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Expression and characterization of recombinant human alpha-galactosidase A

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EXPRESSION AND CHARACTERIZATION OF RECOMBINANT HUMAN ALPHA-GALACTOSIDASE A

by

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ABSTRACT

EXPRESSION AND CHARACTERIZATION OF RECOMBINANT HUMAN ALPHA-GALACTOSIDASE A

by

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Fabry disease, an X-linked inborn error of glycosphingolipid catabolism, results from the deficient activity of the lysosomal hydrolase, α-galactosidase A (α-Gal A). In order to characterize the normal enzyme and to evaluate the clinical effectiveness of enzyme replacement therapy, efforts were directed to produce large quantities of human recombinant α-Gal A. A full-length cDNA clone encoding the 5' signal and untranslated sequence of α-Gal A, was isolated and sequenced. Initial efforts were directed to express the full-length cDNA encoding α-Gal A using various prokaryotic expression vectors. Although microbial expression was achieved, as evidenced by enzyme assays of intact *E. coli* cells and growth on melibiose as the carbon source, the human protein was expressed at low levels and could not be purified from the bacteria. These findings suggest that the recombinant enzyme was unstable due to the lack of normal glycosylation and/or the presence of endogenous cytoplasmic or periplasmic proteases. Therefore, efforts were directed to express the enzyme in eukaryotic systems. The full-length α-Gal A cDNA was inserted into the mammalian expression vector p91023(B) in front of the amplifiable dihydrofolate reductase (DHFR) cDNA. The functional integrity of the cDNA construct (p91-AGA) was confirmed by transient expression of active enzyme in COS-1 cells; 650 U/mg (nmol/hr) vs endogenous levels of ~ 150 U/mg of 4-MU-α-D-galactopyranoside activity. The p91-AGA construct was introduced by electroporation into DG44 *dhfr*$^-$ CHO

cells. Positive selection in media lacking nucleosides resulted in the isolation of clones expressing the active enzyme at levels ranging from 300-2,000 U/mg. Selected subclones were grown in increasing concentrations of methotrexate (MTX, 0.02 to 1.3 μM) resulting in co-amplification of DHFR and α-Gal A cDNAs with intracellular α-Gal A activity ranging from 5,000 to 25,000 U/mg. Subclone DG44.5 secreted approximately 90% of the α-Gal A produced while other lysosomal enzymes were not secreted including β-hexosaminidase, α-mannosidase, β-galactosidase and β-glucuronidase, indicating that the secretion was α-Gal A specific. When the MTX concentration was increased to 1.3 μM, 10^7 cells secreted ~15,000 U/ml culture media/day. Using a hollow fiber bioreactor, up to 10 mg of enzyme protein was secreted per day.

The secreted α-Gal A was purified by affinity chromatography for characterization of various physical and kinetic properties. The recombinant enzyme had a pI of 3.9, a pH optimum of 4.6, a km of 1.9 mM toward 4-methylumbelliferyl-α-D-galactopyranoside and rapidly hydrolyzed globotriaosylceramide, the natural glycosphingolipid substrate, which was targeted with apolipoprotein E to the lysosomes of the enzyme-producing CHO cells. Pulse-chase studies indicated that the recombinant enzyme assumed its secondary structure in <3 min, was in the Golgi by 5 min where it became Endo H resistant, and was secreted into the media by 45-60 min. Labeling studies revealed that both the intracellular and secreted forms were phosphorylated. The secreted enzyme subunit was slightly larger than the intracellular subunit; following *N*-glycanase treatment, both subunits comigrated on SDS-PAGE, indicating differences in the oligosaccharide moieties of the two forms. Treatment of the radiolabeled secreted enzyme with various endoglycosidases revealed the presence of three *N*-linked oligosaccharide chains, two high-mannose type (Endo H sensitive) and one complex type, the latter being endoglycosidase H and F resistant. Analyses of the Endo H released oligosaccharides revealed that one had two phosphate residues and it specifically bound to immobilized mannose-6-phosphate receptors while the

other was a hybrid structure containing sialic acid. These physical and kinetic properties and the presence of complex-type oligosaccharide chains on the recombinant secreted enzyme were similar to those of the native enzyme purified from human plasma. The secreted form of α-Gal A was taken up by cultured Fabry fibroblasts by a saturable process that was blocked in the presence of 2 mM mannose-6-phosphate indicating that binding and internalization were mediated by the mannose-6-phosphate receptor. The binding profiles of the recombinant secreted enzyme and the α-Gal A secreted by NH_4Cl-treated human fibroblasts to the immobilized receptor were identical. The availability of large amounts of soluble, active recombinant α-Gal A which is similar in structure to the native enzyme isolated from plasma will permit further comparison to the native enzyme forms and the clinical evaluation of enzyme replacement in Fabry disease.

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ABBREVIATIONS

α-galactosidase A	α-Gal A
base pair(s)	bp
Chinese hamster ovary	CHO
complementary DNA	cDNA
counts per minute	cpm
deoxyribonucleic acid	DNA
dihydrofolate reductase	dhfr
Dulbecco's Modified Eagle's Medium	DMEM
fetal calf serum	FCS
hour(s)	hr
Immunoglobulin G	Ig G
kilobase pairs	kb
liter	l
mannose-6-phosphate	M-6-P
methotrexate	MTX
micrograms	μg
milligrams	mg
minutes	min
nanograms	ng
polyacrylamide gel electrophoresis	PAGE
polymerase chain reaction	PCR
ribonucleic acid	RNA
sodium dodecyl sulfate	SDS
units	U

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

BACKGROUND

A. Introduction

Of the over 400 inborn errors of metabolism, the specific enzymatic defects are known in more than 200 (1). Identification of the enzymatic defect not only permits precise prenatal and presymptomatic diagnosis, but the knowledge of the defect permits the design of specific therapeutic endeavors. In Fabry disease, a lysosomal storage disease resulting from the deficient activity of α-galactosidase A (α-Gal A), identification of the enzymatic defect in 1967 (2) led to the first *in vitro* (3) and *in vivo* (4) therapeutic trials of α-Gal A replacement in 1969 and 1970, respectively. These and subsequent trials (4-6) demonstrated the biochemical effectiveness of direct enzyme replacement for this disease. Repeated injections of purified splenic and plasma α-Gal A (100,000 U/injection) were administered to affected hemizygotes over a four month period (5). The results of these studies demonstrated that: 1) The plasma clearance of the splenic form was 7 times faster than that of the plasma form (10 min vs 70 min), 2) compared to the splenic form of the enzyme, the plasma form effected a 25-fold greater depletion of plasma substrate over a markedly longer period (48 hours vs 1 hour), 3) there was no evidence of an immunologic response to six doses of either form, administered intravenously over a four month period to two affected hemizygotes and 4) suggestive evidence was obtained indicating that stored tissue substrate was mobilized into the circulation following depletion by the plasma form, but not by the splenic form of the enzyme. Thus, the administered enzyme not only depleted the substrate from the circulation (a major site of accumulation), but also possibly mobilized the previously stored substrate from other depots into the circulation for subsequent clearance. These studies indicated the potential for eliminating, or significantly reducing, the pathological glycolipid storage by repeated enzyme replacement.

However, the biochemical and clinical effectiveness of enzyme replacement in Fabry disease has not been demonstrated due to the lack of sufficient human enzyme for

adequate doses and long-term evaluation. With the advent of recombinant DNA technology it has become feasible to produce large amounts of recombinant human α-Gal A. The use of a eukaryotic expression system that glycosylates the enzyme appropriately is required to produce enzyme suitable for clinical use. Such expression systems should produce sufficient amounts of recombinant enzyme for the comprehensive evaluation of the biochemical and clinical efficacy of enzyme replacement in patients with Fabry disease. In addition, genetic engineering techniques can be employed to alter the physical properties of this protein (i.e. pH optimum, Km, stability). This technology can be adapted to generate novel enzyme molecules that may be more effective for enzyme replacement than the wild type protein. Such engineered molecules might be more stable, have lower Km values, and/or be specifically targeted to critical sites of pathologic substrate accumulation to potentially enhance the effectiveness of enzyme replacement endeavors.

B. Fabry Disease

1. Historical Perspectives

The first patients with angiokeratoma corporis diffusum were independently described in 1898 by two dermatologists, Anderson (7) in England and Fabry in Germany (8). Anderson's original patient was a 39 year old male who had proteinuria, finger deformities, varicose veins, and lymphedema. Fabry's diagnosis of purpura nodularis was based on the observation of a 13 year old male who he followed for the next 30 years. He documented the presence of albuminuria, further described the cutaneous lesions, noting the presence of small vessel aneurysms (9), and subsequently classified his case to be one of angiokeratoma corporis diffusum, a designation that has persisted.

In 1947, Pompen and coworkers (10) made a very significant observation; they described the presence of abnormal vacuoles in the blood vessels of two affected brothers who were brought to autopsy. Based on these findings, they suggested that the disease was a generalized storage disorder. Subsequently, Scriba and Hornbostel (11) and Scriba

(12) noted the lipid nature of the storage material by demonstrating refractile lipid deposits in the blood vessels of a skin biopsy specimen. In 1963, Fabry disease was classified as a sphingolipidosis by Sweeley and Klionsky (14) following the isolation and characterization of two neutral glycosphingolipids, globotriaosylceramide (Gal-Gal-Glc-Cer) and galabiosylceramide (Gal-Gal-Cer) which were accumulated in the kidney of a Fabry hemizygote. Subsequent studies confirmed the systemic accumulation of globotriaosylceramide (15-18), and to a lesser extent galabiosylceramide (14,16,18) in affected males. In addition, the blood group B glycosphingolipid substances also accumulate in patients with B or AB blood types. In 1965, the X-linked recessive inheritance of the disease was documented through pedigree analysis (13).

It was shown in 1967 (2) that the enzymatic defect in Fabry disease was the deficient activity of ceramide trihexosidase, a lysosomal galactosyl hydrolase required for the catabolism of globotriaosylceramide and galabiosylceramide. The enzymatic activity was characterized as an α-galactosyl hydrolase by Kint (19) using synthetic substrates. Subsequently, it was shown that there were two enzymes (α-galactosidases A and B) that hydrolyzed synthetic α-galactosides; α-Gal A was deficient in Fabry hemizygotes. In 1977, α-galactosidase B was shown to be an α-*N*-acetylgalactosaminidase (20, 21).

In affected hemizygous males, the progressive accumulation of globotriaosylceramide, principally in the plasma and vascular endothelial lysosomes, results in progressive vascular narrowing and infraction. The major clinical manifestations of the disease include angiokeratoma, acroparesthesias, and occlusive vascular disease of the kidney, heart and brain, leading to early demise at 30 to 40 years of age (1, 2).

2. Rationale for Enzyme Replacement Therapy in Fabry Disease

Among the inborn errors of metabolism, studies of patients with lysosomal storage disorders have provided basic understanding of the biology of the lysosomal apparatus and its hydrolases, their biosynthesis and processing (22, 23), the mechanism of their transport

to the lysosomes (24-26), and their cofactor requirements (27-30). Of the over 30 lysosomal storage disorders, Fabry disease is an ideal candidate for the application of recombinant DNA techniques to evaluate various therapeutic approaches in model systems, as well as to correlate the effects of site-specific changes on enzyme structure and function. The disease has no central nervous system involvement, thus, the blood/brain barrier does not present an obstacle to enzyme replacement therapy. The defective enzyme, α-Gal A, is a homodimer (31), in contrast to some lysosomal enzymes which have different subunits (e.g., β-hexosaminidase A) (32); therefore, only a single gene product must be obtained. The metabolic defect in cultured fibroblasts from Fabry disease has been corrected *in vitro* by the addition of exogenous enzyme into the culture medium (33). Also, atypical variants with Fabry disease have been identified; these males are clinically asymptomatic, having sufficient residual α-Gal A activity (3 to 10%) to protect them from the major morbid manifestations of the disease (34-39). Finally, as noted above, human trials have demonstrated the biochemical effectiveness of enzyme replacement to deplete the circulating substrate prior to vascular deposition as well as the absence of immunologic complications (6, 40, 41).

C. Lysosomal Enzymes: Biosynthesis and Targeting

1. Lysosomal Hydrolases

Lysosomal enzymes are synthesized on membrane-bound polysomes in the rough endoplasmic reticulum. Each protein is synthesized as a larger precursor containing a hydrophobic amino terminal signal peptide. This peptide interacts with a signal recognition particle, an 11S ribonucleoprotein, which in turn binds to the docking protein and thereby initiates the vectoral transport of the nascent protein across the endoplasmic reticulum membrane into the lumen (42-44). Lysosomal enzymes are cotranslationaly glycosylated by the *en bloc* transfer of a large preformed oligosaccharide, glucose-3, mannose-9, *N*-

acetylglucosamine-2, from a lipid-linked intermediate to the Asn residue of a consensus sequence Asn-X-Ser/Thr in the nascent polypeptide (45). In the endoplasmic reticulum, the signal peptide is cleaved, and the processing of the Asn-linked oligosaccharide begins by the excision of three glucose residues and one mannose from the oligosaccharide chain.

The proteins move, via vesicular transport, to the Golgi stack where they undergo a variety of posttranslational modifications, and are sorted for proper targeting to specific destinations: lysosomes, secretion, plasma membrane. During movement through the Golgi, the oligosaccharide chain on secretory and membrane glycoproteins is processed to the sialic acid-containing complex-type. While some of the oligosaccharide chains on lysosomal enzymes undergo similar processing, most undergo a different series of modifications. The most important modification is the acquisition of phosphomannosyl residues which serve as an essential component in the process of targeting these enzymes to the lysosome (46). This recognition marker is generated by the sequential action of two Golgi enzymes. First, *N*-acetylglucosaminylphosphotransferase transfers *N*-acetylglucosamine-1-phosphate from the nucleotide sugar uridine diphosphate-*N*-acetylglucosamine to selected mannose residues on lysosomal enzymes to give rise to a phosphodiester intermediate (47,48). Then, *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase removes the *N*-acetylglucosamine residue to expose the recognition signal, mannose-6-phosphate (49,50).

Following the generation of the phosphomannosyl residues, the lysosomal enzymes bind to mannose-6-phosphate (M-6-P) receptors in the Golgi. In this way the lysosomal enzymes remain intracellular and segregate from the proteins which are destined for secretion. The ligand-receptor complex then exits the Golgi via a coated vesicle and is delivered to an endosomal compartment where dissociation of the ligand occurs by acidification of this compartment (51). The receptor recycles back to the Golgi while the lysosomal enzymes are packaged into vesicles to form primary lysosomes. Approximately 5-20% of the lysosomal enzymes do not traffic to the lysosomes and are secreted

presumably, by default. A portion of these secreted enzymes may be recaptured by the M-6-P receptor found on the cell surface and be internalized and delivered to the lysosomes (52).

Two mannose-6-phosphate receptors have been identified. A 215 kDa glycoprotein has been purified from a variety of tissues (53,54). The binding of this receptor is divalent cation independent. A second M-6-P receptor also has been isolated which differs from the 215 kd receptor in that it has a requirement for divalent cations. Therefore, this receptor is called the catio ıependent ($M\text{-}6\text{-}P^{CD}$) while the 215 kd one is called cation-independent ($M\text{-}6\text{-}P^{CI}$). The $M\text{-}6\text{-}P^{CD}$ receptor appears to be a three subunit oligomer with a subunit molecular weight of 46 kDa.

2. Biosynthesis and Processing of a-Galactosidase A

The human enzyme has a molecular weight of approximately 101kDa. On SDS gel electrophoresis it migrates as a single band of approximately 49 kDa indicating that the enzyme is a homodimer (55). α-Gal A is synthesized as a 50.5 kDa precursor containing phosphorylated endoglycosidase H sensitive oligosaccharides. This precursor is processed to a mature form of about 46 kDa within 3-7 days after its synthesis. The intermediates of this processing have not been defined (34). As with many lysosomal enzymes, α-Gal A is targeted to the lysosome via the mannose-6-phosphate receptor in fibroblasts. This is evidenced by the high secretion rate of this enzyme in mucolipidosis II cells and in fibroblasts treated with NH_4Cl.

The enzyme has been shown to contain 5-15% Asn linked oligosaccharides (56). The tissue form of this enzyme was shown to have ~52% high mannose and 48% complex type oligosaccharides. The high mannose type coeluted, on Bio-gel chromatography, with $Man_{8\text{-}9}$ GlcNAc while the complex type oligosaccharides were of two categories containing 14 and 19-39 glucose units. Upon isoelectric focusing many forms of this enzyme are observed dep:ıding on the source of the purified enzyme (tissue vs plasma form).

However, upon treatment with neuraminidase a single band is observed (pI ~5.1) indicating that this heterogeneity is due to different degrees of sialyation (55).

D. Enzyme Engineering

1. Introduction

During the last 10 years, dramatic developments in molecular biology and recombinant DNA technology have made it feasible to alter certain physical properties of a protein or even design functionaly improved proteins. The ability to design specificaly altered proteins permits the investigation of the structure/function relationships (57). A requisite for the study of a given protein is information about its three dimensional structure. X-ray diffraction studies yield the most information about a protein's tertiary structure. It is necessary, however, to grow single, high quality crystals (which require large amounts of purified protein) for these studies.

Another approach to obtaining information on the tertiary structure of proteins is nuclear magnetic resonance (NMR) spectroscopy. Both X-ray diffraction and NMR spectroscopy can be used in parallel, but NMR has certain advantages over X-ray crystallography. NMR studies examine the structure of the molecule in solution and can be used to describe dynamic properties of that molecule (58). NMR spectroscopy of small proteins has advanced to the point where one can routinely obtain site-specific information such as conformational changes, apparent pK_a values, hydrogen bonding patterns, hydrogen-exchange rates, ligand-binding geometry, and site-chain mobility (58).

However, one of the fundamental questions in protein design still remains unanswered: given the X-ray structure of the parent protein, how can one predict the changes that a single amino acid change will cause? Obviously there is no simple solution to this problem and powerful computer programs have been designed to predict such changes. As the number of known protein crystal structures increases, certain protein

conformations, favored over others, will become apparent and pave the way to our understanding of protein structure and function.

2. Expression Systems for Human Proteins

The development of expression systems for the high level production of recombinant proteins has been the subject of intense investigation. Countless systems, both prokaryotic and eukaryotic, have been described. Prokaryotic systems offer the distinct advantage of ease of manipulation and low cost of scale-up. However, their major drawback is their lack of proper post-translational modifications of expressed mammalian proteins. Eukaryotic systems allow for proper modification to occur, however manipulation of such systems is time consuming and scale-up becomes a major financial burden.

Eukaryotic vectors based on SV40 are very common (59-62). Two approaches have been followed for the design of these vectors. The first was to replace the SV40 early region with the gene of interest while the second was to replace the late region (64). These constructs were shown to produce recombinant proteins in mammalian cells. Early and late region replacement vectors can also be complimented *in vitro* by the appropriate SV40 mutant lacking the early or late region. Such complementation produces recombinants which are packaged into infectious capsids and which contain the gene of interest. A permissive cell line can then be infected and produce the recombinant protein. SV40-based vectors can also be used in transient expression studies, where best results are obtained when they are introduced into COS-1 (CV-1, origin of SV40) cells, a derivative of CV-1 (green monkey kidney cells) which contain a single copy of an origin defective SV40 genome integrated into the chromosome. These cells actively synthesize large T antigen (SV40), thus initiating replication from any plasmid containing an SV40 origin of replication.

In addition to SV40, almost every molecularly cloned virus or retrovirus has been used as a cloning or expression vehicle (65-82). Of particular interest are vectors based on bovine papilloma virus (65). These vectors have the ability to replicate as extrachromosomal elements. Shortly after entry of this DNA into mouse cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, thereby yielding a high-level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as the neo gene.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. Cells are allowed to grow for 1-2 days in an enriched media following the introduction of the foreign DNA, and then are switched to a selective media. A selectable marker in the recombinant plasmid confers resistance to the selection and allows cells that have stably integrated the plasmid into their chromosomes to grow and form foci which in turn can be cloned and expanded into cell lines. A number of selection systems are available. The herpes simplex virus thymidine kinase (83), hypoxanthine-guanine phosphoribosyltransferase (84), and adenine phosphoribosyltransferase (85) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells respectively. Also, antimetabolite resistance has been used as the basis of selection for the dhfr (confers resistance to methotrexate) (86, 87), gpt (confers resistance to mycophenolic acid) (88), neo (confers resistance to the aminoglycoside G-418) (89), and hygro (confers resistance to hygromycin) (90) genes. Recently, additional selectable genes have been described, namely trpB (91) (allows cells to utilize indole in place of tryptophan), hisD (92) (allows cells to utilize histinol in place of histidine), and ODC (ornithine decarboxylase; confers resistance to the ornithine decarboxylase inhibitor, 2-(Difluoromethyl)-DL-ornithine; DFMO).

In addition, vectors based on a number of retroviruses (avian and murine), adenoviruses, vaccinia virus (93) and polyoma virus have been used for expression. Other cloned viruses, such as JC (94), BK and the human papilloma viruses (95), offer the

potential of being used as eukaryotic expression vectors. High-level expression also has been shown for human growth hormone (96) by utilizing the human metallothionine IIA promoter. Yields of up to 270 mg/liter culture have been reported for this system (96).

3. Prokaryotic Expression systems

Expression of recombinant proteins in prokaryotes offers the advantage of very high yields of recombinant product (mg to g/liter of culture) at less expense. Although a plethora of expression vectors have been reported, the efficient expression of certain mammalian genes (especially glycoproteins) in *E. coli* remains difficult (see below).

An efficient prokaryotic expression system must have a strong promoter. The lactose promoter/operator, the *trp* and synthetic *tac* promoters, the phage λP_L and P_R promoters and the lipoprotein promoter are widely used. Recently, *E. coli* promoters have been reviewed (97,98). A strong promoter will guarantee the transcription of the cloned cDNA, but for efficient translation of the mRNA a good Shine-Dalgarno (SD) sequence is needed, properly spaced from the initiator AUG or GUG. It has been shown that the sequence context surrounding the AUG and the SD sequence is very important in translation initiation (99-101). Lastly, the expression of a cloned cDNA must be regulatable, in order to prevent possible cytotoxicity caused by the expressd gene product. To this effect, the promoters of these expression vectors contain operator sites so that they can be repressed in the presence of the supressor; for example, the lac I gene product for the *lac, trp, tac*, and *lpp-lac* promoters and the cI857 gene product for the λP_L and λP_R promoters.

The abundant information on *E. coli* biology has contributed greatly to the design of novel vectors. Many vectors direct expression of a cloned gene in the cytoplasm of this bacterium (102-112), while others use signals from secretory proteins in *E. coli* (113-115) (or from *Staphylococcus aureus)* to direct the recombinant protein into the periplasmic space or the culture media. For example, Kobayoshi and coworkers (116-118) utilized a

weakly activated *kil* gene in plasmid pMB9 to direct secretion of human growth hormone into the culture media of *E. coli*.

Fusion of a polypeptide to an affinity tail may improve product yield, stabilize the protein and also provide the means for a single step purification of the fusion product. One such vector utilizes the gene encoding the *Staphylococcal* protein A (119,120). This vector will direct the fusion product into the periplasmic space of *E. coli* or into the culture media of Gram positive bacteria such as *Staphylococcus aureus*. Use of an IgG affinity column results in a single step purification of a fusion product that is >99% pure. The protein A moiety can then be removed by enzymatic or chemical treatment.

Although a number of genes, both prokaryotic and eukaryotic, have been expressed in *E. coli*, certain mammalian genes have been difficult to express at reasonable levels or could not be expressed. There are several possible explanations for this situation:

(i) Codon Usage: There are distinct differences between the codons used in *E. coli* genes and mammalian genes. These characteristic patterns of codon usage have been attributed mainly to the availability of isoaccepting tRNAs in the organism (121,122). Also, it was shown that the codon following the AUG initiation codon at the *lac* Z gene of *E. coli* could vary expression of β-galactosidase by a factor of 15 (123).

(ii) mRNA Primary and Secondary Structure: More evidence is coming to light regarding the dramatic effects of mRNA secondary structure on the expression of a gene. Numerous studies have shown that if the SD sequence and/or the AUG are buried in a stable double-stranded loop, translation initiation does not occur or it occurs very inefficiently (124,125). Geiser et al. (126) have shown that more than 150 nucleotides flanking the initiation codon can contribute to the efficiency of the ribosomal binding site from bacteriophage T7 gene *1*. An approach to the problem of inefficient initiation of translation was undertaken by Schoner et al. (127) who used a synthetic two-cistron system to overcome inhibition of translation initiation caused by inhibitory sequences of the cloned cDNA. The concept behind this approach is that if the first mRNA is a short

(24bp), efficiently translated message, it would stimulate translation of the following message since the ribosomes are already loaded onto the bicistron and have melted most of the possible inhibitory secondary structures of the message.

