilsson. 1991. High level expression of functional full-length and truncated glucocorticoid receptor in Chinese hamster ovary cells. J. Biol. Chem. 266:10078-10085.

Andrews, G. K., and E. D. Adamson. 1987. Butyrate selectively activates the metallothionein gene in teratocarcinoma cells and induces hypersensitivity to metal induction. Nucleic Acids Res. 15:5461-5475.

Bishop, D. F., and R. J. Desnick. 1981. Affinity purification of α-galactosidase A from human spleen, placenta and plasma with elimination of pyrogen contamination. Properties of the purified splenic enzyme compared to other forms. J. Biol. Chem. 256:1307-1316.

Bishop, D. F., D. E. Wampler, J. T. Segouris, R. J. Bonefeld, D. K. Anderson, M. C. Hawley, and C. C. Sweeney. 1978. Pilot scale purification of α-galactosidase A from normal serum protein fraction IV-1 of human plasma. Biochim. Biophys. Acta 524:109-120.

Bishop, D. F., R. Korowitch, C. M. Eng, Y. A. Ioannou, T. F. Fitzmaurice, and R. J. Desnick. 1986a. Human α-galactosidase: characterization and eu-karyotic expression of the full-length CDNA and structural organization of the gene. In Lipid Storage Disorders. R. Salvayre, L. Douste-Blazy, S. Gatt.
Bohlen, P., S. Stein, W. Dairman, and S. Udenfriend. 1973. Fluorometric assay of proteins in the nanogram range. Arch. Biochem. Biophys. 155:213-220.

Callow, M. P., J. S. Lebkowski, and M. R. Botchan. 1983. High mutation frequency in DNA transfection into mammalian cells. Proc. Natl. Acad. Sci. USA. 80:3015-3019.

Capyon, F., C. Rougeot, P. Montcourrier, V. Cavailles, G. Salazar, and H. Rochefort. 1989. Increased secretion, altered processing, and glycosylation of pro-cathepsin D in human mammary cancer cells. Cancer Res. 49:3904-3909.

Davis, S. J., H. A. Ward, M. J. Puklavec, A. C. Willis, A. F. Williams, and H. A. Sanger. 1977. DNA sequencing with chain-terminating oligonucleotides. Nucleic Acids Res. 17:4589-4604.

Desnick, R. J., and D. F. Bishop. 1989. Fabry disease, deficient α-galactosidase A activity; Schnider disease, deficient α-N-acetylgalacosaminidase activity. In The Metabolic Basis of Inherited Disease. 6th ed. C. R. Scrivcr, A. L. Beaudet, W. S. SLY, and D. Valle, editors. McGraw-Hill Inc., New York. 1751-1796.

Desnick, R. J., H. S. Bernstein, K. H. Astrin, and D. F. Bishop. 1987. Fabry disease: molecular diagnosis of hemizygotes and heterozygotes. Enzyme. 38:54-64.

Dong, J., and G. G. Sahagian. 1990. Basis for low affinity binding of a lysosomal cysteine protease to the cation-independent mannose 6-phosphate receptor. J. Biol. Chem. 265:4210-4217.

Dong, J., E. M. Pence, and G. G. Sahagian. 1989. Mechanism for selective secretion of a lysosomal protease by transformed mouse fibroblasts. J. Biol. Chem. 264:17500-17507.

Dorner, A. J., L. C. Wasley, and R. J. Kaufman. 1989. Increased synthesis of secreted proteins induces expression of glucose-regulated proteins in butyrate-treated Chinese hamster ovary cells. J. Biol. Chem. 264:20603-20607.

Dorner, A. J., L. C. Wasley, P. Raney, S. Haugejorden, M. Green, and R. J. Kaufman. 1990. The stress response in Chinese hamster ovary cells. J. Biol. Chem. 265:22029-22034.