(iii) Solubility vs Stability of the Gene Product: Many investigators have reported that overproduction of recombinant proteins in *E. coli* usually results in solubility problems, where abnormally folded proteins precipitate to form inclusion bodies in the cytoplasm of the bacterium (128,129). If the protein remains soluble, then proteolysis often occurs, which may be so severe that no native recombinant protein can be isolated. *E. coli* contains at least eight soluble proteolytic activities (130) which are very efficient in degrading abnormally folded proteins (131). Certain proteases have been localized to different *E. coli* subcompartments (cytoplasm or periplasmic space) (132,133). It was also shown that the temperature at which the culture is grown can affect proteolytic activity (i.e., 30 °C vs 42 °C) (128,134,135). In fact, a major cytoplasmic protease synthesized by *E. coli* at high temperatures, and/or after production of abnormally folded proteins, is the lon gene product (136-138). Mutants at the *lon* locus have been isolated and recombinant proteins seem to be more stable in these strains (128,129). Another useful mutant has been isolated that involves the *htpR* locus (136); this locus is believed to encode a heat-shock response regulatory factor that controls transcription of an operon that encodes many gene products, several of which are proteases. Although use of this mutant can increase the yield of recombinant proteins, it has no effect on the yield of proteins directed to the periplasmic space of *E. coli* .

Even though *E. coli* may not be a suitable host for expression studies of many mammalian genes, it is the optimal system for numerous other gene manipulations and molecular studies. This is particularly true for site-directed mutagenesis (i.e., the alteration of a gene sequence in order to study structure/function relationships of the gene product).

4. Protein Engineering by site-specific Mutagenesis

Numerous schemes have been devised for *in vitro* mutagenesis. For random mutations in the region of interest, sodium bisulfide treatment of single-stranded DNA results in deamination of exposed cytosines to form uracil, resulting in cytosine to thymidine transition mutations following DNA replication (139). This procedure was used to mutagenize the overhangs generated by restriction digestion of double-stranded DNA (139). Also, ingeneous schemes have been devised to expose in single-stranded form a predetermined region of a closed circular plasmid. This method involves first nicking the plasmid at a site determined by a synthetic oligonucleotide, using *E. coli* recA protein and S1 nuclease. Following nicking, a gap is generated using any convenient exonuclease (Exo III, λExo). This single-stranded gap can again be mutagenized using sodium bisulfite (140,141).

For site-specific mutagenesis, an oligonucleotide complementary to the site of interest is used. The primer is annealed to a single stranded-template and extended using Klenow polymerase or T_4 polymerase (142). Following extension and ligation with T_4 ligase the product is introduced into *E. coli*. Theoretically, two products, a wild type and a mutant one should arise in a 50:50 ratio after replication of this plasmid. However, the efficiency is low so a number of schemes have been devised to increase the number of mutants obtained; these include the use of phosphorothioate-modified DNA (143,144). In this scheme the mutant oligonucleotide is annealed to the single-stranded target (usually M13) and extension is performed using Klenow polymerase in the presence of phophorothioate-modified dNTPs. DNA modified this way is resistant to many restriction endonucleases, and digestion with any one of them (usually *Nci* I) results in nicking of the wild type strand. This nick can be extended with Exo III and then repaired according to the mutant strand using Klenow polymerase. Yields of up to 90% have been reported for this system.

Another ingenious scheme for mutant strand selection was devised by Kunkel (145). He used *E. coli* strains deficient in the enzymes dUTPase (*dut*$^-$) and uracil glycosidase (*ung*$^-$). The *dut*$^-$ mutation increases the levels of intracellular dUTP while the *ung*$^-$ mutation allows incorporation of occasional deoxyuridine in DNA in place of thymidine. M13 phage grown in a *dut*$^-$ *ung*$^-$ strain contains 20-30 uracil residues/genome. Use of M13 grown in such a host involves priming, extension and ligation as above. However, when these constructs are introduced into a wild type *E. coli* strain, the wild type strand will be degraded (because it contains uracils) and will be repaired according to the mutant strand.

The above methods give excellent results with one major drawback: all mutagenesis has to proceed through M13 intermediates. It would be more desirable to be able to carry out mutagenesis in double-stranded plasmids which are usually the expression vehicles that will be used to study the effects of these mutations. A number of different approaches have been reported to accomplish this objective (146,147). In general, a single-stranded gap is generated and the mutant oligonucleotide annealed and extended to fill the gap. Double-stranded mutagenesis is still at an infant stage and no methods have been devised, yet, for selection of the mutant strand. Efficiencies as low as <0.5% have been reported (146). However, new approaches are constantly being reported (148-153), and it should not be long before more efficient schemes of double-stranded mutagenesis are devised.

With these tools at hand, protein engineering is now a feasible endeavor and the design of new and novel proteins and peptides is within sight. As the secrets of protein folding and protein structural domains begin to be elucidated, the modification and improvement of a protein will become a reality. There will be many uses for such improved proteins including use for the targeted treatment of specific diseases.

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Chapter 1

Expression of Human α-galactosidase A in E. coli and S. aureus

ABSTRACT

The expression of human α-galactosidase A (α-Gal A; EC 3.2.1.22) was evaluated in the *E. coli* K12 *melA* strain M2701, using various vectors and the chromogenic substrate, X-α-Gal, to detect enzymatic activity. Using the pKK223-3 vector for cytoplasmic expression, no α-Gal A activity or enzyme protein was detected. In contrast, the pIN III-C vector which targets the expressed protein to the periplasmic space, produced active α-Gal A detected with X-α-Gal and by incubation of the bacteria with the fluorogenic substrate, 4-methylumbelliferyl-α-D-galactopyranoside (4MU-α-Gal). However, active enzyme could not be purified from the periplasm, possibly due to the fusion of the lipoprotein octapeptide outer membrane anchor to the amino-terminus of the enzyme. Similarly, active α-Gal A was expressed by the pIN III-ompA vector, which lacks the octapeptide anchor; enzymatic activity was detected in intact cells with X-α-Gal and the fluorogenic substrate. In addition, IPTG induced clones expressed sufficient α-Gal A to grow on melibiose or α-methylgalactoside as the sole carbon source whereas uninduced cells did not survive. However, only 1-5 units/ml of α-Gal A was recovered from the periplasmic fraction, suggesting that the expressed enzyme was unstable.

To further evaluate this possibility, the α-Gal A cDNA was subcloned into the pRIT5 and pRIT2T vectors which express protein A fusion products into the periplasm or cytoplasm of *E. coli*, respectively. The 76 kDa fusion protein expressed by both constructs was rapidly degraded to inactive species of about 68 kDa and 57 kDa, consistent with potential Gly-Gly-X sites for proteolytic degradation at residues 261 and 361 in the α-Gal A subunit, respectively. Expression of the pRIT5-AGA construct in *S. aureus* resulted in similar degradation products. When the pRIT2T-AGA construct was expressed in *E. coli lon* strains, the 76 kDa fusion protein was observed in the cytoplasm but not in the periplasmic space. These results provide the first demonstration that active human α-Gal A

can be expressed in *E. coli*, and indicate that the non-glycosylated enzyme polypeptide is unstable and rapidly degraded, presumably due to proteases in the periplasmic space.

INTRODUCTION

Human α-galactosidase A (α-Gal A; EC 3.2.1.22) is a lysosomal hydrolase which cleaves the terminal α-galactosyl moieties of glycolipids and glycoproteins (1). The mature lysosomal enzyme, purified from a variety of sources, is a 101 kDa homodimeric glycoprotein with a subunit molecular weight of about 46 kDa (2). Biosynthetic studies indicated that the enzyme subunit is synthesized as a 45 kDa precursor polypeptide which is co-translationaly glycosylated in the endoplasmic reticulum to a 50 kDa species. After cleavage of the signal peptide, carbohydrate modification, and phosphorylation in the Golgi apparatus, the mature 46 kDa subunit forms the active homodimer (3). The cDNA for the α-Gal A subunit encodes 429 residues including a 31 amino acid leader sequence (4).

The deficient activity of α-Gal A is the enzymatic defect in Fabry disease, an X-linked lysosomal storage disorder (1). In affected hemizygous males, the deficient α-Gal A activity results in the accumulation of the neutral glycosphingolipid, globotriaosylceramide, in the plasma and in endothelial cell lysosomes of blood vessels throughout the body. Previous studies demonstrated the biochemical and immunologic efficacy of enzyme replacement in Fabry disease using enzyme purified from human plasma and spleen (5). However, complete evaluation of this therapeutic approach was not possible due to the lack of sufficient quantities of purified human α-Gal A. Therefore, efforts were directed to develop a suitable expression system for the efficient production of recombinant human α-Gal A.

Prokaryotic expression systems have been shown to produce large amounts of many prokaryotic and eukaryotic proteins (6). Prokaryotic systems offer many advantages over eukaryotic systems, including simplicity of genetic manipulation and expression of the target gene, ease of scale-up and low cost of production. A wide range of expression

vectors and hosts are available for the targeted delivery of the recombinant protein into the bacterial cytoplasm, periplasm or across the outer membrane into the growth medium (7-14). However, experience with the expression of eukaryotic proteins in *E. coli* has been variable; the expression of active enzymes, particularly glycoproteins, has been troublesome (15,16). Therefore, the expression of each protein requires evaluation in different prokaryotic vector/host systems. Factors influencing the successful prokaryotic expression of a eukaryotic protein include molecular weight, number of sulfhydryl bridges and the functional importance of glycosylation or other post-translational events for folding, stability or activity (for review, see 6). Additional factors include the mRNA secondary structure and the efficiency of mRNA translation, which depend strongly on the codon usage of a particular message, as well as the stability of the eukaryotic gene product in the bacterial host (e.g.17-21).

A common procedure for improving the expression of a foreign protein in *E. coli* is to fuse its cDNA to a gene that is efficiently expressed. Many such constructs have been described including fusions to phage genes, *E. coli* β-galactosidase and to the *S. aureus* protein A genes (22,23). The latter construct offers the added advantage of simple and efficient purification of the fusion protein by IgG affinity chromatography (23). For example, a protein A~β-galactosidase construct expressed a fusion protein which retained both activities upon purification (24).

A previous effort to express α-Gal A in *E. coli* employed the maxicell strain CSR 603 which reportedly did not hydrolyze X-α-Gal and did not grow in media containing melibiose or α-methylgalactoside as the sole carbon source (25). However, evaluation of this strain revealed that, in fact, *E. coli* strain CSR 603 had endogenous α-Gal A activity detectable with 4-MU-α-Gal as the substrate, grew on melibiose and α-methylgalactoside, and a few colonies hydrolyzed X-α-Gal (Ioannou, Y.A., unpublished). These findings prompted the use the *E. coli melA* strains M2701 and M2508 (26) which do not hydrolyze 4-MU-α-Gal or X-α-Gal and do not grow on either melibiose or α-methylgalactoside. In

this chapter, I describe the construction and evaluation of selected prokaryotic expression vectors and their success in the expression of human α-galactosidase A in *E. coli*. These studies demonstrate that active human α-Gal A can be expressed in *E. coli*, however, the unglycosylated enzyme is rapidly inactivated by endogenous proteases.

MATERIALS AND METHODS

Materials.

The pIN *E. coli* expression vectors were the generous gift of Dr. M. Inouye, Medical and Dental College of New Jersey, Piscataway, NJ. The pRIT fusion vectors, pKK233-2 and IgG-Sepharose 6 Fast Flow were purchased from Pharmacia LKB (Piscataway, NJ). The pGEM plasmids were purchased from Promega (Madison, WI). Restriction endocucleases and DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA); T4 DNA ligase was purchased from IBI (New Haven, CT). All reagents for microbial growth were purchased from DIFCO (Detroit, MI). X-α-Gal was purchased from Boeringer Manheim (Indianapolis, IN).

Bacterial Strains and Transformations.

The *melA E. coli* strains M2701 (F^- *melA lacZ galK melB met*1 str^r λ^-) and M2508 (Hfr-1 *melA lacZ melB met*1 λ^+ λ^s) were the generous gift of Dr. R. Schmitt, Institut für Biochemie, Regensburg, F.R.G (26). BNN 103 (Δ[*lac* IPOZYA]U^{169} *pro*A Δ*lon ara*D^{139} *str*A *thi*) was purchased from ATCC (Rockville, MD). The *lon* strain LC 137 (*htp*R^{165} *lon*^{R9} lac_{am} trp_{am} pho_{am} rpsL sup C^{ts} mal_{am} tsx:Tn 10) was the generous gift of Dr. L. Rosenberg, Harvard University, Cambridge, MA) (27). The *S. aureus* strain SA 113 was purchased from Pharmacia LKB (Piscataway, NJ).

E. coli was grown in LB media or M9 media supplemented with the appropriate carbon source and amino acids. *S. aureus* was grown as described (28).

E. coli was transformed by a standard $CaCl_2$ protocol (29). *S. aureus* transformations were performed essentially as described (28).

Construction of pG-AGA.

Plasmid pcDAG126 (30) containing the full-length α-Gal A cDNA was digested with *Bam* HI and *Pst* I and the 1.45 kb insert fragment was purified on an 0.8 % agarose gel. The cDNA was then force-cloned into plasmid pGEM4 at the *Bam* HI and *Pst* I sites resulting in plasmid pG-AGA. As shown in Figure 1, plasmid pG-AGA was digested with *Pst* I and *Bam* HI and the 1.5 kb α-Gal A cDNA was gel purified. This cDNA was treated with *Alu* I methylase to add a methyl group to the 3' *Hgi* AI site (nt 302) in the α-Gal A cDNA. Subsequent digestion with *Hgi* AI removed the 5' untranslated sequence and the α-Gal A 93 nt signal sequence, thereby leaving the nucleotide sequence encoding the mature α-Gal A subunit of 398 residues. Following *Hgi* AI digestion, the cDNA was treated with T_4 polymerase in the presence of all four nucleotides in order to remove the overhang and expose the first CTG of the mature sequence. Plasmid pGEMBlue was digested with *Eco* RI, the 5' overhangs filled-in with Klenow, and then digested with *Bam* HI. The truncated cDNA was subcloned into the pGEMBlue vector. The correct construct was identified by the presence of a reconstructed *Eco* RI site, since this restriction site could only result from blunt-end ligation of the cytidine (the CTG from the α-Gal A cDNA) to the blunt end of the truncated *Eco*RI site of the vector. This construct was confirmed by sequencing across the junction and was designated pG6-AGA (Fig.1).

Construction of pKK233-2-AGA.

For the construction of this vector, plasmid pG-AGA was digested with *Nco* I and *Hind* III. Following purification of the 1.3 kb fragment on 0.8 % agarose, it was ligated

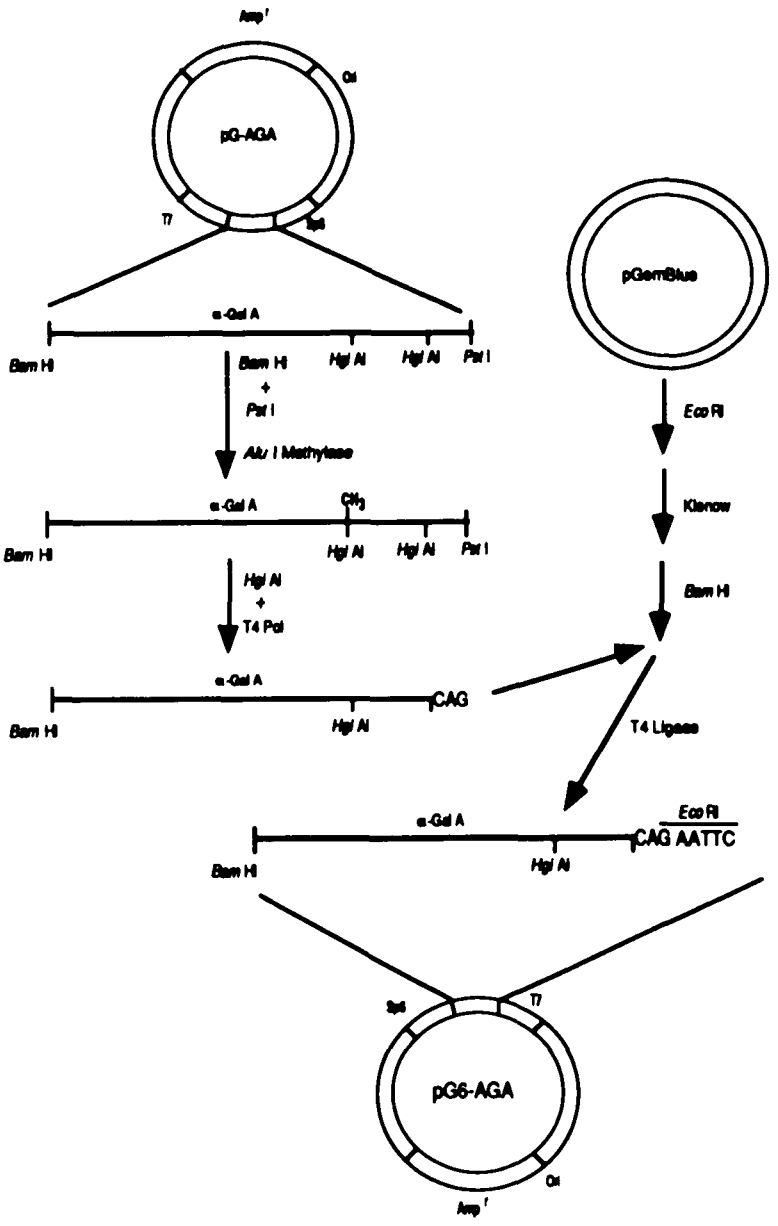

Figure 1: Constuction of plasmid pG6-AGA which contains the mature α-Gal A nucleotide sequence with Leu 32 as the first codon in the sequence.

into the *Nco* I, *Hind* III digested pKK233-2 vector. This manipulation eliminated 10 amino acids from the amino terminus of the mature sequence, therefore, initiation occurred at Met 42 instead of Leu 32. Positive clones were identified by digestion with *Nco* I and *Hind* III and were designated pKK-AGA.

Construction of pIN III and pRIT Expression Plasmids.

The *Bam* HI α-Gal A fragment from plasmid pG6-AGA was purified and ligated into the *Bam* HI site of the *E. coli* expression vector pIN III-C to construct plasmid pIN-C-AGA (Fig. 2). This construct directs expression of a fusion protein consisting of the signal peptide and eight amino-terminal amino acids from the *E. coli* lipoprotein, (lpp), and eight amino acids (encoded by the polylinker) proceeding the first codon (Leu 32) of the mature α-Gal A sequence. The lpp signal peptide directs the transfer of the nascent fusion protein into the periplasmic space, while the eight amino-terminal extension anchors the protein to the outer membrane. Plasmid pG6-AGA was used to construct pIN-ompA-AGA using the same *Bam* HI fragment as above (Fig 2). Expression of the cloned cDNA in both vectors is controlled by the IPTG inducible *lpp-lac* hybrid promoter (lipoprotein-lactose) (12).

For the construction of the protein A fusion vectors, the *Sma* I α-Gal A fragment from pG6-AGA was cloned into the *Hinc* II site of pRIT5 (Fig. 2). This construct,designated pRIT5-AGA, directs the expression of a fusion protein consisting of the IgG binding domains of protein A and the mature sequence for α-Gal A. pRIT5-AGA was digested with *Bam* HI and the α-Gal A fragment was subcloned into the *Bam* HI-digested vector, pRIT2T, which is designed for cytoplasmic expression in *E.coli* (Fig. 2).

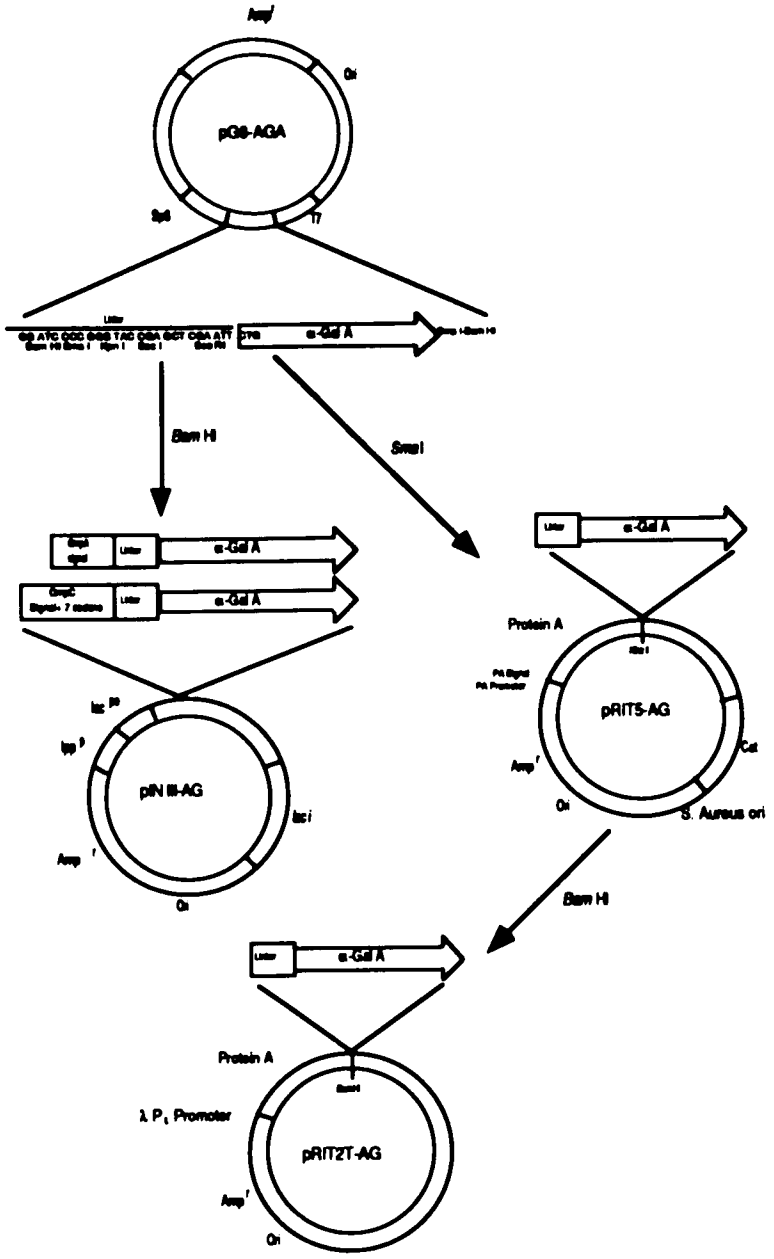

Figure 2: Construction of expression plasmids pIN III and protein A fusion vectors pRIT, using the α-Gal A mature sequence.

Construction of bicistronic construct pIN-NX-AGA.

An oligonucleotide linker encoding the sequence described by Shroner et al (31,32), was synthesized using an Applied Biosystems oligonucleotide synthesizer. A *Xba* I site was engineered at the 5' of this oligonucleotide while the 3' end had an *Nco* I site. Plasmid pIN-C-AGA (see above) was digested with *Xba* I and *Nco* I. The large fragment was purified on 0.8 % agarose gel and the linker was ligated into these sites. The construct was confirmed by sequencing.

α-Gal A Activity Assays.

α-Gal A activity toward the synthetic fluorogenic substrate, 4-methylumbelliferyl-α-D-galactopyranoside (4-MU-α-Gal), was determined as previously described (33). Briefly, a stock solution of 5 mM 4-MU-α-Gal was prepared in citrate-phosphate buffer, pH 4.6, with gentle solubilization in an ultrasonic bath. The reaction mixture containing 10-50 µl of the enzyme source and 150 µl of substrate was incubated at 37 °C for 30 min. The reaction was terminated with the addition of 2.3 ml of 0.1 M ethylenediamine. One unit of activity equals that amount of enzyme which hydrolyzed 1 nanomole of substrate/hr.

α-Gal *A Activity in Intact Bacteria.*

For determination of α-Gal A activity in intact cells of *E. coli melA*, the bacterial cells were centrifuged in a microfuge for 2 min at room temperature. The supernatant was discarded and the pellet was washed once in 20 mM $NaPO_4$ buffer, pH 6.0, and then the cells were gently resuspended in 200 µl of the same buffer. Assays were carried out with 20 µl of cell suspension and 150 µl of 4-MU-α-Gal at 37 °C for 10 min as described above.

Protein Assays and SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Protein concentration was determined by the fluorescamine method (34) as modified by Bishop et al.(35). Polyacrylamide gel electrophoresis was carried out under reducing conditions as described by Laemmli in a slab gel containing 10% acrylamide (36).

Preparation of E. coli Extracts.

(1) Isolation of the Cytoplasmic Fraction: Cells were centrifuged at 6,000 rpm for 10 min at 4 °C in a Sorvall RC-B5 refrigerated centrifuge. The pellet was resuspended in 3 ml of lysis buffer (50 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 100 mM NaCl) and then 80 µl of 50 mM phenylmethylsulfonyl fluoride (PMSF) was added followed by 80 µl of lysozyme (10 mg/ml). The cells were incubated at 4 °C for 20 min with occasional stirring. Deoxycholate (4 mg) was added with stirring and the suspension was incubated in a 37 °C water bath where it was stirred with a glass rod until it became viscous. Then, 200 µl of deoxyribonuclease A (1 mg/ml) was added and the mixture was incubated at room temperature for 60 min. Soluble and insoluble fractions were separated by centrifugation in a refrigerated Sorvall RC-B5 centrifuge at 10,000 rpm for 30 min. Alternatively, after the addition of lysozyme and incubation at 4 °C for 20 min, cells were sonicated on ice in a Branson cup sonicator by three 30 sec bursts at 70% power with 30 sec intervals. Soluble and insoluble fractions were separated as above (37).

(2) Isolation of the Periplasmic Fraction: Cells were harvested by centrifugation at 5,000 rpm in a refrigerated Sorvall RC-B5 centrifuge for 10 min. The pellet was resuspended in a one-fourth volume of sucrose solution (0.3 M Tris-HCl buffer, pH 8.0, containing 20% sucrose, 1 mM EDTA and 0.5 mM $MgCl_2$) and incubated at room temperature for 10 min. Then, the cells were centrifuged at 5,000 rpm for 5 min at 4 °C and the supernatant was discarded. The pellet was resuspended in a one-fourth volume of ice-cold 0.5 mM $MgCl_2$ and after a 10 min incubation on ice the cells were centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant contained the periplasmic fraction (38).

Affinity Chromatogrphy on IgG-Sepharose.

Affinity chromatography on IgG-Sepharose 6 Fast Flow was performed according to the manufacturer's recommendations (Pharmacia). Briefly, the resin was packed by gravity in a 10 x 2 cm glass column and then washed with 5 bed volumes of TST buffer (50 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl and 0.05% Tween 20). The column was equilibrated with 2 to 3 bed volumes each of 1) 0.5 M HAc buffer, pH 3.4, 2) TST, 3) 0.5 M HAc buffer, pH 3.4 and 4) TST. The sample was applied to the column and the gel was washed with 10 bed volumes TST and 2 bed volumes of 5 mM NH_4-acetate buffer, pH 5.0. Elution was performed with 0.5 M HAc buffer, pH 3.4. Aliquots were collected and assayed for protein (A_{280} = 1.0 for 2.6 mg protein /ml). The aliquots containing the fusion protein were concentrated to dryness with a Speed-Vac evaporator (Savant).

Construction of lon, melA Strains of E. coli.

(1) Hfr matings: Transfer of the *melA* mutation was accomplished through strain M2508 (an Hfr strain with the origin of chromosome transfer at the *lac* locus). Male (M2508) and female (RR1, a rec^+ derivative of HB101) cells were grown to an exponential phase (OD_{600} = 0.5 - 1.0). Aliquots (0.2 ml of each) were mixed and incubated at 37 °C for 3 hr and then a 0.1 ml aliquot of the mating mixture was spread on selective plates (M9-minimal plates plus galactose). Following plating, four *melA* strains were identified and isolated (39).

(2) P1 Transduction: P1 transduction was performed essentially as described by Miller (40). However, since there is no adequate selection for *melA* recombinants, the following approach was undertaken. The *Amp*C locus lies 1 min away from the *melA* locus. The *Amp*C locus is inactive in *E. coli*, but cells resistant to low amounts of ampicillin (~10μg/ml) arise spontaneously. Strain M2701 (*melA*) was first made ampicillin resistant by plating cells on LB plates containing 10 μg/ml ampicillin. The amp^r clones

were grown overnight in YT media. $CaCl_2$ and $MgSO_4$ were added to a final concentration of 0.01 M and 0.005 M, respectively. P1 stock phage (obtained from ATCC, Rockville, MD) was added (0.5 ml stock to 0.5 ml of overnight culture) and the mixture was incubated at 37 °C for 20 min. Soft agar (8 ml, containing 0.01 M $MgSO_4$ and 0.005 M $CaCl_2$) was added and the mixture was poured onto YT plates containing 0.01M $CaCl_2$. Plates were placed at 37 °C until there was a confluent lysis of cells (~5-7 hours). YT medium (5 ml) containing 0.01 M $MgSO_4$ was added to each plate and the top agar was scraped into a 150 ml glass Corex bottle. Chloroform (10 drops) was added, the mixture vortexed, and allowed to sit at room temperature for 5 min. The mixture was then centrifuged at 7,500 rpm for 20 min in a refrigerated Sorvall RC-B5 centrifuge and the supernatant containing the phage was saved.

For transduction, BNN 103 or LC 137 *lon* acceptor cells, were grown overnight in YT media to stationary phase. $CaCl_2$ and $MgSO_4$ (0.01 M and 0.005 M respectively) were added to the cells and 0.5 ml aliquots were mixed with 0.5 ml of phage supernatant above. Ten-fold dilutions of this mixture were made and then were incubated at 37 °C for 15 min before they were spread onto selective plates containing 0.01 M sodium citrate which removes the Ca^{2+} ions and prevents phage adsorption onto new cells. Recombinants were selected on LB agar plates containing 10 μg/ml ampicillin and X-α-Gal. Twenty to thirty colonies appeared 48 hr later, approximately 30% were white. Ten white colonies of BNN 103 and ten from LC137 were picked and found to be *melA* negative.

RESULTS

Cytoplasmic Expression of pKK-AGA in E. Coli melA M2701.

The cytoplasmic expression vector pKK-AGA was introduced into *E. coli* M2701. This vector contains the strong *tac* hybrid promoter which can be derepressed by the addition of IPTG to the growth media. The recombinant clones containing the plasmids did not express active α-Gal A based on their inability to hydrolyze the X-α-Gal chromogenic substrate in the indicator plates. In order to determine whether the recombinant protein was produced, cells were grown to an OD_{590}=0.4 and IPTG was added (1 mM) to derepress the promoter. Following growth for an additional 3 hr, the cells were collected, lysed and cytoplasmic and membrane fractions isolated. Analysis of the fractions from induced, uninduced, or non-transformed cells on SDS-PAGE revealed no new protein bands when stained with Coomasie blue.

Evaluation of Human α-Gal A pIN Vector Constructs.

The pIN vectors contain the strong lipoprotein (lpp) promoter and sequences from the *E. coli* outer membrane proteins A and lipoprotein (ompA, lpp) encoding signal peptides that direct secretion of the expressed product into the periplasmic space. The pIN-C-AGA construct was introduced into *E. coli* M2701 and positive clones expressing active α-Gal A were identified by their ability to hydrolyze X-α-Gal on selective plates. Cultures of positive clones were induced with IPTG and following an osmotic shock procedure, the supernatant and cells were assayed separately for α-Gal A activity toward the 4-MU-α-Gal substrate. No activity was released into the periplasmic fraction, as expected. Since it was possible that the lpp anchor was inhibiting the release of soluble α-Gal A, the pIN-ompA-AGA vector, which lacked the peptide anchor, was constructed (Fig. 2). Following transformation of the *E. coli melA* strain, positive clones were isolated and tested for α-Gal A activity by an intact cell assay designed to measure active α-Gal A before cell lysis. α-

Gal A activity was detected and was induced 3 to 5-fold upon addition of IPTG to the culture media (Table 1). However, the osmotic shock procedure failed to release any active α-Gal A in the periplasmic fraction. In addition, Coomasie-blue stained SDS-PAGE of control and expressing pIN-ompA-AGA cells did not reveal the appearence of a new protein band, indicating that the human enzyme either was made at very low levels and/or was unstable when targeted to the periplasmic space.

Table 1
Expression of active α-Gal A by pIN-ompA-AGA constructs in *E. coli* M2701. The high level of α-Gal A activity in the uninduced cultures results from growth of these cells in rich LB media.

pIN-OmpA-AGA subclone	IPTG	
	-	+
	U/ml	U/ml
M2701	0	0
-AGA.1	230	300
-AGA.2	340	1540
-AGA.14	260	1380
-AGA.3B	626	1900

In order to estimate the amount of α-Gal A made by these cells the activity was normalized for the number of *E. coli* cells expressing the human enzyme (Table 2). Since 1 OD unit is equal to about $2x10^8$ cells/ml then a liter of culture at this density will produce approximately 0.15mg of α-Gal A.

To further confirm the expression of active α-Gal A in *E. coli*, the parental strain M2701 and positive clone pIN-ompA-AGA3B were plated on selective plates and their

IPTG-induced growth was monitored with melibiose or α-methylgalactoside as the sole source of carbon (Table 3). The *melA* bacteria transformed with the pIN-ompA-AGA3B construct used these α-galactosides as carbon sources only in the presence of IPTG indicating the induced expression of sufficient amounts of the human enzyme to support growth.

Table 2
Amount of active α-Gal A produced by 2 x 10^8 cells/ml. An average of 300 U/ml represents approximately 0.15 μg of enzyme protein.

pIN-OmpA-AGA subclone	IPTG -	IPTG +
	U/OD*$_{590}$	U/OD*$_{590}$
-AGA.3B.1	23.5	334
-AGA.3B.2	14.5	237

*One Optical Density unit at 590 nm is ~ 2 x 10 ^{8} cells/ml.

Table 3
Use of α-galactosides as sole carbon source by *melA* strains harboring constucts expressing active human α-Gal A.

	LB	M9+Melibiose		M9+α-methyl-gal		M9+glucose -Met
		IPTG				
Clone		-	+	-	+	
M2701	+	-	-	-	-	-
-AGA.3B	+	-	+	-	+	-

Expression of RIT2T and RIT5 α-Gal A-Protein A Fusion Constructs in E. coli.

To further investigate the stability of the expressed human α-Gal A, plasmids pRIT5-AG and pRIT2T-AG were used to determine if a protein A fusion construct would increase the stability and expression levels of the human enzyme. These plasmids direct expression of a truncated protein A (IgG binding domains) fused through a polylinker sequence to the amino terminus of α-Gal A. Expression in plasmid pRIT2T is driven by the λ P_L promoter whereas pRIT5 is driven by the protein A promoter.

Expression of pRIT5-AGA resulted in no detectable α-Gal A activity, using selective plates with X-α-Gal or the whole cell assay. The periplasmic fraction and media

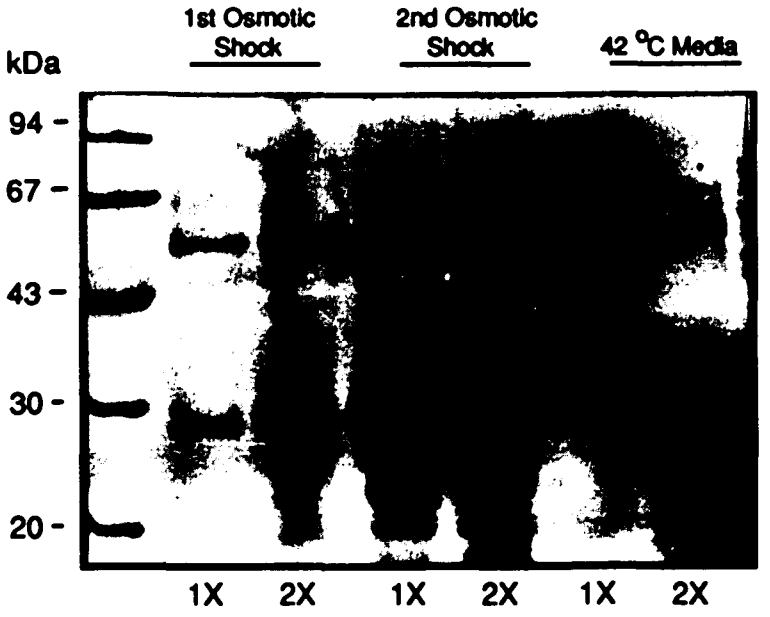

Figure 3: Isolation of periplasmic fraction from *E. coli* expressing the α-Gal A~protein A fusion. Polypeptides isolated from the first and second osmotic shock are shown. Cells grown at 42 °C allow periplasmic proteins to be secreted into the media.

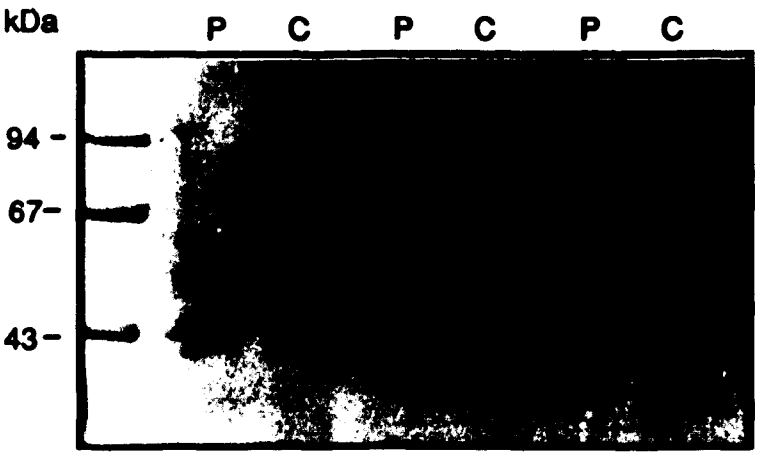

Figure 4: Protein A fusion constructs expressed in a *lon* negative strain of *E. coli*. Fusions expressed in the cytoplasm (C) and periplasm (P) of this bacterium are shown.

from cells grown at 42 °C were purified on IgG-Sepharose and visualized on SDS-PAGE (Fig. 3). The expected fusion product of ~76 kDa (31 kDa protein A and 45 kDa α-Gal A) was not observed. Instead, the SDS-PAGE of the IgG-affinity purified protein revealed the presence of ~57 and 67 kDa polypeptides as well as smaller fragments of 30 kDa and 23 kDa. These findings were consistent with the synthesis of a fusion protein that was unstable in the periplasm, presumably due to proteolytic degradation. Therefore, the *E. coli* strain BNN 103, which is deficient in lon protease activity, was used as a host for the pRIT5-AGA and pRIT2T-AGA constructs. The expressed fusion protein from *lon* extracts cells was purified, and the amount of enzymatic activity was determined. In the absence of the *lon* protease the 76 kDa fusion protein expressed by pRIT2-AGA, in the cytoplasm, was detected. In contrast, the fusion protein was not detected in the periplasmic space of cells containing pRIT5-AGA (Fig. 4).

Expression in S. aureus.

To test whether a different host could spare α-Gal A from proteolytic degradation, the pRIT5-AGA plasmid was introduced into *S. aureus* strain SA 113. In addition to an *E.coli* origin of replication this plasmid contains a *S. aureus* origin allowing it to replicate in this host. The culture media from transformed cells was collected and purified as above (Fig. 5). As with *E. coli*, the 76 kDa fusion product was not detected, presumably due to its instability. However, as with *E. coli* a 57 kDa polypeptide was detected.

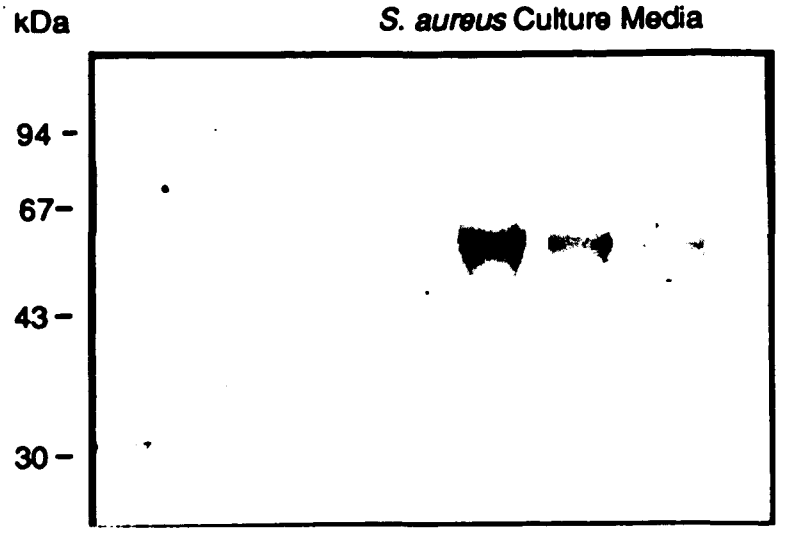

Figure 5: α-Gal A~protein A fusion expressed in *S. aureus*. The culture media from clones containing the expression plasmid was collected, concentrated and purified on IgG-Sepharose.

DISCUSSION

To facilitate efforts to express human α-Gal A in *E. coli*, I employed *melA* bacterial strains which were deficient in α-galactosidase and therefore, unable to use either melibiose

or α-methylgalactoside as the sole carbon source (26). In addition, rapid, sensitive assays using the chromogenic substrate, X-α-Gal, and the fluorogenic substrate, 4-MU-α-Gal, were employed to detect α-Gal A activity in intact cells. With this system initial efforts using the pKK-AGA construct to express active enzyme into the bacterial cytoplasm resulted in no detectable enzymatic activity. Moreover, the failure to detect the human enzyme protein suggested that the non-glycosylated enzyme polypeptide was markedly unstable, or more likely, that the construct which contained a truncated α-Gal A sequence (with Met 42 as the initiation AUG), was poorly translated due to codon usage (41,42). In support of the latter concept was the fact that the second codon in the truncated construct, GGC (Gly), has been shown to markedly reduce translation inititation of the *lacZ* gene in *E. coli* when present as the second codon (41). Therefore, constructs designed to express the entire mature enzyme subunit into the periplasmic space were evaluated.

Transformation of *melA* strains with the pIN-C-AGA or pIN-ompA-AGA constructs resulted in detectable α-Gal A activity in whole cell assays and in the periplasmic fraction, albeit at very low levels such that the enzyme protein subunit was not detectable in Coomassie-stained denaturing polyacrylamide gels. The low level expression of α-Gal A by the pIN-ompA-AGA and pIN-C-AGA constructs presumably was due to the fact that the enzyme polypeptide was expressed as a fusion protein with the leader sequence of the highly expressed *E. coli* lipoproteins (lpp and ompA). In order to determine if the low level expression was due to the poor translation efficiency of the α-Gal A mRNA, a short, highly expressed cistron (32) was inserted into the pIN vector 5' to the initiation AUG and directly infront of the mature α-Gal A *N*-terminal codon (Leu 32) . The lack of α-Gal A expression suggested that even enhancing the interaction of ribosomes with the ribosomal binding site through the efficiently expressed cistron did not increase translation of the α-Gal A message, presumably due to the presence of inhibitory sequences or mRNA secondary structure.

The inability to recover active enzyme in the periplasmic fractions from the pIN expression constructs may have resulted from subunit instability and proteolytic degradation. To evaluate this possibility, protein A fusion constructs were prepared so that the protein A~α-Gal A fusion protein could be efficiently purified by affinity chromatography on IgG-Sepharose. The fusion protein is directed to the *E. coli* cytoplasm by RIT2T and the periplasmic space by RIT5, which can also direct secretion of the fusion construct into the culture media when expresed in *S. aureus*. Expression of RIT2T-AGA and RIT5-AGA constucts resulted in 76 kDa products that were rapidly degrated to 57 kDa and 68 kDa in the cytoplasm and periplasm, respectively. The finding of distinct proteolytic fragments indicates that the 76 kDa fusion protein is cleaved at distinct sites in the protein sequence. Analysis of expression of the RIT5-AGA construct in *S. aureus* resulted in secretion of fusion protein into the culture media from which the same proteolytic fragments were purified. In addition, expression of both protein A fusion constructs in an *E. coli lon* (protease deficient) strain resulted in the detection of the 76 kDa species in the cytoplasm, whereas the intact polypeptide was not detected in the periplasmic space, further indicating that the expressed fusion protein was clipped at the carboxyl terminal end by proteolytic enzymes that presumably rendered the native enzyme inactive.

Analysis of the mature α-Gal A polypeptide (M.W.~45,356 Da) revealed that cleavage around residues 260 and 360 would result in a fusion polypeptide subunits of 68 and 57 kDa. Of note, only these two α-Gal A regions have the concensus sequence Gly-Gly-X which has been shown to be a site for a specific viral protease (43). It is intriguing to speculate that these proteolytic sites are cleaved by an *E. coli* protease, thereby rendering that human enzyme protein inactive. Perhaps the native glycosylated enzyme has carbohydrate residues in these regions which normally protect the enzyme from proteolysis. Sequencing these proteolytic fragments could confirm this hypothesis and also, identify these sensitive sites which in turn could be altered by site-specific

mutagenesis. This approach has been used successfully for the stabilization of a protein A~β-galactosidase fusion (44).

Thus, the studies described here indicate that expression of human α-Gal A in *E. coli* is limited by inefficient translation possibly due to inhibitory sequences and that the non-glycosylated enzyme is very unstable and susceptible to proteolytic degradation, presumably due to the lack of oligosaccharide moieties which are involved in protein folding and stabilization. Therefore, further efforts to produce large quantities of the human recombinant glycoprotein should evaluate various mammalian expression systems which are cabable of proper co- and post-translational modifications of this lysosomal enzyme.

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Chapter 2

A protein A~α-Gal A Fusion Expressed in Mammalian Cells Application to the Study of Mutations

ABSTRACT

The purification of proteins expressed in *E. coli* and *S. aureus* has been facilitated by the use of fusion constructs with staphylococcal protein A which offers a quick single-step purification of the fusion product. A fusion construct of the human α-galactosidase A cDNA and the staphylococcal protein A IgG binding domain E was expressed in COS-1 cells and then purified to apparent homogeneity by IgG affinity chromatography. The fusion construct was engineered using PCR techniques to insert the 16 nucleotide collagenase cleavage recognition sequence between the α-Gal A and the protein A domain E sequence. In addition, the termination codon was deleted from the α-Gal A cDNA and inserted at the terminus of the domain E sequence. Transient expression of the fusion construct in COS-1 cells resulted in a 6- to 7-fold increase over endogenous levels of α-Gal A activity and significant secretion in the media (4,000 units; nmoles/hr). The fusion protein, from the culture media, was purified to homogeneity on IgG sepharose chromatography. After collagenase treatment, the liberated α-Gal A was sepatated from the protein A peptide by IgG chromatography. By this method over 85% of secreted α-Gal A fusion protein was purified as the active, glycosylated homodimeric protein. This method should be useful for the expression and rapid purification of normal and mutant proteins.

INTRODUCTION

In addition to the production of large amounts of various proteins, prokaryotic expression systems have proven useful for structure/function analysis of expressible proteins by site-specific or saturation mutagenesis techniques (1-3; for review see 4). Such studies have provided invaluable information to determine the functional role of specific protein domains for biologic activity, substrate, cofactor, and/or subunit binding, stability, and other functional properties. In addition, the expression of various mutant proteins in prokaryotic systems has provided a relatively expedient method to produce, isolate and characterize large amounts of altered proteins. However, the expression of mammalian proteins in prokaryotic systems often is unsuccessful due to a variety of diffictulties including poor translation of the eukaryotic message associated with prokaryotic codon usage (5, for review see 6) and the lack of crucial post-translational modifications required for protein folding, stability and functional activity (7). Therefore, a variety of eukaryotic expression systems have been developed to overcome these obstacles. These include transient and stable expression systems, the latter providing the means to produce large quantities of mammalian proteins (for review see 8). Although these systems permit the stable, high level expression and post-translational modification of mammalian proteins, they require significant time and effort expenditures to establish, and thereby preclude their routine use for the structure/function studies which require the expression of a large number of transcripts engineered by mutagenic techniques. Thus it would be desirable to develop a eukaryotic expression system that permitted the facile expression of a given cDNA and rapid purification of the gene product. Such a system would permit the evaluation of the structure/function relationships of multiple cDNA alterations with significantly less effort than presently required for eukaryotic proteins that have not been successfully expressed in prokaryotic systems. In addition, the development of a more convenient system for the production and isolation of specific human proteins would

facilitate the analysis of naturally occuring mutations that result in disease. Towards this goal, efforts were directed to engineer a fusion construct that could be expressed transiently in COS-1 cells such that sufficient protein could be rapidly purified for initial structure/function analyses. In this chapter, I describe the synthesis and transient expression of an α-Gal A~protein A fusion constuct which is designed for the rapid purification of active human α-Gal A.

METHODS

Materials.

Restriction endonucleases, Taq polymerase, T4 ligase and pGEM plasmids were obtained from Promega (Madison, WI). Vector pRIT5 and IgG-Sepharose were purchased from Pharmacia (Piscataway, NJ). Sequenase sequencing kits were purchased from United States Biochemical Corp (Cleveland, OH). Collagenase was obtained from Sigma (St. Louis, MO). Oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer model 380B.