Dorner, A. J., R. Brady, S. R. Hibbert, and P. G. Penchve. 1975. A practical chromogenic procedure for the detection of homoglycoses and heteroglycoses carriers of Niemann-Pick disease. N. Engl. J. Med. 293:632-636.

Griffiths, G., and K. Simons. 1986. The transf Golgi network: sorting at the exit site of the Golgi complex. Science (Wash., DC). 234:438-443.

Halper, L. A., and P. A. Sreer. 1977. Interaction between citrate synthase and mitochondrial malate dehydrogenase in the presence of polyethylene glycol. Arch. Biochem. Biophys. 184:529-534.

Hand, A. R., and C. Oliver. 1983. Cell Biology of the Secretory Process. Academic Press Inc., New York. 185-198.

Hand, A. R., and C. Oliver. 1984. Effects of secretory stimulation on the Golgi apparatus and GERL of rat parotid acinar cells. J. Histochem. Cytochem. 32:403-442.

Helms, J. B., A. Karrenbauer, K. W. Wirtz, J. E. Rothman, and F. T. Wieland. 1988. Use of a cloned multidrug resistance gene for coamplification and secretion of a lysosomal protease by transformed mouse fibroblasts. J. Biol. Chem. 263:20027-20032.

Horst, M., and A. Hasilik. 1991. Expression and maturation of human cathepsin D in breast cancer: a study of multidrug resistance gene expression. J. Histochem. Cytochem. 39:3377-3382.

Kaufman, R. J. 1990. Selection and coamplification of heterologous genes in mammalian cells. Proc. Natl. Acad. Sci. USA. 87:5393-5397.

Kaufman, R. J. 1990a. Selection and coamplification of heterologous genes in mammalian cells. Methods Enzymol. 185:537-566.

Kaufman, R. J. 1990b. Vectors used for expression in mammalian cells. Methods Enzymol. 185:387-411.

Kaufman, R. J., L. C. Wasley, A. J. Spiliotes, S. D. Gossels, S. A. Latt, G. R. Larsen, and R. M. Kay. 1985. Coamplification and coexpression of human tissue-type plasminogen activator and murine dihydrofolate reductase cDNA in Chinese hamster ovary cells. Mol. Cell. Biol. 5:1750-1759.

Kaufman, R. J., L. C. Wasley, and A. J. Dorner. 1988. Synthesis, processing, and secretion of recombinant human factor VIII expressed in mammalian cells. J. Biol. Chem. 263:6352-6362.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680-685.

Lazarus, D., and C. A. Gallo. 1985. Protein determinants impair recognition of procasphein L phosphorylated oligosaccharides by the cation-independent mannose 6-phosphate receptor. J. Biol. Chem. 265:11864-11871.

Lemansky, P., F. P. Bishop, R. J. Desnick, A. Hasilik, and K. von Figure. 1989. Induction of synthesis and processing of α-galactosidase A in human fibroblasts: Evidence for different mutations in Fabry disease. J. Biol. Chem. 262:2062-2065.

McCormick, F., M. Trahey, M. Innis, B. Dieckmann, and G. Ringold. 1984. Inducible expression of amplified human beta-interferon genes in CHO cells. Mol. Cell. Biol. 4:166-172.

McCracken, A. A. 1991. Trafficking of secreted proteins. In Intracellular Trafficking of Proteins. C. S. Steer, and J. A. Hanover, editors. Cambridge University Press, New York. 461-485.

Papoff, J. 1989. Inducible overexpression and secretion of int-1 protein. Mol. Cell. Biol. 9:3377-3384.

Rochefort, H., V. Cavailles, P. Augereau, F. Capony, T. Maudelonde, I. Touitou, and M. Garcia. 1989. Overexpression and hormonal regulation of pro-cathepsin D in mammary and endometrial cancer. J. Steroid. Biochem. 34:177-182.

Shinnick, R. J., and M. Garcia. 1990. Cathepsin D in breast cancer: from molecular and cellular biology to clinical applications. Cancer Cell. 2:383-388.