Cell Culture and Transfections.

COS-1 cells were obtained from ATCC (Rockville, MD). The cells were cultured by standard technique in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum and antibiotics.

Exponentially growing COS-1 cells (5×10^6 cells / T75 flask) were detached from the plastic by trypsinization, collected by centrifugation at 3,000 xg, and then washed once in ice-cold electroporation buffer (phosphate buffered sucrose: 272 mM sucrose, 7 mM sodium phosphate, pH 7.4, containing 1 mM $MgCl_2$). Following centrifugation at 3,000

xg, the cells were resuspended in 0.8 ml of electroporation buffer and placed in an electroporation cuvette with a 0.4 cm gap. Ten to fifteen μg of plasmid DNA was added and cells were kept on ice for 5 min. The cell-containing cuvette was placed in a Gene Pulser electroporation apparatus (Bio-Rad) and the cells were pulsed at 350 V, 25 μF. The cells were kept on ice for an additional 10 min and then plated into a 100 mm culture dish containing 10 ml of growth medium.

PCR, DNA Sequencing and Vector Constructions.

The fusion construct was synthesized using a recently described PCR technique (9,10). Briefly, the full-length α-Gal A cDNA was subcloned into the pGEM plasmid and the resulting pG6-AGA plasmid was used for PCR amplification of the α-Gal A sequence with primers designed to delete the termination codon, to add a collagenase cleavage consensus sequence at the 3' end and to include an *Eco* RI recognition sequence at the 5' end of the cDNA (Fig.1). The sense primer was 5'-CCGAATTCATGCTGTCCGGTC A CCGTG-3' and the antisense primer was 5'-CGCCGGACCAGCCGGAAGTAAGTCT T TTAATG-3'. The protein A domain E (11) was similarly amplified with the collagenase consensus sequence in the 5' oligonucleotide; the sense and antisense oligonucleotides were 5'-CCGGCTGGT CCGGCGCAACACGATGAAGCT-3' and 5'-GGCCGAATTCC GGGATCCTTATTTTGGAGCTTGAGA-3', respectively. The 1323 nt and 201 nt products of the α-Gal A and protein A PCR reactions were gel-purified on an 0.8% agarose gel and mixed together for the fusion PCR reaction. The sense primer from the α-Gal A reaction and the antisense primer from the protein A reaction were used for the final fusion reaction. The product of this reaction was digested with *Eco* RI and ligated into the *Eco* RI digested plasmid pGEM4Z. The protein A domain E and junctions between the linker and α-Gal and protein A were confirmed by the dideoxynucleotide chain termination sequencing method of Sanger (12). The confirmed fusion sequence then was digested

with *Eco* RI and subcloned into the eukaryotic expression vector p91023(B), generously provided by Dr. R. Kaufman, Genetics Institute, Cambridge, MA.

RESULTS

Construction of α-Gal A~Protein A (AGA~PA) Fusion.

Figure 1 shows the strategy used for the construction of the α-Gal A~Protein A domain E fusion sequence. The full-length α-Gal A cDNA (1323 nt) and protein A domain E sequence (201 nt) were amplified separately and then fused by a second PCR amplification (Fig. 1) using the 5' α-Gal A cDNA sense primer (P1) and the 3' Protein A antisense primer (P4). The primers were designed to: 1) eliminate the α-Gal A TAA stop codon, 2) insert the 16 nt collagenase cleavage consensus recognition sequence encoding Pro Ala Gly Pro between the α-Gal A and protein A cDNA sequences, and 3) introduce a TAA stop codon at the 3' end of protein A domain E. The integrity of this construct was confirmed by sequencing the protein A domain, linker and 3' end of the α-Gal A cDNA (Fig. 2).

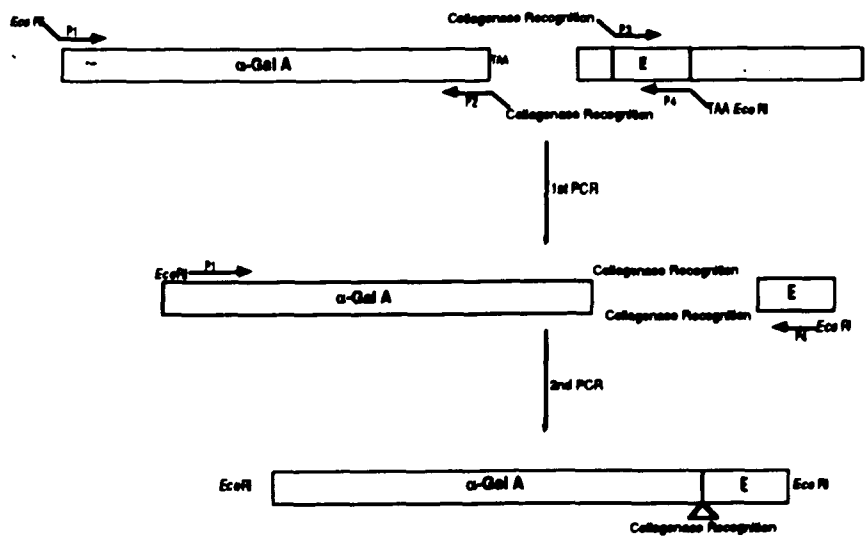

Figure 1: Construction scheme of the α-Gal A~protein A fusion. The fusion was accomplished in two separate PCR reactions as described in "methods".

Table 1
Transient expression of AGA~PA construct in COS-1 cells. Following transfection a 7-fold increase in endogenous α-Gal A activity was observed. Also, an increase of α-Gal A in the culture media was observed.

COS-1 cells	α-Gal A Activity*	
	Cells	Media
	(U/mg)	(U/ml)
Control	210	0
Transfected	1300	400

*Assayed using 4MU-α-Gal as substrate

Expression of pAGA~PA in COS-1 Cells.

Seventy-two hr after transfection with the pAGA~PA constuct, maximal levels of 4MU-α-Gal activity were detected in cell extracts and in the spent culture media (Table 1). Compared to the endogenous α-Gal A activity in COS-1 cells of 210 U/mg, the transfected cells expressed 1300 U/mg. No α-Gal A activity was detected in the spent culture medium of untransfected COS-1 cells whereas 72 hr after transfection, 400 units of activity were secreted into the media.

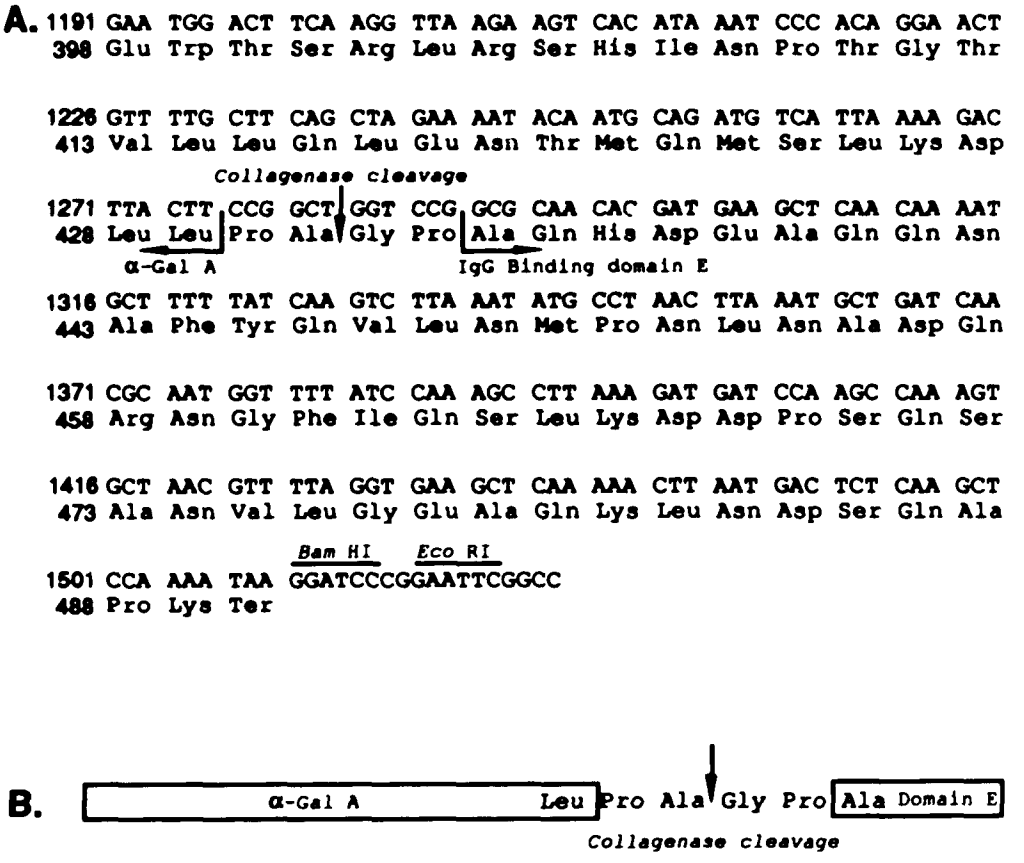

Figure 2: Nucleotide sequence of the protein A domain E, collagenase cleavage sequence and 3' α-Gal A sequence (A). Schematic of the fusion construct showing the collagenase consensus in relation to the α-Gal A and protein A domains (B).

Affinity Purification of α-Gal A.

The spent media from a single 100 mm dish of COS-1 cells was collected 72 hr after transfection and applied to a column of IgG-Sepharose. Minimal activity of α-Gal A passed through the column during sample application (flow-through), or during the buffer wash (Table 2). However, more than 95% of the bound α-Gal A~protein a fusion protein was eluted by the addition of 0.5 M acetic acid (elution buffer).

Table 2
IgG-Sepharose chromatography of the α-Gal A~Protein A fusion product from the culture media of transfected COS-1 cells. Ten ml of culture media were applied to the column, washed and eluted as described in "methods".

Purification Step	α-Gal A Activity*
	(U/ml)
Medium	4,400
Flow-Through	10
Buffer Wash	0
Elution**	4,200

*Assayed using 4MU-α-Gal as substrate
**0.5 M HAc, pH 3.4

Table 3
Treatment of α-Gal A~Protein A fusion with collagenase. Upon treatment the binding of α-Gal A to the IgG column decreased from 69% to 11%.

	% α-Gal A activity * recovered	
Step	Collagenase**	
	-	+
Flow-Through	31	89
Elution	69	11

*Assayed using 4MU-α-Gal as substrate; a total of 4,200 units of α-Gal A activity was applied.
** Collagenase treatment for 1 hr at 25 °C.

Release of the Protein A Domain from the AGA~PA Fusion Protein.

The affinity purified fusion protein was treated with collagenase for 1 hr and the reaction products were rechromatographed on the IgG affinity column. The Protein A domain E was readily bound to the IgG column, whereas the human α-Gal A was eluted in the flow-through. Almost 90% of the applied activity was eluted. Based on the specific activity of the purified enzyme, it was estimated that this procedure resulted in 90% pure enzyme.

DISCUSSION

The overlap extension method (9,10) has been used to fuse the full-length α-Gal A cDNA to the protein A domain E of *Staphylococcus aureus*. Following transfection by electroporation, the α-Gal A activity in COS-1 cell extracts was increased 6- to 7-fold. In addition, the transfected cells secreted significant amounts of the fusion protein into the culture media (400 U/ml). The secreted fusion protein was rapidly purified by a single IgG affinity purification step. The engineering of a collagenase cleavage recognition consensus sequence between these two polypeptides facilitated the cleavage of the fusion protein so that the purified human α-Gal A polypeptide could be readily separated from the protein A domain by a second IgG purification step. Of interest was the fact that the fusion construct retained α-Gal A activity, presumably indicating that the enzyme polypeptide formed the active homodimeric configuration even though the carboxy terminus was joined to an additional 56 residues of the protein A domain. Since COS-1 cells transfected with an α-Gal A construct exhibit similar levels of expression and distribution between cells and media it appears that the protein A domain does not interfere with either the folding or the

proper processing of this lysosomal enzyme. Furthermore, the presence of the dimerized α-Gal A polypeptide did not inhibit the binding of the protein A domain to the IgG affinity column. The insertion of the four residue collagenase cleavage recognition sequence between the α-Gal A and protein A polypeptides permitted cleavage of the fusion protein leaving only two of the collagen residues on each of the peptides.

The ease of cDNA construction using the polymerase chain reaction, transfection and purification of the expressed protein permits the isolation of small, but sufficient amounts of α-Gal A for characterization of the enzyme's physical and kinetic properties. Using site-directed mutagenesis or naturally occurring mutant sequences, this system provides a reasonable approach to determine the effects of the altered primary structure on the function of the protein.

Extension of this method to other human and/or mammalian proteins will determine the general applicability of this strategy. Fusion constructs with the protein A domain preceeding the amino terminus and/or following the carboxy terminus should be engineered to evaluate which fusion construct will interfere the least, if at all, with the protein's biologic function and the ability to bind IgG.

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Chapter 3

Overexpression and Specific Secretion of α-galactosidase A in Chinese Hamster Ovary Cells

ABSTRACT

Fabry disease, an X-linked inborn error of glycosphingolipid catabolism, results from the deficient activity of the lysosomal hydrolase, α-galactosidase A (α-Gal A). Previous studies demonstrated the biochemical effectiveness of α-Gal A replacement in patients with Fabry disease; however, long-term clinical evaluation was precluded by the inability to purify sufficient enzyme from human plasma or tissues. Therefore, in order to produce large quantities of recombinant human α-Gal A, the full-length cDNA was inserted into the mammalian expression vector p91023(B) in front of the amplifiable dihydrofolate reductase (DHFR) cDNA. The functional integrity of the expression construct (p91-AGA) was confirmed by transient expression of active enzyme in COS-1 cells; 650 U/mg (nmol/hr) versus endogenous levels of ~150 U/mg of 4-MU-α-D-galactopyranoside activity. Then, p91-AGA was introduced by electroporation into DG44 *dhfr*$^-$ Chinese hamster ovary (CHO) cells. Positive selection in media lacking nucleosides resulted in the isolation of clones expressing the active enzyme at levels ranging from 300 to 2,000 U/mg. Selected subclones grown in increasing methotrexate concentrations (0.02 to 1.3 μM) to coamplify the DHFR and α-Gal A cDNAs, expressed intracellular levels of α-Gal A activity ranging from 5,000 to 25,000 U/mg. Notably, subclone DG44.5, which expressed high intracellular levels of α-Gal A, secreted approximately 90% of the total recombinant enzyme produced. At a methotrexate concentration of 1.3 μM, 10^7 DG44.5 cells secreted ~15,000 U/ml culture media/day. Of note, endogenous CHO lysosomal enzymes were not secreted (ie, β-hexoseaminidase, α-mannosidase, β-galactosidase, and β-glucuronidase) indicating that α-Gal A secretion was specific and not due to saturation of the mannose-6-phosphate receptor mediated pathway. Using a hollow-fiber bioreactor, up to 10 mg of recombinant α-Gal A enzyme was produced/day. Thus, the overexpression and selective secretion of human α-Gal A should provide sufficient quantities of this recombinant protein

for purification, characterization and crystallization as well as for the clinical evaluation of α-Gal A replacement in patients with Fabry disease.

INTRODUCTION

Fabry disease, an X-linked recessive disorder results from the deficient activity of the lysosomal hydrolase, α-galactosidase A (α-Gal A, EC 3.2.1.22). The enzymatic defect leads to the accumulation of neutral glycosphingolipids with terminal α-galactosyl moieties, the primary substrate being globotriaosylceramide (1-4). In affected males, the progressive glycosphingolipid deposition in the plasma and in lysosomes of the vascular endothelium causes the major clinical manifestations of the disease, including angiokeratoma, acroparesthesias, and cardiac, cerebral and renal vascular disease. Death occurs in the third to fifth decades of life due to vascular disease complications. Heterozygous females are usually clinically asymptomatic or only mildly symptomatic and live a normal lifespan (3-6).

Previously, *in vitro* and *in vivo* efforts to replace the defective α-Gal A with normal enzyme have been reported (7-10). Addition of exogenous α-Gal A to the media of Fabry fibroblasts demonstrated the ability of the active enzyme to gain access to and metabolize the accumulated substrate (7). These *in vitro* studies indicated the feasability of enzyme replacement and, in particular, demonstrated that low levels (<5%) of exogenous enzyme were capable of normalizing substrate metabolism (11,12). Subsequent clinical trials demonstrated the biochemical effectiveness and lack of immune response to intravenously administered α-Gal A isolated from plasma and tissue sources (8-10). The plasma form was retained in the circulation longer and depleted significantly more of the accumulated circulating substrate than the splenic form (5). Although the amounts of enzyme administered were small, these studies demonstrated the feasibility of enzyme therapy for Fabry disease. However, this approach has been limited by the inability to purify sufficient amounts of the human enzyme.

The recent isolation of the full-length cDNA and entire genomic sequence encoding α-Gal A (13,14) has permitted the evaluation of prokaryotic and eukaryotic expression

systems for the production of the human enzyme. Prokaryotic expression of the human enzyme proved unsuccessful (15,16) due to inefficient translation and instability of the unglycoslyated protein (16). Therefore, various eukaryotic vectors were considered since glycosylation may be essential for proper folding, stability and lysosomal targeting of the enzyme. Of eukaryotic expression systems, two of the most widely used are the baculovirus insect cell system and SV40-based vectors containing dominant selectable markers for amplifiable expression in mammalian cells (for review see 17). Since glycosylation in insect cells is known to differ from that in mammalian cells (18,19), investigators have preferred to express human glycoproteins in mammalian cells such as Chinese hamster ovary (CHO) cells, since recent reports have shown the post-translational carbohydrate modifications of the CHO-derived proteins to be similar to those of the native human glycoproteins (20). Therefore, the p91023(B) eukaryotic expression vector was chosen to express human α-Gal A in CHO cells. This vector contains the pBR322 origin of replication and tetracycline resistence gene allowing propagation in *E. coli*, but lacks the "poison" sequences shown to inhibit replication in COS-1 cells (21). The SV40 origin of replication and enhancer sequences allow for plasmid amplification and efficient transcription of the cloned cDNA in mammalian cells. Translation is enhanced by the adenovirus "tripartite" leader sequence (22) and VA genes (23). Also, the presence of a downstream dihydrofolate reductase gene, *dhfr*, has been shown to stabilize hybrid transcripts while providing a selectable marker for stable transformation and the potential for amplification in the presence of step-wise increments of methotrexate (MTX) (17,24).

In this chapter, I describe the use of the p91023(B) vector for the amplification of the human α-Gal A cDNA in *dhfr*$^-$ CHO cells. Of particular interest was the finding that clones which produced high intracellular levels of human α-Gal A also selectively secreted the lysosomal enzyme into the culture media, providing a convenient and highly enriched source for purification.

MATERIALS AND METHODS

Materials.

Restriction endonucleases, the Klenow fragment of DNA polymerase I, T4 polymerase and T4 ligase were purchased from New England Biolabs (Beverly, MA); α- and γ-32[P] dNTPs (3000 Ci/mole) and α35[S] dATP (100 Ci/mole) were purchased from Amersham (Arlington Heights, IL). The COS-1 and DG44 CHO *dhfr*⁻ cell lines were purchased from ATTC, Rockville, MD. The p91023(B) vector was kindly provided by Dr. R.J. Kaufman, Genetics Institute, Cambridge, MA.

Construction of Expression Vector p91-AGA.

Plasmid pcDAG126 (25) containing the full-length α-Gal A cDNA was digested with *Bam* HI and *Pst* I and the 1.45 kb insert fragment was purified by agarose gel electrophoresis. The cDNA was then force-subcloned into plasmid pGEM-4 at the *Bam* HI and *Pst* I sites resulting in pGEM-AGA126. This plasmid was then digested with *Hind* III, end-filled using Klenow and ligated to *Eco* RI linkers. After digestion with *Eco* RI, the 1.45 kb fragment was purified as above and cloned into the *Eco* RI site of the mammalian expression vector p91023(B) resulting in p91-AGA (Fig. 1).

Cell Culture, Electrotransfection, and Gene Amplification.

COS-1 and DG44 CHO cells were maintained at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and antibiotics; DG44 (*dhfr*⁻) cells were maintained by addition of 0.05 mM hypoxanthine and 0.008 mM Thymidine to the media. Following transfection, the recombinant CHO lines were grown in DMEM supplemented with 10% dialyzed FCS in the absence or presence of MTX.

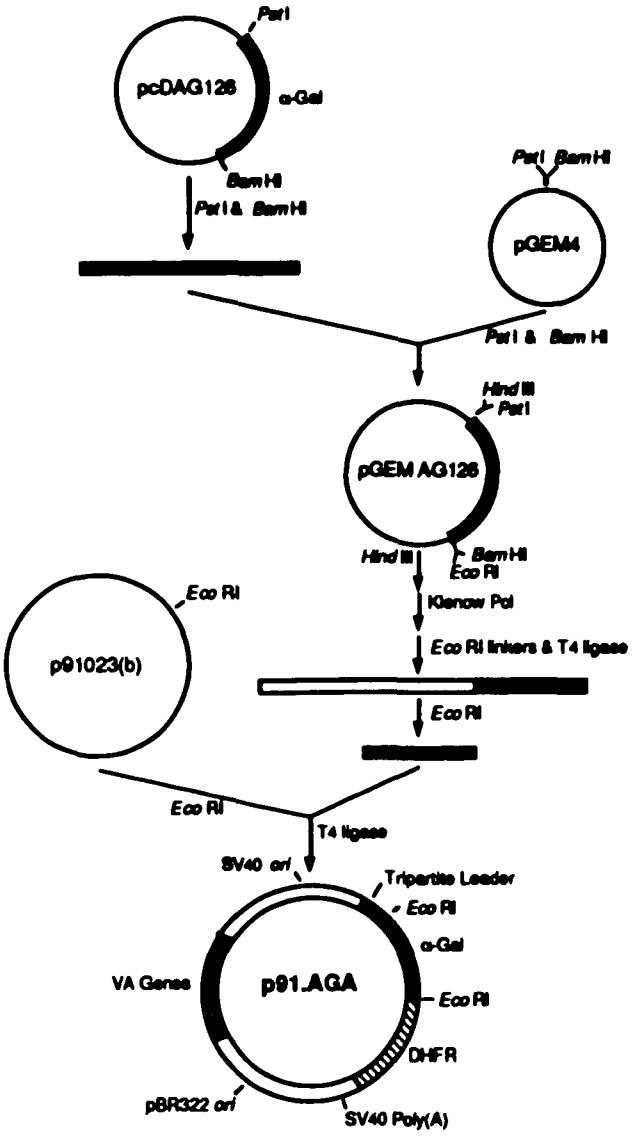

Figure 1: Construction of the α-Gal A mammalian expression vector p91-AGA. The full-length cDNA was excised from plasmid pcDAG126, adapted by the addition of *Eco* RI linkers and subsequently cloned into the *Eco* RI site of expression vector p91023(B).

For electroporation, cells were trypsinized and centrifuged at 2000 rpm at room temperature for 10 min. The pellet was washed once with DMEM supplemented with 10% FCS serum and twice in 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 to 1.0 x 10^7/ml. The cell suspension (0.8 ml) was placed in a 0.4 cm gap cuvette (Bio-Rad), 5-20 μg of plasmid DNA was added and kept on ice for 10 min. The cuvette was placed in the "Gene Pulser" chamber (Bio-Rad) and pulsed once at 25 μF with 300 V for COS-1 cells or 400 V for CHO DG44 (*dhfr*$^-$) cells, the optimized settings for the respective cell lines. The cuvette containing the pulsed cells was placed on ice for 10 min and then the cells were removed from the cuvette and placed in 15 ml of DMEM supplemented with 10% FCS.

For transient expression, COS-1 cells were harvested at 72 hr and assayed immediately. For stable expression, the transfected DG44 cells were grown for 48 hr and then were removed from the culture dish by trypsinization and replated at a 1:15 ratio in DMEM supplemented with 10% dialyzed FCS. Media was replaced every four days. After two weeks of growth, cell foci became visible and individual clones were isolated with cloning rings. Clones which expressed the highest levels of α-Gal A were subjected to amplification *en masse* by step-wise growth in increasing concentrations of methotrexate (MTX), 0.02, 0.08, 1.3, 20, 40, 80, 250, and 500 μM.

Enzyme and Protein Assays.

For enzyme assay, the cells in a 100 mm culture dish were washed twice with 5 ml of phosphate buffer saline (PBS) and scraped into a 12 ml conical tube using a rubber policeman. Following centrifugation at 2,500 rpm for 10 min, the cells were resuspended in 1 ml of 25 mM $NaPO_4$ buffer, pH 6.0, and then disrupted in a Branson cup sonicator with three 15 sec bursts at 70% output power. The sonicate was centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatant was removed and assayed immediately. Alternatively, for rapid screening, cells were washed as above and 1 ml of lysis buffer (50 mM sodium phosphate buffer, pH 6.5, containing 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 0.2 mM PMSF) was added to the dish. The lysed cells were incubated at 4 °C for

30 min, the lysates collected and transfered to a 1.5 ml tube, centrifuged in a microfuge, and then the supernatant was removed for assay.

The α-Gal A activities in the cell lysates and media were determined using 50 mM 4-methylumbelliferyl-α-D-galactopyranoside (4MU-α-Gal) as previously described (26). Briefly, a stock solution of 5 mM 4MU-α-Gal was prepared in 0.1M citrate/ 0.2M phosphate buffer, pH 4.6, in an ultrasonic bath. The reaction mixture, containing 10 to 50 µl of cell extract and 150 µl of the stock substrate solution, was incubated at 37 ^{o}C for 10 to 30 min. The reaction was terminated with the addition of 2.3 ml of 0.1 M ethylenediamine. The fluorescence was determined using a Turner model 111 Fluorometer. One unit of activity is the amount of enzyme which hydrolyzes one nmol of substrate per hour. The activities of α-mannosidase, β-galactosidase, β-hexosaminidase, β-glucuronidase and acid phosphatase were determined using the appropriate 4-methylumbelliferyl substrate. Protein concentrations were determined by the fluorescamine method (27) as modified by Bishop et al. (28).

RESULTS

Expression of Human α-Gal A in COS-1 Cells.

The full-length human α-Gal A cDNA was cloned into the expression vector p19023(B) (29) and the construct, designated p91-AGA, was introduced into COS-1 cells by electroporation. Increased levels of α-Gal A activity were detected at 24, 48 and 72 hr after transfection (Fig. 2), indicating the functional integrity of the p91-AGA construct. At

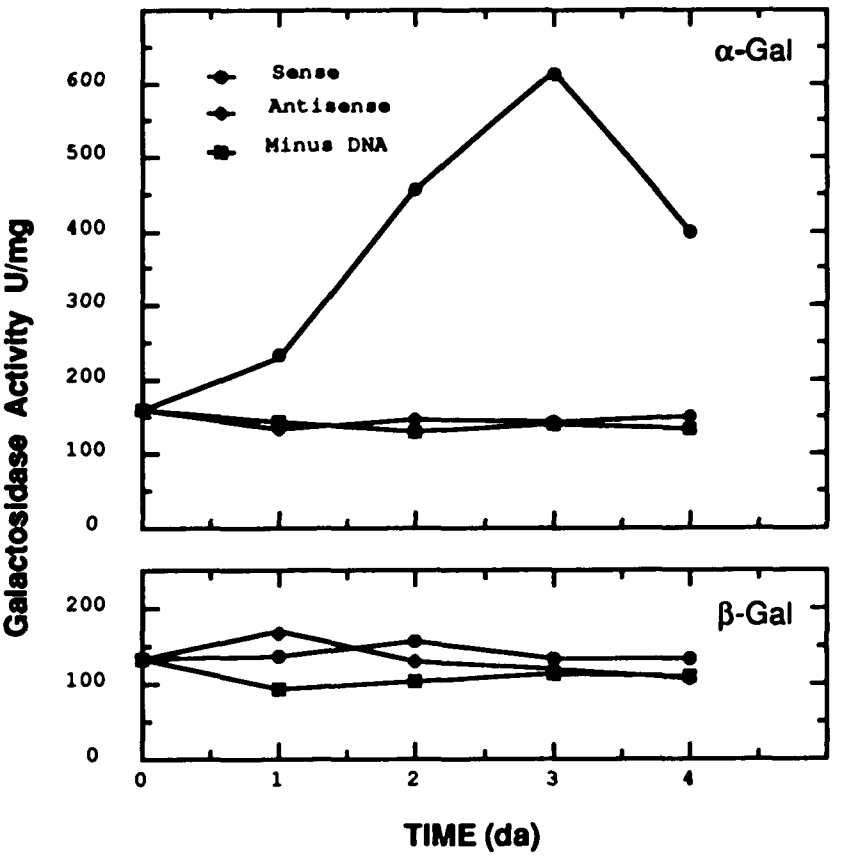

Figure 2: Transient expression of human α-Gal A in COS-1 cells. Maximum activity (U/mg) was reached 72 hr post-transfection in cells receiving the p91-AGA construct. No increase in α-Gal A activity was observed in cells receiving no plasmid DNA nor in cells receiving the p91 vector with the α-Gal A cDNA in the reverse orientation.

72 hr after transfection, the α-Gal A activity increased about four-fold, while no increase in α-Gal A activity was observed in cells transfected with the p91023(B) vector containing the α-Gal A cDNA in the antisense orientation, nor in the cells that received no DNA. In addition, the β-galactosidase levels, determined as a lysosomal enzyme control, were not changed.

Transfection and Amplification of α-Gal A in dhfr⁻ CHO Cells.

Recombinant clones stably expressing human α-Gal A were obtained by electrotransfection of the p91-AGA constuct into DG44 *dhfr⁻* CHO cells and amplification of the integrated vector DNA with selection in increasing MTX concentrations. Initial growth in media lacking nucleosides resulted in the identification of over 100 clones expressing α-Gal A at levels ranging from 200 to 1,800 U/mg protein (Table 1). Clones

Table 1
Intracellular α-galactosidase A activity in DG44 (*dhfr⁻*) CHO cells following electrotransfection with p91-AGA.

Clone	α-Gal A Activity
	(U/mg)
Parental DG44:	497
Transfected:	
4	493
5	1,243
7	108
8	524
9	1,155
11	1,115
20	624
24	1,864
46	720
52	180

Table 2
Intracellular α-galactosidase A activities in p91-AGA transfected DG44 (*dhfr*⁻) CHO cells following initial amplification in methotrexate.

Clone	α-Gal A
0.02 μM MTX:	(U/mg)
5	4,990
9	2,900
11	3,170
46.1	1,237
46.5	4,570
46.12	4,100
0.08 μM MTX:	
5.7	7,950
5.9	14,680
5.11	3,070
9.1	10,290
9.4	7,950
9.6	3,860

with the highest α-Gal A levels were grown in the presence of 0.02 to 0.08 μM MTX to amplify the integrated p91-AGA DNA. Table 2 shows the intracellular α-Gal A levels in representative amplified clones increased 2 to 6 fold in 0.02 μM MTX and up to 10 fold when further amplified in 0.08 μM MTX.

High Expression Clones Secrete Human α-Gal A.

Among the positive clones amplified in the presence of 0.08 μM MTX, clone 5.3 had the highest intracellular α-Gal A level (Table 2) and therefore was chosen for further amplification. When grown in the presence of 1.3 μM MTX, the α-Gal A activity in the growth media of clone DG5.3 was determined to be 2,500 U/ml, or 25-fold greater than the level in untransfected parental DG44 cells (50 to 100 U/ml). Growth in the presence of

Table 3
Intracellular and secreted α-galactosidase A activities in 91-AGA transfected CHO line DG5.3 following step-wise amplification in methotrexate. Data were obtained on clones after three weeks of growth in the absence of methotrexate.

Methotrexate Concentration	CHO Cells*	Media*
(μM)	(U/mg)	(U/ml)
Untransfected DG44:	250	100
Transfected p91AGA5-3:		
0.00	375	150
0.02	550	265
0.08	600	560
1.3	2,560	2,090
20	6,270	6,530
40	5,795	6,855
80	6,365	8,750
250	5,720	9,945
500	12,560	18,140

* 10^7 cells and 10 ml of media for each Methotrexate concentration

increasing concentrations of MTX, resulted in increased intracellular and secreted α-Gal A activities (Table 3). Interestingly, over 80% of the total α-Gal A produced was secreted and growth in increasing MTX concentrations continued to increase the percentage of enzyme secreted. Note that the data shown in Table 3 were obtained after the cells were amplified in the presence of the indicated MTX concentration and then assayed for α-Gal A

Table 4
Lysosomal enzyme activities secreted in the culture media of transfected and parental CHO cells.

Lysosomal Enzyme	CHO Cell line	
	DG44* Control	5-3250* α-Gal A
α-Galactosidase A	56	16,900
α-Arabinosidase	2.4	0.9
α-Fucosidase	341	358
β-Galactosidase	35.2	8.9
β-Glucuronidase	90.9	53.7
β-Hexosaminidase	2,290	2,090
α-Mannosidase	147	82.8
Acid Phosphatase	30.6	6.1

* Average of Triplicate Determinations in Two Independent Experiments

activity after growth for three weeks in the absence of MTX, which accounts for their lower intracellular activities than during growth under selective pressure (30,31,32).

Specific Secretion of Overexpressed Lysosomal Enzymes.

To determine whether the secretion of α-Gal A was due to saturation of the receptors for lysosomal targeting, the culture media from clone DG5.3 was assayed for the presence of other lysosomal enzymes. As shown in Table 4, the activities of seven

Table 5
Butyrate effect on α-Gal A secretion in CHO DG11.

Clone	α-Gal A Activity	
	Cells	Media
	(U/mg)	(U/ml)
Control	259	102.6
Butyrate	687	675
Butyrate+5mM M6P	604	700

representitive lysosomal enzymes were not increased or were lower than those in the media of the DG44 parental cell line, indicating that the DG5.3 secretion of α-Gal A was specific.

To determine if the secretion was specific to clone DG5.3, another clone, DG9, which was not secreting α-Gal A (i.e., activity in media was 120 U/ml), was subjected to step-wise growth in increasing MTX concentrations (i.e., from 0.02 to 20 μM MTX). After amplification in 20 μM MTX, clone DG9 had intracellular and secreted levels of α-Gal A activity of 9,400 U/mg and 7,900 U/ml, respectively, or 89% of the total α-Gal A activity produced was secreted.

Since treatment of recombinant CHO cells with 50 mM butyrate has been shown to specifically increase transcription of the stably integrated p91023(B) vector in CHO cells (33,34), another transfected clone, DG11, which was not amplified, was grown in the presence of 5 mM butyrate. The intracellular levels of α-Gal A activity increased from 259 U/mg to 687 U/mg. Noteably, in the presence of butyrate, increased α-Gal A activity was secreted into the media (103 to 675 U/ml), suggesting that secretion occured when the gene copy number increased (or more precisely the steady state α-Gal A mRNA was increased).

Effect of Serum Concentration on Secretion.

To determine if the serum concentration of the growth media had an effect on the levels of recombinant α-Gal A secretion, clone DG5.3 was grown in 100 mm culture dishes at a density of 5 x 10^6 cells per dish, in the presence of 0% to 10% dialyzed FCS for 5 days. There was no apparent effect on α-Gal A secretion in cells grown with 2.5% to 10% serum (Fig 3). The decreased level of secretion by DG5.3 cells cultured in 0% and 1% serum presumably reflected the poor growth of these cells.

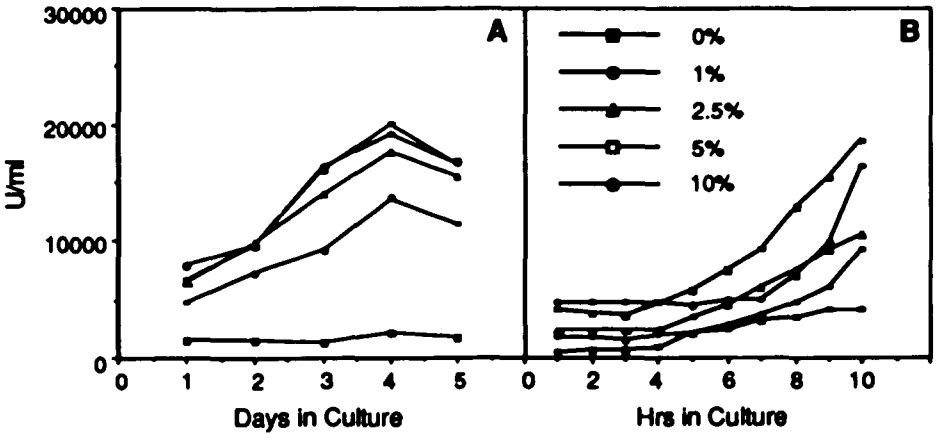

Figure 3: Serum effect on secretion of recombinant α-Gal A by CHO DG5.3. Cells were plated in DMEM supplemented with the appropriate serum concentration (A). Cell were plated in DMEM supplemented with 10% FCS. Following confluency (~4 days), the media was replaced with fresh DMEM supplemented with the appropriate serum concentration (B).

Production in Bioreactors.

To produce large quantities of recombinant human α-Gal A, 10^8 cells of clone DG5.3 which had been grown in the presence of 500 μM MTX ($DG5.3_{500}$), were used to seed a hollow fiber bioreactor. As shown in Figure 4, the level of α-Gal A produced increased to about 10,000 U/ml per day and remained constant for about three months (not

shown). In addition, the serum concentration required by these cells in the bioreactor was step-wise decreased to 1% without seriously decreasing α-Gal A production (Fig 4). A single 90-day run of this bioreactor resulted in >350 mg of active recombinant α-Gal A secreted into the culture media.

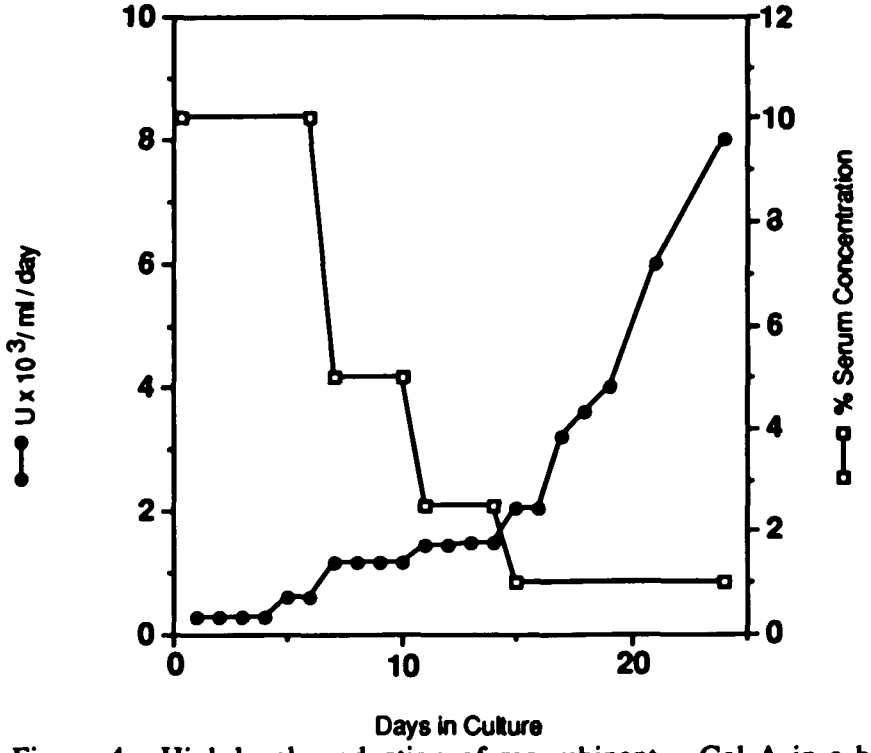

Figure 4: High-level production of recombinant α-Gal A in a hollow fiber bioreactor. The amount of fetal bovine serum required by this system for optimal cell-growth and protein secretion could be decreased to about 1%.

DISCUSSION

Mammalian expression systems already have proven invaluable for the high-level expression of human proteins which require various post-translational modifications (e.g., glycosylation, phosphorylation, γ-carboxylation) for folding, stability, activity and/or subcellular targeting (35-39). Among the available systems, SV40-based vectors containing dominant selectable markers for gene amplification in marker-deficient CHO cells permit stable integration and high-level expression of the selectable marker and the gene of interest (31,32). Notable examples of biologically functional recombinant glycoproteins produced in CHO cells with amplifiable vectors include the human tissue plasminogen activator (40), human factor VIII (20), β Interferon (41), adenosine deaminase (42), transforming growth factor β1 (43), and others. For human α-Gal A, post-translational modifications appear to be essential for stability and activity, as evidenced by the fact that the unglycosylated enzyme expressed in *E.coli* was unstable and rapidly degraded (15,16). In addition, the α-Gal A subunit, which has four potential *N*-glycosylation sites, undergoes carbohydrate modification and phosphorylation for lysosomal delivery (44,45). Previous characterization of α-Gal A purified from plasma and tissue identified their different carbohydrate compositions, the plasma glycoform having more sialic acid residues (28,46). Moveover, clinical trials of enzyme therapy revealed that compared to the tissue-derived form, the plasma glycoform had a prolonged retention in the circulation and was more effective in depleting the circulating accumulated substrate following intravenous administration to patients with Fabry disease (9). Thus, the amplified expression of human α-Gal A in CHO cells was a reasoned choice for the expression of this recombinant enzyme whose native composition includes galactosyl and sialic acid residues (47).

Although this is the first human lysosomal hydrolase to be successfully overexpressed, an unexpected finding was the secretion of over 80% of the enzyme produced. This could result from several different mechanisms including 1) saturation of the mannose-6-phosphate receptor pathways, 2) a mutation that alters a critical glycosylation site, 3) failure to expose the mannose-6-phosphate moiety for receptor binding, or 4) an unusually low affinity of recombinant α-Gal A for the mannose-6-phosphate receptor (48-50; for review see 51). If the secretion of α-Gal A was due to the saturation of the receptor-mediated pathway, then it would be expected that the other endogenous lysosomal enzymes also would be secreted. However, the levels of secreted CHO hydrolases were unchanged, or decreased (Table 4). To rule out a possible mutation in the α-Gal A cDNA introduced during construction and integration of the vector (52), the integrated vector DNA was amplified by the polymerase chain reaction. Ten subclones were completely sequenced in both orientations, and no mutations were identified. In the following chapter (45), it will be shown that the mannose-6-phosphate moiety is present on the enzyme and that the enzyme binds efficiently to the immobilized mannose-6-phosphate receptor. Furthermore, to prove that the secretion of this protein was not α-Gal A dependent, the cDNA encoding another lysosomal hydrolase α-*N*-acetylgalactosaminidase, was inserted into p91023(B) and amplifed in CHO cells. Analagous to the observations with α-Gal A, cells that were high expressors of α-*N*-acetylgalactosaminidase also secreted the recombinant enzyme in the medium.

The presence of functional mannose-6-phosphate moieties on the secreted enzyme implied that perhaps a different mechanism was responsible for its secretion. In fact, many other secreted proteins have been shown to contain mannose-6-phosphate. Some of these proteins include lysosomal proteins while the location of others is not clear. These proteins include, proliferin (53) secreted by proliferating mouse placental cell lines, epidermal growth factor receptor in A-431 cells (54), transforming growth factor β1 (55), uteroferrin, an iron containing acid phosphatase secreted in large amounts by the uterine endometrium

of pigs (56), and cathepsin L (MEP), a mouse lysosomal cysteine protease secreted by mouse NIH 3T3 fibroblasts (57). Of interest, transformation of NIH 3T3 cells with Kirsten virus results in a 25-fold increase in the synthesis of MEP causing this enzyme to be selectively secreted even though it contains functional mannose-6-phosphate moieties (57). Recently, the mechanism for the selective secretion of MEP has been identified and it involves an inherent low affinity of MEP for the mannose-6-phosphate receptor (58).

It is also notable that the overexpression of yeast vacuolar carboxypeptidase Y in yeast results in over 50% of the normally glycosylated protein secreted as the precursor form (59). Similar findings were observed for the yeast proteinase A gene (60). Studies have suggested that the precursor glycoproteins have subcellular localization signals located within the *N*-terminal propeptide that are recognized by the secretion pathway, thereby precluding delivery to the lysosome-like vacuole. It is notable that the secretion of these yeast genes is gene-dosage dependent and that a similar phenomenon is observed for the expression in CHO cells of human α-Gal A. Also, it is of interest that the precursor form of the yeast enzymes was secreted. The plasma form of α-Gal A is more sialyated and secreted, and others have shown that the lysosomal enzymes in human urine are the precursor forms (61). Thus, it is possible that the high-level expression of human lysosomal hydrolases results in their secretion due to the inability to modify the precursor and/or inability of the subcellular localization machinery to accommodate the intracellular delivery of the overexpressed glycoprotein. However, this again would result in the secretion of other lysosomal enzymes. Since no other lysosomal enzymes are detected in the culture media, it is unlikely that secretion of α-Gal A results from saturation of a component of the subcellular localization machinery.

Further studies, directed to determine the amino acid and carbohydrate differences between the secreted and intracellular forms of recombinant α-Gal A may provide insights into the mechanism underlying the mislocalization and selective secretion of human α-Gal A. In addition, efforts to evaluate the generality of this observation should include the

overexpression of other human lysosomal enzymes. The fact that large amounts of recombinant human α-Gal A are secreted by CHO cells permits the convenient production of the recombinant enzyme. Chapter 4 describes a method for the purification of the recombinant enzyme and the characterization of its physical and kinetic properties including its receptor-mediated uptake by Fabry fibroblasts.

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Chapter 4

Purification, Characterization and Processing of Recombinant α-galactosidase A

ABSTRACT

Human α-galactosidase A cloned into the amplifiable eukaryotic expression vector, p91023(B), has been overexpressed in Chinese hamster ovary (CHO) cells. The recombinant enzyme protein, was selectively secreted into the culture media and over 200 mg was purified to homogeneity by a Fast Protein Liquid Chromatographic procedure including affinity chromatography on α-galactosylamine-Sepharose. The purified secreted enzyme was a homodimeric glycoprotein with native and subunit molecular weights of 101 and 46 kDa, respectively. The recombinant enzyme had a pI of 3.7, a pH optimum of 4.6, and a km of 1.9 mM toward 4-methylumbelliferyl-α-D-galactopyranoside. It rapidly hydrolyzed pyrene dodecanoyl sphingosyl trihexoside, a fluorescently labeled analogue of the natural glycosphingolipid substrate, which was targeted with apolipoprotein E to the lysosomes of the enzyme-producing CHO cells. Pulse-chase studies indicated that the recombinant enzyme assumed its disulfide-defined secondary structure in <3 min, was in the Golgi by 5 min where it became Endo H resistant and was secreted into the media by 45-60 min. Both the intracellular and secreted forms were phosphorylated. The secreted enzyme subunit was slightly larger than the intracellular subunit. However, following endoglycosidase treatment, both subunits co-migrated on SDS-PAGE, indicating differences in the oligosaccharide moieties of the two forms. Treatment of the radiolabeled secreted enzyme with various endoglycosidases revealed the presence of three *N*-linked oligosaccharide chains, two high-mannose types (Endo H sensitive) and one complex type, the latter being Endo H and F resistant. Analyses of the Endo H released oligosaccharides revealed that one had two phosphate residues which specifically bound to immobilized mannose-6-phosphate receptors while the other was a hybrid structure containing sialic acid. These physical and kinetic properties and the presence of complex-type oligosaccharide chains on the recombinant secreted enzyme were similar to those of the

native enzyme purified from human plasma. The secreted form of α-Gal A was taken up by cultured Fabry fibroblasts by a saturable process that was blocked in the presence of 2 mM mannose-6-phosphate indicating that binding and internalization were mediated by the mannose-6-phosphate receptor. The binding profiles of the recombinant secreted enzyme and the α-Gal A secreted by NH_4Cl-treated human fibroblasts to the immobilized receptor were identical. The availability of large amounts of soluble, active recombinant α-Gal A which is similar in structure to the native enzyme isolated from plasma will permit further comparison to the native enzyme forms and the clinical evaluation of enzyme replacement in Fabry disease.

INTRODUCTION

Human α-Gal A is a lysosomal hydrolase encoded by a housekeeping gene localized to the chromosomal region Xq21.33-Xq22 (1,2). The mature lysosomal form of the enzyme is a homodimeric glycoprotein with a native molecular mass of 101 kDa (3). The chromosomal gene and full-length cDNA encoding human α-Gal A have been isolated and characterized (4,5). The full-length 1437-bp cDNA encodes 429 amino acids including a signal peptide of 31 residues (5). The processed 1.4 kb poly $(A)^+$ mRNA is translated and co-translationally glycosylated to a 50 kDa precursor glycopeptide in the rough endoplasmic reticulum (6,7). After the 31 amino acid signal peptide is cleaved, the glycopeptide undergoes modification of its *N*-linked oligosaccharide moieties in the Golgi apparatus, and then is transported to the lysosome where it functions as an α-galactosyl exohydrolase. The human enzyme from plasma and tissue sources are differentially glycosylated resulting in their distinct isoelectric points, migration in electropheretic gels, and Km values (8). Neuraminidase treatment of both enzymes indicated that the plasma form was more highly sialylated (3).

In affected males with this X-linked recessive disorder, the substrate primarily accumulates in their plasma and subsequently is deposited in their vascular endothelial cells; the progressive substrate deposition results in vascular narrowing and eventual occlusion leading to early demise (30s to 40s)(9).

Efforts to treat Fabry disease have focused on enzyme replacement. Previously, the results of a clinical trial involving multiple injections of purified tissue and plasma forms of α-Gal A into two brothers with Fabry disease was reported (10). These trials demonstrated differences in enzyme clearance rates and substrate depletion and reaccumulation kinetics for enzyme purified from tissue versus plasma sources (11). The differential plasma clearance of the glycoprotein enzymes was presumably related to differences in their post-translational modifications. The splenic form, which was rapidly cleared from the

circulation ($t_{1/2}$ 10 min), contained few sialic acid residues. The plasma form, however, was higly sialylated and was retained in the circulation ($t_{1/2}$ 70 min) (11). These results are in accordance with the Ashwell/Morell model for the prolonged retention of sialylated glycoproteins in the circulation and the rapid clearance of desialylated proteins (12). Compared to the splenic enzyme, the plasma form effected a 25-fold greater depletion of the circulating substrate over a markedly longer period (48 hr versus 1 hr) and the stored tissue substrate was mobilized into the circulation following depletion by the plasma form, but not by the splenic enzyme (11). Thus, the administered enzyme not only depleted the substrate from the circulation, but also mobilized the previously stored substrate from other depots into the circulation for subsequent clearance. These studies demonstrated the potential for eliminating, or significantly reducing the progressive glycolipid storage by repeated enzyme replacement. However, clinical improvement has not been documented to date due to the lack of sufficient quantities of purified enzyme for assessment of long term clinical efficacy.

In chapter 3 the high level expression of human α-Gal A in Chinese hamster ovary cells has been reported (13). Notably, over 90% of the α-Gal A produced in these cells was secreted into the media providing a convenient source for the purification and characterization of the recombinant enzyme. In this chapter, I describe a method for the efficient purification of the secreted enzyme, report the physical and kinetic properties of the homogeneous enzyme, the characterization of the biosynthesis and post-translational processing of the recombinant enzyme and the receptor-mediated uptake by cultured Fabry fibroblasts.

MATERIALS AND METHODS

Materials.

Endo-β-*N*-acetylgulcosaminidase H (Endo H), endo-β-*N*-acetylgulcosaminidase D (Endo D), endoglycosidase F (Endo F) and peptide:*N*-glycosidase F (PNGase F) were purchased from Boeringer Mannheim, Indianapolis, IN. [^{35}S]-methionine (>1,000 Ci/mmol), D-[2,6-^{3}H]-mannose (60 Ci/mmol), ^{32}P-Phosphorus (10 mCi/ml) and Amplify were obtained from Amersham, Arlington Heights, Il. Pansorbin was purchased from Calbiochem, San Diego, CA. 4-MU glycosides were purchased from Genzyme, Cambridge, MA. Freund's adjuvants, sphingomyelin (from brain) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma, St Louis, MO. QAE-Sephadex, Sephadex G-25, Octyl-Sepharose and Superose 6 were purchased from Pharmacia-LKB, Piscataway, NJ. The TLC silica plates (cat. 5626) were purchased from EM Science, Gibbstown, NJ. The CHO *dhfr*$^{-}$ cell line DG 44 was purchased from ATCC, Rockville, MD. All tissue culture reagents were from Gibco, Grand Island, NY. Sinti Verse I scintillation coctail was from Fisher, Pittsburgh, PA. The immobilized mannose-6-phosphate receptor was the generous gift of Dr. Stuart Kornfeld, Washington University, St. Louis, MO. The pyrene dodecanoyl sphingosyl trihexoside (P-C_{12}STH) was the generous gift of Dr. Shimon Gatt, Hebrew University, Israel. Apolipoprotein E was the generous gift of BTG Inc., Ness-Ziona, Israel.

Cell Culture.

Cells were maintained at 37 °C in 5% CO_2 in Dulbecco's Modification of Eagle's Medium (DMEM) with 10% fetal calf serum (FCS) and antibiotics. The DG44 line was cultured in DMEM supplemented with HT (hypoxanthine, thymidine, Sigma) while the recombinant CHO line DG5.3 received DMEM supplemented with 10% dialyzed FCS (14).

Purification of Recombinant α-Gal A.

Recombinant CHO culture media was collected (20 L) and concentrated to 500 ml using a Pellicon cassette tangential-flow concentrator, with a molecular weight cutoff of 10,000 daltons (Millipore, MA). The pH of the concentrate was adjusted to 4.7 to 5.0 with 10 N HCl and subsequently clarified by centrifugation at 10,000 rpm in an RC-B5 refrigerated centrifufge for 10 min.

All chromatographic steps were automated on an FPLC apparatus (Pharmacia) and were performed at room temperature. Approximately 100 ml of the media concentrate (~20 mg of α-Gal A enzyme protein)(15) was applied to an α-Gal A affinity column (α-GalNH_2-Sepharose; 2.5 x 8 cm) pre-equilibrated with buffer A (0.1 M citrate-phosphate, pH 4.7, 0.15 M NaCl). The column was washed with buffer A until the protein concentration in the eluate returned to the pre-application level (~200 ml) and was eluted with 150 ml of buffer B (0.1 M citrate-phosphate, pH 6.0, 0.15 M NaCl, 70.4 mM galactose). The eluate was collected, concentrated to about 20 ml using an ultrafiltration cell, M.W. cutoff 30,000 daltons, under positive nitrogen pressure (Amicon). The concentrate was mixed with an equal volume of buffer C (25 mM Bis-Tris, pH 6.0, 3 M $(NH_4)_2SO_4$) and then centrifuged at 10,000 rpm for 10 min. The pellet, which contained up to 40% of the activity, was redissolved in buffer A and mixed with an equal volume of buffer C and centrifuged as above. The combined supernatants were applied to a column of Octyl-Sepharose (1.5 x 18 cm) pre-equilibrated with buffer D (25 mM Bis-Tris, pH 6.0, 1.5 M $(NH_4)_2SO_4$). The column was washed as above until the eluting protein concentration returned to pre-application levels (~100 ml) and the column was eluted with buffer E (5 mM sodium-phosphate, pH 6.0, 50% ethylene glycol). The product from three Octyl-Sepharose elutions, totaling approximately 75 ml, was concentrated as above to about 2 ml using an Amicon concentrator. The concentrate was finally applied to a column of Superose 6 (20-40 μ, Pharmacia, 1.6 x 100 cm) equilibrated in buffer F (25 mM

sodium phosphate, pH 6.5, 0.1 M NaCl). The α-Gal A peak was collected, ~ 20 ml, concentrated as above and stored in buffer F at 4 °C.

Enzyme and Protein Assays.

α-Gal A was assayed using 4-methylumbelliferyl-α-D-galactopyranoside (4-MU-α–Gal) as previously described (16). Briefly, a stock solution of 5 mM 4-MU was prepared in 0.1 M citrate-phosphate buffer, pH 4.6, and was solubilized in an ultrasonic bath. The reaction mixtures containing 10-50 µl of enzyme preparation or cell extracts and 150 µl substrate, were incubated at 37 °C for 10-30 minutes. The reactions were terminated with the addition of 2.33 ml of 0.1 methylenediamine. One unit of activity is that amount of enzyme which hydrolyzes 1 nmol of substrate/hr.

Endo H, Endo D, Endo F and PNGase F digestions were performed as described (17). Samples were diluted to 0.2-0.5% SDS before digestion. All reaction volumes were 50 µl. A drop of toluene was added to each reaction tube to prevent bacterial growth. Briefly, Endo H digestions (5 mU/ reaction) were performed at 37 °C overnight in 5 mM sodium citrate, pH 5.5 and 0.2 mM PMSF. Endo D digestions (10 mU/ reaction) were performed at 37 °C overnight in 0.2 M citrate phosphate buffer, pH 6.0 and 0.2 mM PMSF. Endo F digestions (50 mU/ reaction) were performed overnight at 30 °C in 0.17 M sodium acetate, pH 6.0, 1.6% NP-40 and 0.2 mM PMSF. PNGase F digestions (100 mU/reaction) were carried out overnight at 30 °C in 0.17 M potassium phosphate, pH 8.6, 1.6 % NP-40, 0.2 mM PMSF.

Protein concentration was determined by the fluorescamine method (18) as modified by Bishop et al. (19).

In Vivo Natural Substrate Assay.

For this assay, 30 nmoles of P-C_{12}STH and 70 nmoles of sphingomyelin were mixed in a chloroform:methanol solution (1:1), evaporated under nitrogen and dried in a

Speed-Vac (Savant). The pellet was resuspended in 2 ml of saline, sonicated using a Heat Systems Ultrasonics, Inc., Microson sonicator for 3-5 min at 40% output power and allowed to stand at room temperature for 1 hr. Apolipoprotein E (80 μg) was added and the mixture was incubated for an additional 15 min at room temperature. The liposomes were added to the culture media of recombinant CHO cells and incubated at 37 °C in a CO_2 incubator for 1-4 hrs. Cells were removed from the culture dishes by trypsinization, washed once in DMEM supplemented with 10% fetal calf serum and twice with saline. The cell pellet was resuspended in chloroform-methanol and heated to 60 °C for 10 min and centrifuged at 600 xg for 10 min. The supernatant was dried under nitrogen and the pellet resuspended in 100 μl of chloroform:methanol. Samples were spotted on a silica thin layer chromatography plate and chromatographed in chloroform:methanol:water (90:10:1) for 45 min followed by chromatography in chloroform:methanol:water (75:25:4) for 30 min. Products were visualized under UV light (330 nm), excised from the plate by scraping, resuspended in chloroform:methanol, and their fluorescence quantitated on a Farrand spectrofluoremeter (343 nm excitation, 378 nm emission).

Polyclonal Antibodies.

A New Zealand white rabbit (2 kg) was injected with 150 μg of purified splenic α-Gal A in Freund's complete adjuvant prepared as follows: 150 μg of α-Gal A was added to 0.5 ml of PBS in a glass syringe. Using a stainless steel 21 guage needle, the PBS/α-Gal A solution was mixed with 0.5 ml of Freund's complete adjuvant, in a second glass syringe, until a homogenous emulsion was obtained. The emulsion was injected into 8 different subcutaneous sites (back) and 1 intramuscular site (thigh). Two months following the inital injection, the rabbit was boosted with 50 μg of α-Gal A in Freund's incomplete adjuvant as above. Serum was collected from an ear vein at days 8 and 12 following the boost. The titer was checked using a standard ELISA assay (20).

Subsequent boosts were given approximately every two months followed by a bleeding 10 days later. A typical bleed yielded 30-40 ml of blood.

SDS-PAGE and Autoradiography.

Polyacrylamide gel electrophoresis was carried out under reducing conditions (where appropriate) as described by Laemmli in a 1.5 mm thick slab containing 10% acrylamide (21). The gel was fixed in 10% acetic acid and 20% methanol for 30 min and then soaked in Amplify for 30 min with agitation. Gels were vacuum dried for 90 min (Hoffer) and exposed to Kodak X Omat AR for 4-72 h.

Isoelectric Point, and pH Optimum Determination.

The isoelectric point was dermined using QAE-Sephadex essentially as described by Yang and Langer (22). The pH optimum was determined in 25 mM sodium phoshate buffer at 37 °C.

Mannose-6-Phosphate Receptor Affinity Chromatography and QAE-Sephadex Chromatography.

The 215 kDa mannose-6-phosphate receptor (M-6-P receptor) coupled to Affigel-10 was at a concentration of 0.4 mg/ml of packed gel. Samples, in 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), were applied to a 1.5 x 0.8 cm column at a flow rate of 0.3 ml/min. Following sample application (5 ml) the column was washed with 5 ml of binding buffer and eluted with a nonlinear gradient of mannose-6-phosphate in binding buffer (0-5 mM). This experimental gradient (23) was formed by a homemade apparatus consisting of two chambers of 2.5 cm diameter and 1 cm diameter. Fractions were collected (0.5 ml) and 10 µl assayed for α-Gal A activity, using 4-MU-α-Gal, and radioactivity, using 10 ml of Sinti Verse I scintillation cocktail.

QAE-Sephadex chromatography in a 3 x 0.8 cm column was performed as described (24,25). Briefly, following digestion with Endo H, the released oligosaccharides (labeled with [^{3}H]-mannose) were isolated and desalted on an 18 x 0.8 cm column of Sephadex G-25. Samples were applied to the column of QAE-Sephadex and eluted with successive 5 ml aliquots of 2 mM Tris, pH 8.0 containing 0, 20, 40, 80, 100, 120, 140, 160, 200, 400 and 1,000 mM NaCl. Oligosaccharides eluted according to number of their negative charges; 0 charge at 0 mM NaCl, 1 at 20 mM NaCl, 2 at 70 mM NaCl, 3 at 100 mM NaCl and 4 at 140 mM NaCl.

Labeling of Cells with [^{35}S]-Methionine, [^{3}H]-Mannose and [^{32}P]-Phosphorus.

Confluent cultures in 100 mm dishes were washed once with 5 ml of methionine-free DMEM. A fresh aliquot of this medium (5 ml) was placed in each dish and cultures were incubated in a 37 °C incubator for 30 min. The media was removed from the dishes and a fresh aliquot of methionine-free DMEM (1 ml), supplemented with 10% dialyzed FCS and 50-100 µCi of [^{35}S]-methionine was added. Cells were incubated at 37 °C for 3-5 min, the radioactive media was removed and cells washed twice with DMEM plus FCS. Cells were chased for the indicated times in 5 ml of DMEM plus FCS containing 2 mM methionine. For overnight labeling, cultures received 5 ml of methionine-free DMEM supplemented with dialyzed FCS, glutamine, antibiotics, 10 mM NH_4Cl and 200 µCi [^{35}S]-methionine.

For [^{3}H]-mannose labeling, cultures were grown as above in supplemented DMEM. Cells were washed with 5 ml of low-glucose DMEM and a fresh aliquot of media was added. [^{3}H]-mannose (250 µCi; dried under nitrogen and resuspended in DMEM), was added and cells were incubated in a 37 °C incubator for 24 hr.

For ^{32}P labeling, cultures were switched to phosphate-free DMEM supplemented with 10% dalyzed FCS. Following addition of [^{32}P]-orthophosphate (1 mCi) cultures were incubated in a 37 °C, CO_2 incubator for 24 hr.

Cell Lysis and Immunoprecipitation.

Cells grown in 100 mm culture dishes were washed twice with 5 ml of phosphate buffered saline (PBS) and scraped into 12 ml conical tubes using a rubber policeman and 10 ml of PBS. Following centrifugation at 2,500 rpm for 10 min cells were resuspended in 1 ml of 25 mM $NaPO_4$, pH 6.0, and received three 15-sec bursts in a Branson cup sonicator. Cell debris was removed by centrifugation (10,000 rpm for 15 min at 4 °C). Alternatively, cells were washed as above and 1 ml of lysis buffer (50 mM sodium phosphate, pH 6.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.2 mM PMSF) was added to the dish. The culture dish was incubated at 4 °C for 30 min and cells were transfered to a 1.5 ml eppendorf microcentrifuge tube. Cell debris was removed as above.

Immunoprecipitation was carried out as described (26). Briefly, 0.5 ml of cell lysate or culture media was placed in a 1.5 ml eppendorf microfuge tube and 50 µl of preimmune rabbit serum was added. The mixture was incubated at 4 °C for 1 hr with gentle agitation. Fifty µl of Pansorbin was added and incubation was continued for 30 min. The mixture was clarified by centrifugation at 10,000 rpm for 5 min, 100 µl of anti-α-Gal A polyclonal antibody added and incubation continued for 1 hr at 4 °C with gentle rocking. Pansorbin (100 µl) was added and incubation continued for 30 min as above. The tertiary *S. aureus* cells-antibody-antigen complex was collected by centrifugation as above. The supernatant was discarded and the pellet washed successively in NET 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, in NET buffer with 0.1% SDS and in TN buffer (10 mM Tris, pH 7.5, 0.1% NP-40). The immunoprecipitated protein was denatured by heating at 100 °C for 5 min in the presence of 2% SDS, 100 mM DTT (DTT was not used for experiments involving secondary structure conformations). *S. aureus* cells were removed by centrifugation at 10,000 rpm for 5 min at room temperature.

RESULTS

Purification.

Recombinant α-Gal A produced in a cell bioreactor was purified from the crude media by affinity chromatography on α-GalNH_2-C_{12}-Sepharose (15) followed by hydrophobic chromatography on Octyl-Sepharose and gel filtration on a 100 cm Superose 6 column (see "Methods"). Table 1 shows a typical purification of a 20 mg lot of recombinant α-Gal A and the specific activities of the enzyme at each stage of purification. The recombinant enzyme was essentialy homogeneous, following the gel filtration step, and was >98% pure as judged by SDS-PAGE (Figure 1). A minor contaminant of bovine serum albumin was removed by an additional gel filtration step on a column of Blue-Sepharose (27) resulting in an enzyme preparation which was greater than 99% pure as judged by loading 20 mg of α-Gal A on SDS-PAGE (data not shown).

Table 1
FPLC Purification of recombinant α-Gal A. The table lists a typical purification run starting with 20 mg of α-Gal A.

Step	U x 10^3	U x 10^3/ mg	Fold Purification	Yield (%)
Media	39,750	5	1	100
α-GalNH_2-Sepharose	36,500	680	136	91
Octyl Sepharose	31,750	3,400	680	79
Superose 6	30,800	4,150	830	78

That the purification of recombinant α-Gal A would be facilitated by growth of the CHO cells in serum-free media was demonstrated by metabolic labelling of total cellular

Figure 1: SDS-PAGE of each step of the α-Gal A purification scheme. 1, 6, molecular weight markers; 2, crude media; 3, affinity chromatography; 4, Octyl-Sepharose chromatography; 5, Superose 6 chromatography.

and secreted protein. In contrast to the result seen in Figure 1, radiolabeled α-Gal A was essentially the only protein seen in the media of the high-expressor line, DG5.3 (Fig. 2).

Physicokinetic Properties.

Recombinant α-Gal A was found to have a subunit molecular weight of ~57 Kd based on SDS-PAGE (Fig. 1). The Km towards the artificial substrate 4-MU-α-D-galactopyranoside was 1.9 mM (Fig. 3A) and the pH optimum and isoelectric point were 4.6 and 3.7 respectively (Fig. 3B & 3C).

In order to determine whether the recombinant enzyme recognized and hydrolyzed its natural substrate, liposomes containing the fluorescently-labeled α-Gal A substrate P-C_{12}STH and apolipoprotein E (for lysosomal targetting) were incubated with CHO cells overexpressing α-Gal A (clone DG5.3). As shown in Figure 4, recombinant lysosomal α-Gal A rapidly hydrolyzed the substrate to P-C_{12}STH rendering the second enzyme in this

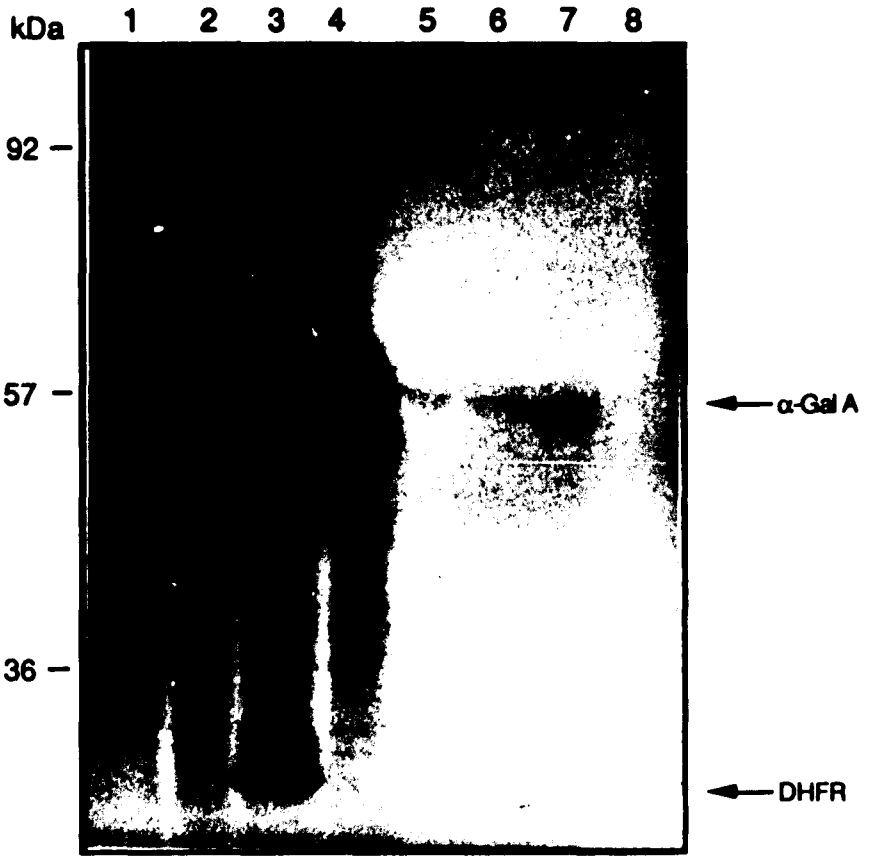

Figure 2: Total cellular (lanes 1-4) and media (lanes 5-8) from control DG44 cells (lane 1-5), DG5 cells (lanes 2,6), DG5.3 cells (lanes 3,7) and DG11 cells (lanes 4,8), labeled with [^{35}S]-methionine.

pathway, β-galactosidase, limiting since it is only found at normal levels in this system. The rapid hydrolysis of P-C_{12}STH indicates that recombinant α-Gal A can recognize this natural substrate analog and very efficiently hydrolyze it. Also, since this substrate is targeted to the lysosome, cell associated recombinant α-Gal A must be correctly targeted to this location. These results indicate that recombinant α-Gal A produced and secreted by CHO cells is essentially identical to the enzyme purified from human plasma (Table 2).

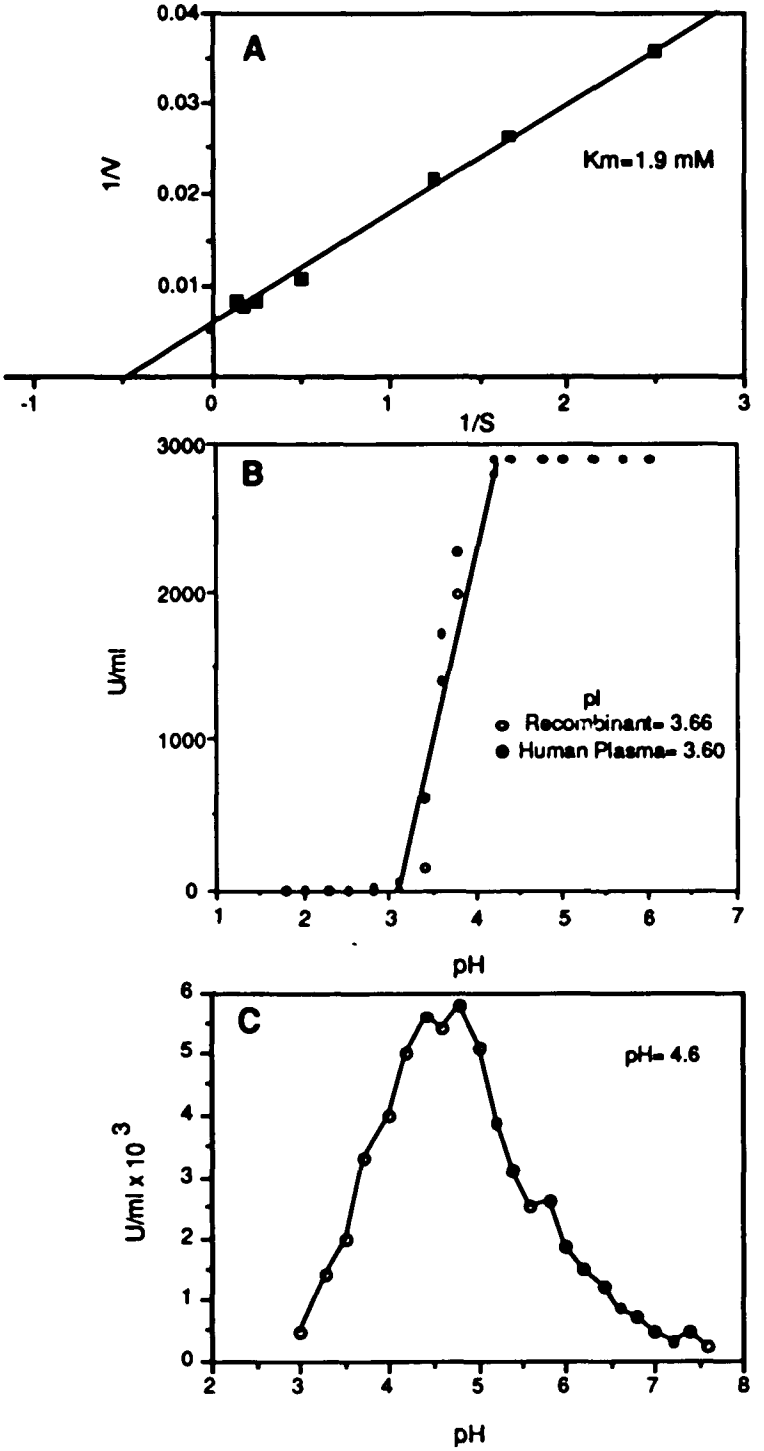

Figure 3: Physicokinetic properties of recombinant α-Gal A. Km towards the artificial substrate 4-MU-α-D-galactopyranoside (A). Isoelectric point of recombinant and human plasma purified enzyme (B). pH optimum of the recombinant enzyme.

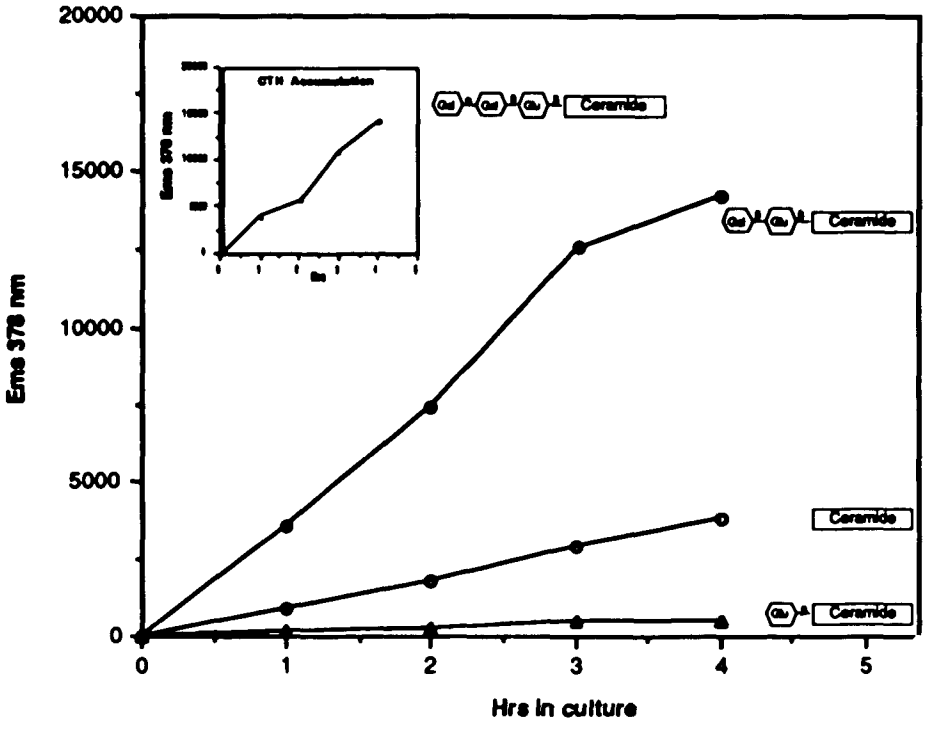

Figure 4: P-C_{12}STH degradation by CHO DG5.3 cells overproducing human α-Gal A. Rapid degradation of this substrate is observed by the accumulation of P-C_{12}SDH.

Table 2
Property comparison of recombinant α-Gal A and enzyme purified from human tissue.

Property	α-Gal A		
	Spleen	Plasma	Recombinant
MW-Subunit, (KDa)	53	57	57
pH Optimum	4.5	4.6	4.6
Isoelectric point, pI	4.3	3.7	3.7
Km (4-MU-α-D-Gal), mM	2.5	1.9	1.9
Phosphorylation (M-6-P)	+	?	+
Natural Substrate Hydrolysis (GL-3)	+	+	+

Processing and Rate of Secretion of Recombinant α-Gal A.

Nascent polypeptides, transversing the endoplasmic reticulum, assume secondary structure conformations cotranslationally or soon after their synthesis is completed (28). α-Gal A was labeled with [^{35}S]-methionine for three minutes and then chased with cold methionine for the indicated times. Immunoprecipitated α-Gal A was visualized on SDS-PAGE. The samples were prepared without DTT in order to maintain disulfide bridges that might have formed during the chase, indicative of a secondary structure conformation. A control (DTT) was prepared by boiling an aliquot of the 60 min sample in the presence of DTT to destroy disulfide bonds and the secondary structure. At 0 min of chase (after 3 min of labeling) there was already a change in the mobility of this enzyme indicating that conformational changes occur cotranslationally or soon after completion of synthesis (Fig. 5).

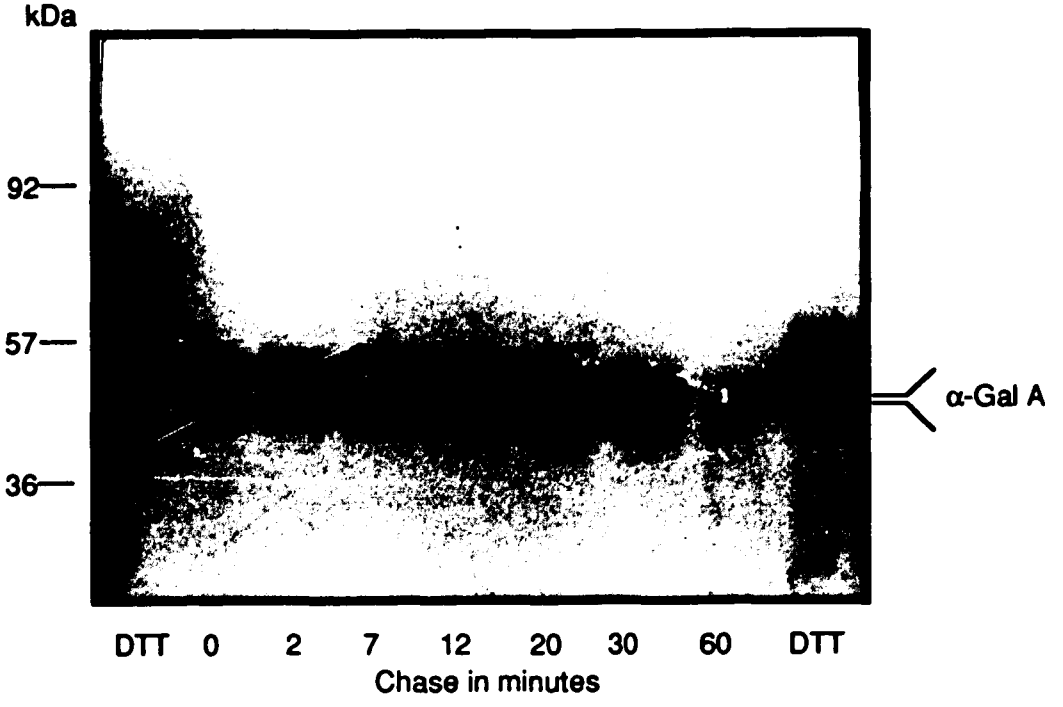

Figure 5: Aquisition of disulfite bridges by recombinant α-Gal A. CHO DG5.3 cells were labeled with [^{35}S]-methionine and chased for the indicated times. SDS-PAGE in the absence of a reducing agent reveals the formation of secondary structure via disulfite bonds.

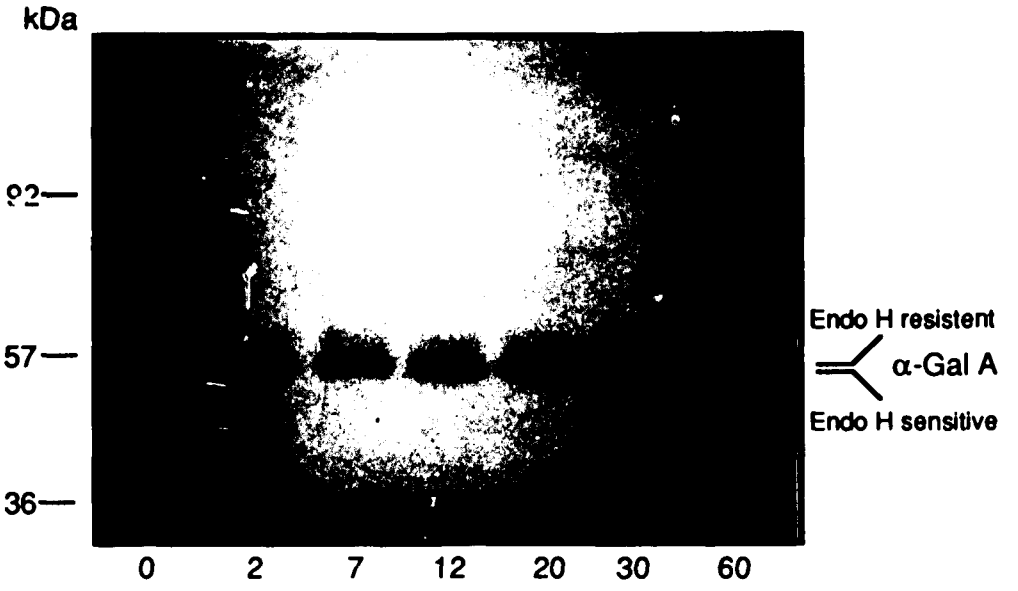

Figure 6: Arrival of newly synthesized α-Gal A to the Golgi network detected by the aquisition of Endo H resistent oligosaccharides.

Arrival of the new polypeptide to the Golgi network was detected by the acquisition of Endo H resistant oligosaccharides (28). Radiolabeled α-Gal A (3 min pulse) was chased with nonradioactive methionine and immunoprecipitated as above. The immunoprecipitates were then treated with Endo H and visualized on SDS-PAGE. Between 2 and 7 min of chase the first Endo H resistant form of α-Gal A could be detected, indicative of arrival of the recombinant enzyme at the Golgi, about 5-10 min following its synthesis (Fig. 6). The majority of the Endo H sensitive form was rendered resistant by 60 min of chase.

This enzyme transverses the Golgi network and is secreted at 45-60 min of chase (Fig. 7). Analysis of total media, from [^{35}S]-methionine labeled cells, revealed that >95% of the secreted protein by the recombinant CHO cells was α-Gal A (Fig. 8).

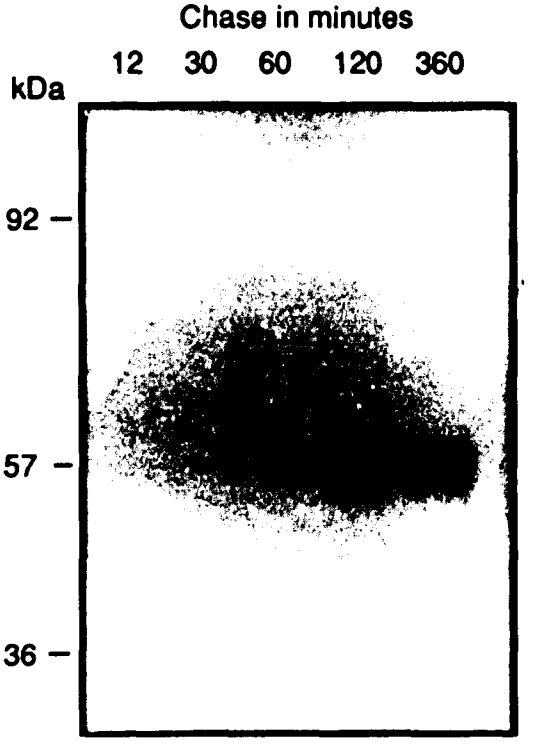

Figure 7: Secretion rate of recombinant α-Gal A. CHO DG5.3 cells were labeled with [^{35}S]-methionine for 5 min and chased with cold methionine. Culture media aliquots were removed at the indicated times and immunoprecipitated with anti-α-Gal A polyclonal antibody.

Analysis of Carbohydrate Moieties on Recombinant α-Gal A.

There are four *N*-glycosylation consensus sequences (Asn-X-Ser/Thr) in the α-Gal A subunit predicted by the cDNA sequence. The fourth site is probably not utilized since it contains a proline residue in the X position. Recombinant α-Gal A was digested with Endo H, Endo F, Endo D and PNGase F. Digestion with PNGase F caused an ~ 7 kDa shift in mobility on SDS-PAGE of half of the α-Gal A (Fig. 9). This change in molecular weight can be attributed to the removal of 3 *N*-linked carbohydrate moieties. Digestion of the recombinant enzyme with a cocktail of Endo H, Endo F and PNGase F did not result in

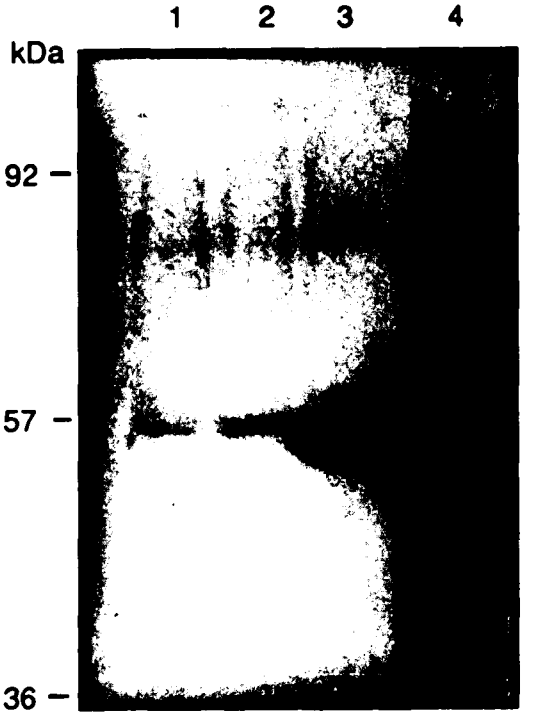

Figure 8: SDS-PAGE of culture media from DG44 (lane 1; control), DG5 (lane 2) and DG5.3 (lanes 3,4) cells labeled with [^{35}S]-methionine for 1 hr (lanes 1-3) and 24 hr (lane 4).

any further decrease in molecular weight, indicating that all of the enzyme contains 3 *N*-linked carbohydrate moieties.

Endo D, a glycosidase with a strict specificity for the lower Manα 1-3 branch of the high-mannose core pentasaccharide (17), did not have an effect on the mobility of α-Gal A, indicating that the recombinant enzyme does not contain this type of oligosaccharide (Fig. 9). Endo H and Endo F together resulted in a 4 kDa shift indicating that 2 out of the 3 oligosaccharides on this enzyme are of the high-mannose type (25).

Interestingly, intracellular α-Gal A was completely sensitive to PNGase F while half of the secreted enzyme was partially resistant to PNGase F (Fig.10). Since this

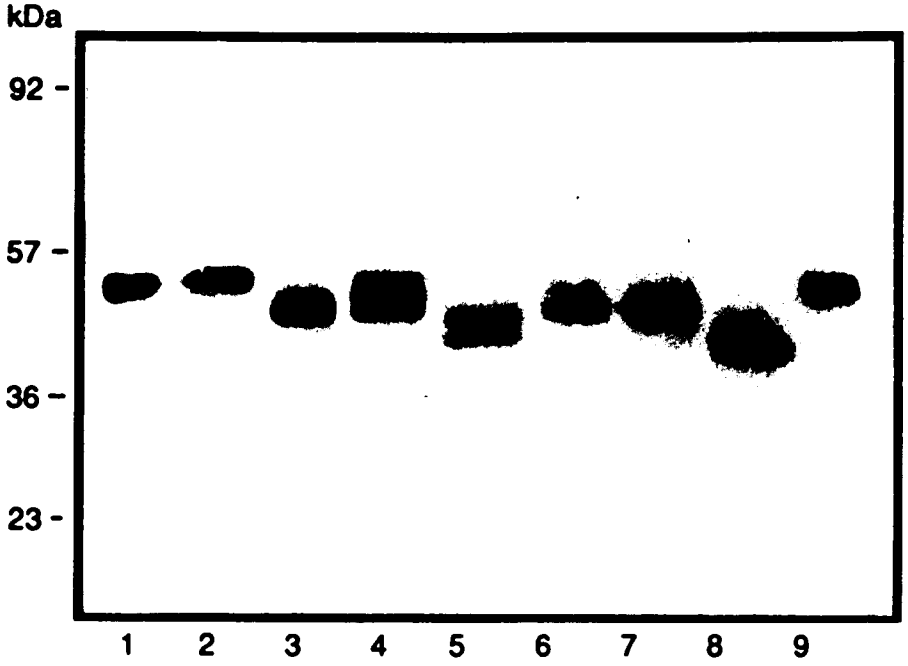

Figure 9: Analysis of the carbohydrate moieties on recombinant α-Gal A. CHO DG5.3 cells were labeled with [^{35}S]-methionine for 24 hr, the culture media collected and the recombinant enzyme immunoprecipitated. Aliquots were digested with Endo D (lane 2), Endo H (lane 3), Endo F (lane 4), PNGase F (lane 5), Endo D and H (lane 6), Endo H and F (lane 7), and Endo H, F and PNGase F (lane 8). Untreated samples (lanes 1,9).

resistance was eliminated by co-treatment with Endo H and Endo F (Fig. 9), further studies are necessary with Endo H and Endo F separately to determine the molecular parameters engendering either selective inhibition of PNGase F or resistance to denaturation of secreted recombinant α-Gal A.

Having determined that the recombinant enzyme contains 3 oligosaccharides, two of which are of the high-mannose type, the effect of inhibition of glycosylation was investigated (29). Processing and secretion of recombinant α-Gal A is not affected by selected inhibitors of oligosaccharide processing. In the presence of deoxynojirimycin (an inhibitor of glucosidase I and II), deoxymannojirimycin (an inhibitor of mannosidase I),

and swainsonine (an inhibitor of mannosidase II) α-Gal A secretion rate remains the same as the controls (Fig. 11). However, tunicamycin (an inhibitor of oligosaccharide addition)

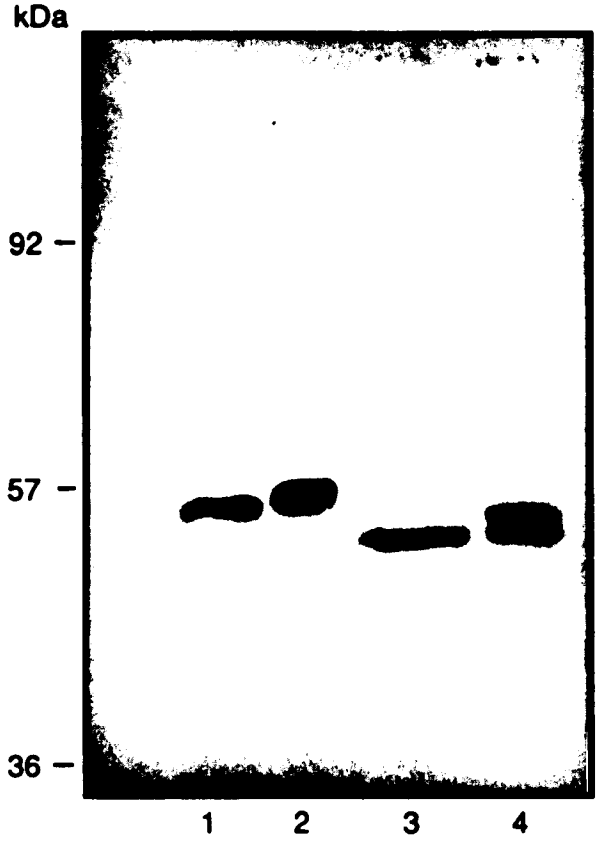

Figure 10: Cellular (lanes 1,3) and secreted (lanes 2,4) forms of recombinant α-Gal A treated with PNGase F (lanes 3,4). Controls (lanes 1,2).

inhibits secretion of α-Gal A by as much as 80% (Fig. 11). The secreted enzyme from tunicamycin-treated cultures could bind to a Con A Sepharose column (not shown) indicating that this enzyme is partially glycosylated, probably due to incomplete inhibition of glycosylation by tunicamycin. These results demonstrate that oligosaccharide addition but not processing is necessary for proper maturation and secretion of the recombinant enzyme.

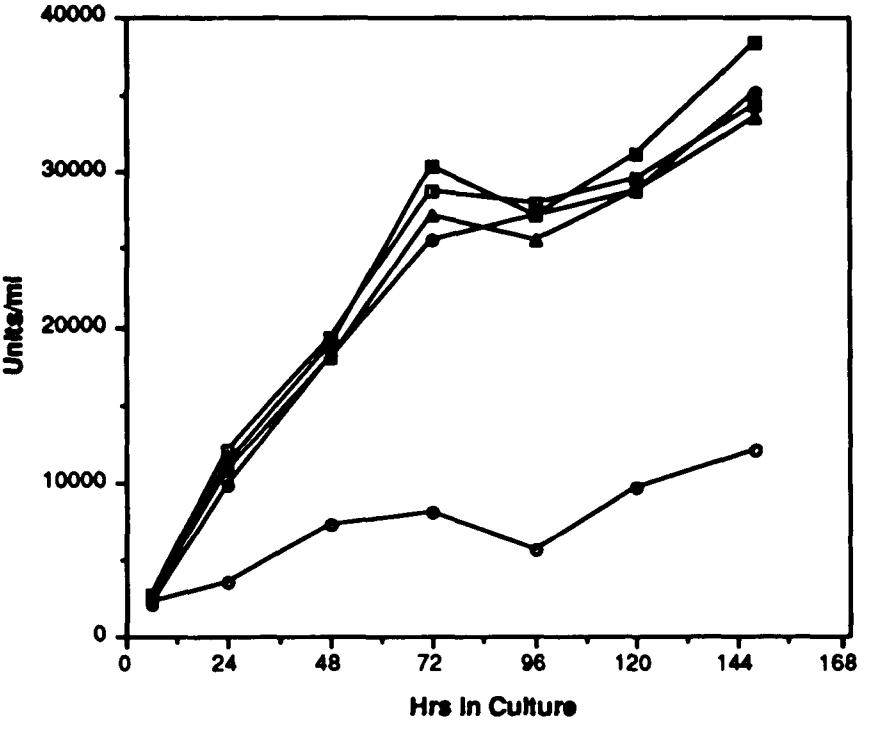

Figure 11: Effect of glycosylation inhibitors on the secretion of recombinant α-Gal A. ■——■ Control, o——o Tunicamycin, ●——● Deoxynojirimycin, ▲——▲ Swainsonine, ■——■ Deoxymannojirimycin.

Phosporylation.

Since the recombinant α-Gal A contained high-mannose moieties, the recombinant enzyme could contain M-6-P and be competent for receptor mediated uptake. Cells from clone DG5.3 were metabolically labeled with [^{32}P] orthophosphate for 12 hr and then the cell extracts and media immunoprecipitated and visualized on SDS-PAGE. As shown in Figure 12, both cell-associated and secreted α-Gal A were phosphorylated, presumably at their carbohydrate moieties as suggested by the *in vitro* experiments described above.

Figure 12, both cell-associated and secreted α-Gal A were phosphorylated, presumably at their carbohydrate moieties as suggested by the *in vitro* experiments described above.

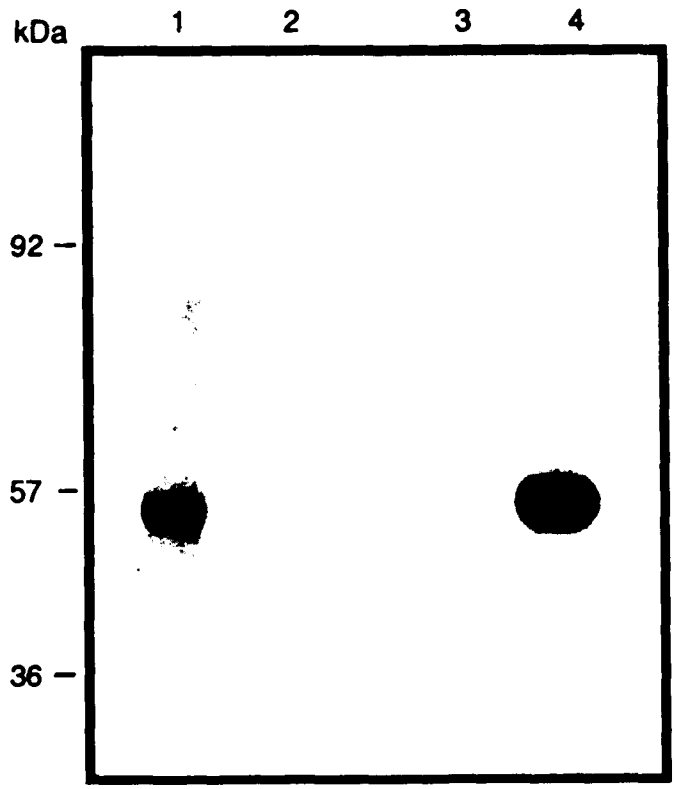

Figure 12: ^{32}P labeling of CHO DG44 (lanes 2,3) and DG5.3 (lanes 1,4). α-Gal A was immunoprecipitated from cells (lanes 1,2) and media (lanes 3,4).

Analysis of Endo H Sensitive Oligosaccharides.

The high-mannose oligosaccharides were removed by treating immunoprecipitated [^3H]-mannose labeled α-Gal A with Endo H. These oligosaccharides were analyzed by chromatography on QAE-Sephadex (24,25). Two major forms of these oligosaccharides were detected, a form with 2 negative charges and one with 4 negative charges (Fig. 13A). The negative charge can be contributed by a phosphodiester bond (-1), a phosphomonoester bond (-2) or sialic acid (-1). Treatment of these sugars with dilute HCl did not shift the profile of any of the peaks indicating that there are no phosphodiester

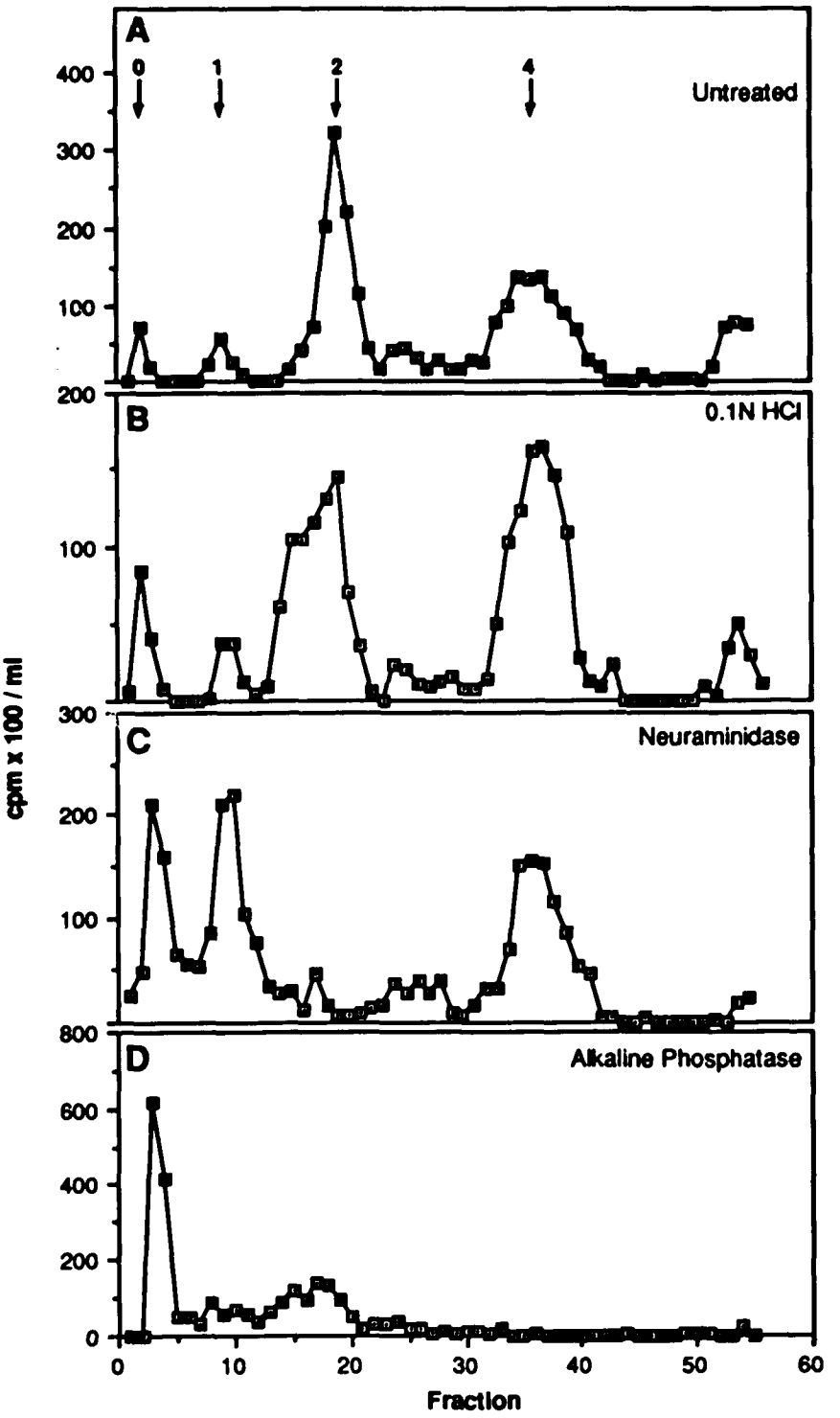

Figure 13: QAE-Sephadex chromatography of Endo H sensitive oligosaccharides of recombinant α-Gal A. Untreated (A), dilute HCl (B), neuraminidase treated (C) and alkaline phosphatase treated (D) oligosaccharides.

groups on these sugars (Fig. 13B) (24). Treatment with neuraminidase causes a shift of the -2 peak resulting in two new peaks at 0 and -1 negative charges (Fig. 13C). Therefore, the charge of the -2 peak is contributed by sialic acid, most likely two moieties. The resulting -1 peak following neuraminidase treatment is probably a partial digestion of the -2 peak by the enzyme. Treatment of these oligosaccharides with alkaline phosphatase caused a shift of the -4 peak to 0 negative charge (Fig. 13D). There was no effect on the -2 peak, indicating that the charge of the -4 peak is contributed by two phosphomonoester bonds while the -2 peak does not contain any such bonds. Thus, it is evident from these results that Endo H releases two types of high-mannose oligosaccharides from recombinant α-Gal A, one containing sialic acid (possibly a hybrid oligosaccharide) and the other containing 2 phosphomonoester bonds (presumably as mannose-6-phosphate).

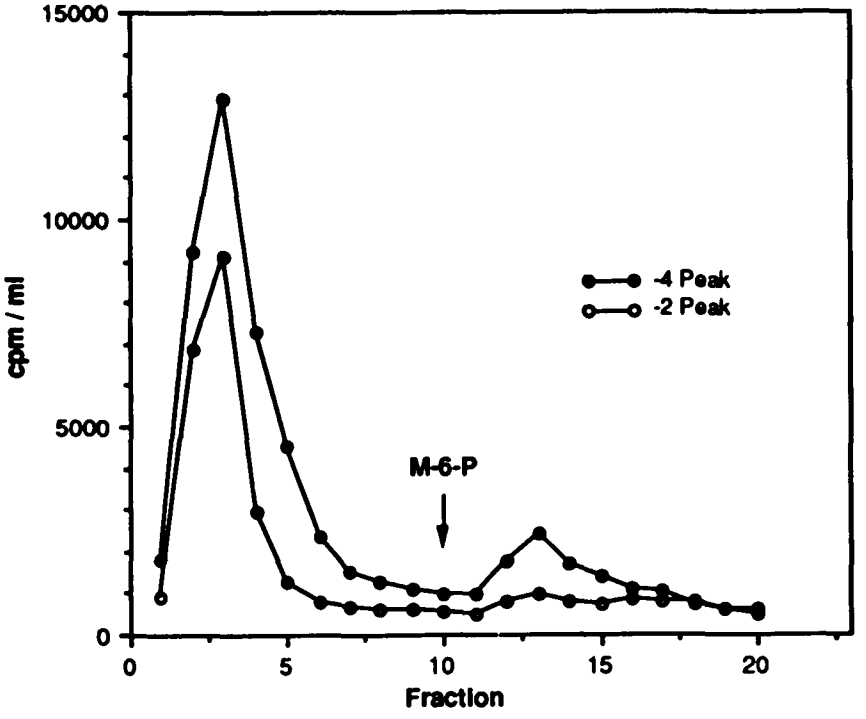

Figure 14: Endo H sensitive oligosaccharides of recombinant α-Gal A chromatographed on M-6-P receptor. Solid circles, peak minus 4, open circles, peak minus 2.

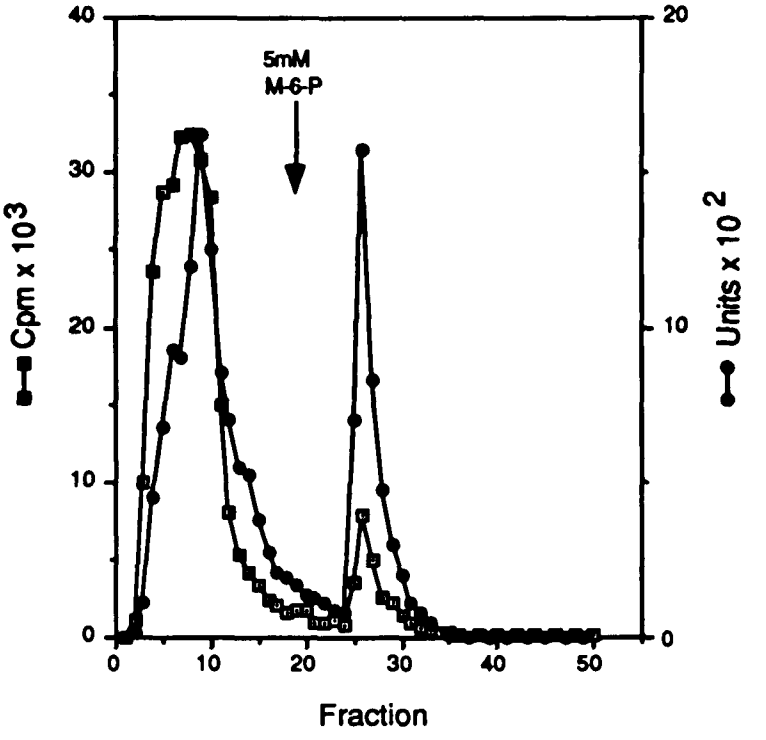

Figure 15. Recombinant α-Gal A chromatography on M-6-P receptor. DG5.3 cells were labeled with [^{35}S]-methionine for 24 hr and the media collected for chromatography. Solid circles, α-Gal A activity; open boxes, total radioactivity.

To further confirm these findings peaks -2 and -4 were chromatographed on an immobilized mannose-6-phosphate receptor column (Fig. 14). Although peak -4 interacted weakly with the receptor, it could be bound to the column and required the addition of mannose-6-phosphate for elution. A very weak interaction was observed between the receptor column and the -2 peak, suggesting that a portion of these hybrid oligosaccharides may contain M-6-P.

The weak interaction of the high-mannose oligosaccharides with the M-6-P receptor could be explained by the absence of the protein core (24). DG5.3 cells were labeled with [^{35}S]-methionine and the secretions chromatographed on a column of immobilized

mannose-6-phosphate receptor. Notably, the recombinant enzyme bound strongly to the column and was eluted specifically by the addition of 5 mM M-6-P (Fig. 15).

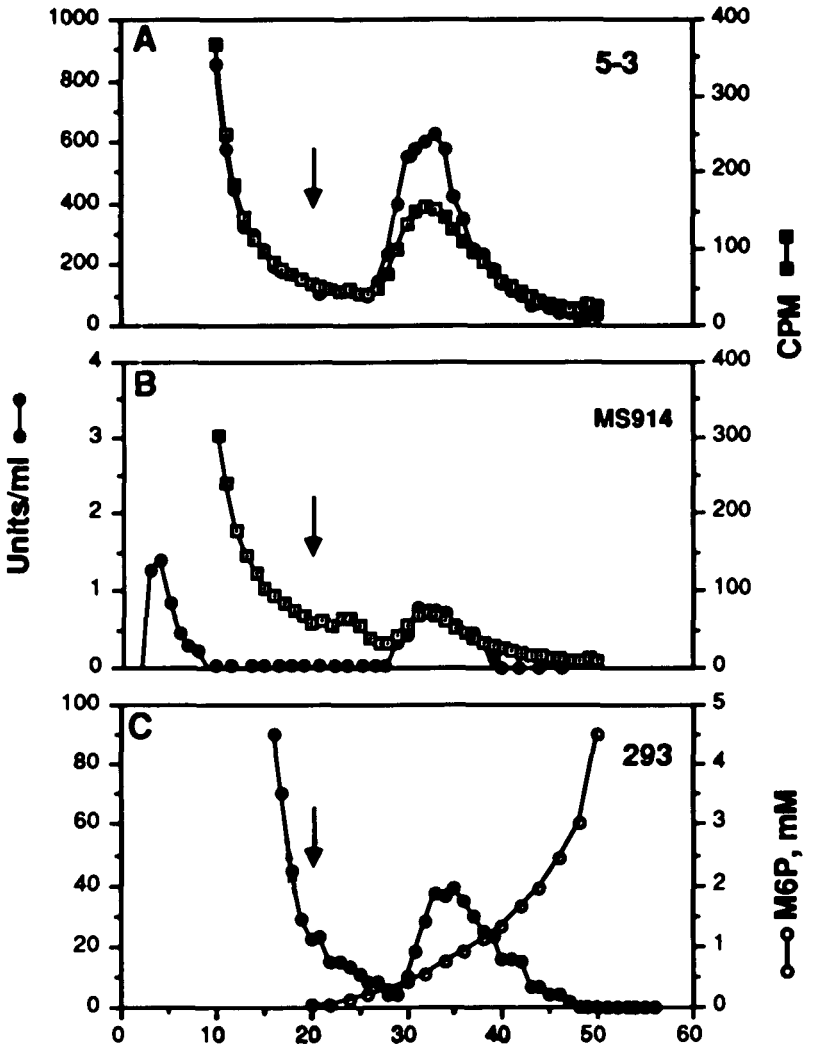

Figure 16. Recombinant and human α-Gal A affinity chromatography on M-6-P receptor. Cells were labeled with [^{35}S]-methionine for 24 hr in the presence of NH_4Cl and the culture media collected. DG5.3 secretions (A), MS914 secretions (B) and 293 secretions (C). Solid circles, α-Gal A activity. Squares, total radioactivity. Open circles, M-6-P gradient used for elution. Arrows indicate the start of the the M-6-P gradient.

Interaction of α-Gal A with the Mannose-6-Phosphate Receptor.

Since recombinant α-Gal A has been shown to contain mannose-6-phosphate moieties, it was important to establish whether this was also true for normal human α-Gal A. CHO proteins were labeled with [^{35}S]-methionine in the presence of NH_4Cl, to cause quantitative secretion of newly synthesized lysosomal enzymes (30). The media was collected and chromatographed on a column of immobilized mannose-6-phosphate receptor. The column was eluted with a gradient of mannose-6-phosphate as described under "Methods". This elution protocol can separate lysosomal enzymes into low and high affinity receptor-binding ligands (23).

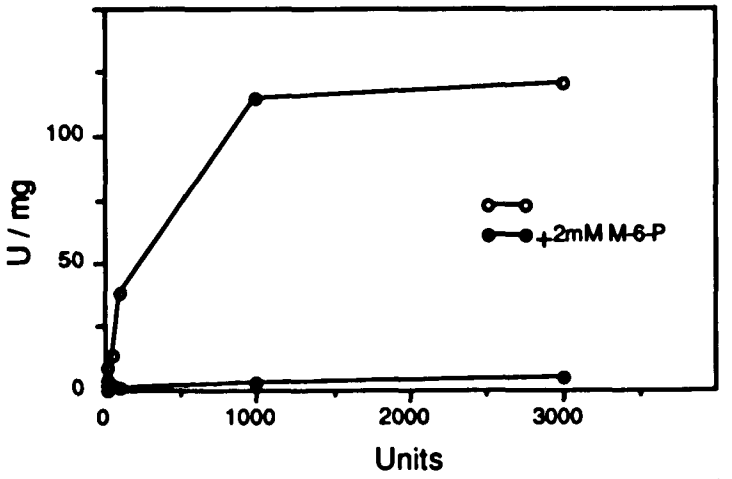

Figure 17. Uptake of recombinant α-Gal A by Fabry fibroblasts. Cells were incubated with the indicated amounts of α-Gal A for 6 hr. Open circles, α-Gal A uptake, closed circles, uptake in the presence of 2 mM M-6-P.

The recombinant enzyme co-eluted with the bulk of the lysosomal enzymes at an M-6-P concentration indicative of high affinity forms (Fig.16A). The same experiment was performed with secretions of MS914 (normal diploid human fibroblasts) cells (Fig. 16B) and 293 cells (human adenovirus transformed embryonic kidney cells) (Fig. 16C).

When the same M-6-P gradient was applied, human α-Gal A also co-eluted with the bulk of the lysosomal enzymes, demonstrating that the recombinant enzyme exhibits affinity to the M-6-P receptor similar to that of the normal human enzyme.

Receptor Mediated Uptake of Recombinant α-Gal A in Fabry Fibroblasts.

Fabry fibroblasts were incubated with varying amounts of the recombinant enzyme for 6 hrs (Fig. 17). The enzyme uptake was saturatable and was specifically inhibited by the addition of 2 mM M-6-P in the uptake media, implying that the uptake was via the cell surface M-6-P receptor.

DISCUSSION

Human recombinant α-Gal A was purified to homogeneity from the media of the CHO cell line, DG5.3, which was shown to secrete most of the recombinant enzyme synthesized. The culture media from this clone was highly enriched for α-Gal A when serum-free medium was used, constituted greater than 95% of the total extracellular protein. Thus, purification to homogeneity could be accomplished in only three chromatographic steps. Over half a gram of enzyme was produced in three months and from a portion of this, 280 mg was purified with a yield of 80% using only laboratory-scale equipment. Notably, the recombinant enzyme had full enzymatic activity with a specific activity equal to that of the previously purified human enzyme (8). The recombinant enzyme was able to recognize and effectively cleave an analog of the natural substrate, globotriaosylceramide.

The purified enzyme had molecular weight, pH optimum, km and isoelectric point values which were essentially identical to those of the enzyme purified from human plasma (8). Analysis of the carbohydrate moieties on this enzyme revealed the presence of three oligosaccharide chains on the α-Gal A polypeptide. These chains were a mixture of complex, hybrid and high-mannose types as evidenced by endoglycosidase and QAE-Sephadex studies. Most importantly, the recombinant enzyme was also similar to the native plasma form of α-Gal A in having terminal sialic acid moieties (3). Since the plasma form of the enzyme was shown to be much more effective in degrading circulating $GbOse_3Cer$ than the splenic form, the recombinant enzyme may be the most appropriate form for enzyme replacement therapy of Fabry disease. The saturable uptake of recombinant α-Gal A by Fabry and normal fibroblasts was demonstrated and specifically inhibited by 2 mM mannose-6-phosphate. Enzyme which was secreted from cells incubated with NH_4Cl behaved identically in these uptake studies, further indicating that the recombinant α-Gal A is most similar to the plasma form of the enzyme. Thus, the

prerequisites for therapy have been achieved and a promising drug is now available for human clinical trials.

In addition, the CHO cell system has great promise for studies of the cell biology of lysosomal biogenesis and glycohydrolase processing. Light microscopy revealed highly vaculated cytoplasm in the DG5.3 CHO cells suggesting a proliferation of lysosomal membranes and offering the potential for analysis of lysosomal biogenesis. Preliminary studies have indicated that the recombinant enzyme is synthesized very rapidly, exits the endoplasmic reticulum in 5-10 min following its synthesis and is secreted 45-60 min later. These fast kinetics of recombinant α-Gal A biosynthesis allow for intersting studies involving lysosomal enzyme biosynthesis and offer a methodology that, to date, is only rivaled by viral systems. In fact, recombinant α-Gal A is synthesized so rapidly that a single radioactive pulse of 3 min is sufficient to label enough enzyme for these studies. The unexpectedly specific secretion of only the overproduced recombinant α-Gal A and not other lysosomal enzymes appears analogous to "gene dosage-dependant secretion" described by Rothman, et al. (31,32) and poses interesting questions relating to protein transport and targeting.

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SUMMARY

The study of the structure and function of proteins has facilitated the elucidation of the molecular mechanisms involved in catalysis as well as provide insights into the nature of protein folding, stability and subcellular localization. These studies have been markedly enhanced by the ability to genetically engineer and modify the protein's structure and by the development of systems for the efficient high-level production of the desired protein. These expression systems offer the ability to isolate sufficient quantities of otherwise difficult to obtain proteins, which in turn simplifies their purification, characterization and crystallization. In cases where the lack of a particular enzyme protein results in disease, therapeutic endeavors can be evaluated by administering the recombinant enzyme. Also, a mammalian expression system which rapidly synthesizes a recombinant protein can be utilized in the study of protein trafficking and targeting as well as in the dissection of the molecular mechanisms involved in the post-translational modifications of proteins.

In chapter one, the prokaryotic expression of human α-Gal A was evaluated, using various vectors in *E. coli* and *S. aureus*. Although this human lysosomal enzyme could be expressed in a catalytically active form, its expression was transient and the protein product was unstable. This instability was shown to be due to proteolytic degradation, presumably due to the lack of glycosylation, the nonreducing environment of these prokaryote and pH differences between the bacterial compartments and the natural milieu of the human enzyme (lysosome, pH <5).

In chapter two, elements evaluated in the prokaryotic expression systems were utilized to express a catalytically active enzyme which could be purified quickly and efficiently using affinity chromatography. A nucleotide sequence encoding one of the IgG binding domains of the staphylococcal protein A, domain E, was fused to the 3' end of the

sequence encoding α-Gal A. This fusion construct was expressed very efficiently in mammalian cells and retained both activities, α-Gal A and IgG binding activities. This allowed for the quick purification of the fusion product using IgG-Sepharose affinity chromatography. The protein A affinity tail could be removed by incubation of the purified product with collagenase which recognized the consensus sequence Pro-Ala-Gly-Pro engineered between the α-Gal A and protein A moieties. Following digestion with collagenase the affinity tail could be removed from the enzyme preparation by a second IgG-Sepharose step.

In chapter three, the α-Gal A cDNA was used to construct a eukaryotic expression vector. Following confirmation of the functional integrity of the engineered construct in COS-1 cells, it was introduced into *dhfr*⁻ CHO cells and amplified using *dhfr* as the selectable marker in the presence of increasing concentrations of methotrexate. Amplified clones were isolated and expressed active α-Gal A at 100 to 200 fold over background. A clone expressing high levels of active α-Gal A was isolated and was found to secrete large amounts of the expressed enzyme into the culture media. This secretion was not due to saturation of the lysosomal trafficking pathway and was specific to α-Gal A. In fact, no other lysosomal enzymes were elevated in the growth media. This clone, DG5.3, was grown in a hollow fiber bioreactor and the culture media containing ~400 mg of α-Gal A was collected for 90 days (~100 L).

Finally, in chapter four, the recombinant enzyme produced in the bioreactor was purified to homogeneity using a procedure which included sequential affinity, hydrophobic and gel filtration chromatography. This purification scheme resulted in a protein preparation that was essentially homogeneous as judged by SDS-PAGE with a final yield of ~80%. The physical and kinetic properties of the recombinant secreted enzyme were found to be essentially identical to those of the enzyme previously purified from human

plasma. Furthermore, labeling studies using clone DG5.3 revealed that the recombinant enzyme received three oligosaccharide moieties, one being Endo H resistant and two being Endo H sensitive. Further analysis of the Endo H sensitive oligosaccharides showed two types of carbohydrate structures, one containing two phosphomonoester bonds and one containing sialic acid. The oligosaccharide containing two phosphomonoester groups interacted weakly with the mannose-6-phosphate receptor. However, the native recombinant enzyme interacted strongly with the mannose-6-phosphate receptor and required the addition of 5 mM mannose-6-phosphate for elution indicating the optimal interaction of the phosphorylated oligosaccharides with the receptor requires the presence of the protein core. Also, the recombinant enzyme was efficiently taken up by Fabry fibroblasts and this uptake was completely inhibited by the addition of 2 mM mannose-6-phosphate into the uptake media, indicating that the uptake was facilitated via the mannose-6-phosphate receptor. Metabolic labeling studies revealed that the folding of nascent α-Gal A is cotranslational, the enzyme reaches the Golgi complex in about five minutes and is secreted in 45-60 min. Both the intracellular and secreted forms of the enzyme were phosphorylated.

These studies demonstrate the feasibility of overproducing a human lysosomal enzyme in CHO cells. These cells can perform all post-translational modifications necessary for proper enzyme function and catalysis. Further, this recombinant system offers the ability to effectively study protein transport and secretion as well as factors governing the handling of overproduced proteins in mammalian cells. The observation that the overexpressed protein is specifically secreted while other lysosomal enzymes are retained opens up new questions relating to the trafficking pathways of secreted and lysosomal proteins. Is there a specific pathway for handling overproduced proteins? Does the overproduction of a particular enzyme induce the increased expression of other proteins involved in enzyme folding, processing and secretion or does it inhibit the expression of

other cellular proteins? Is the secretion of this enzyme constitutive or are there signals necessary for its secretion?