Molecular consequences of the pathogenic mutation in feline G_{M1} gangliosidosis

Douglas R. Martin^{a,*}, Brigitte A. Rigat^b, Polly Foureman^a, G.S. Varadarajan^a, Misako Hwang^a, Barbara K. Krum^a, Bruce F. Smith^{a,c}, John W. Callahan^b, Don J. Mahuran^b, and Henry J. Baker^{a,c}

^a Scott-Ritchey Research Center, College of Veterinary Medicine, Auburn University, Auburn, AL 36849, USA

^b Hospital for Sick Children, Division of Neurosciences, Toronto, Ont., Canada M5G 1X8

^c Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849, USA

Abstract

G_{M1} gangliosidosis is an inherited, fatal neurodegenerative disease caused by deficiency of lysosomal β -D-galactosidase (EC 3.2.1.23) and consequent storage of undegraded G_{M1} ganglioside. To characterize the genetic mutation responsible for feline G_{M1} gangliosidosis, the normal sequence of feline β -galactosidase cDNA first was defined. The feline β -galactosidase open reading frame is 2010 base pairs, producing a protein of 669 amino acids. The putative signal sequence consists of amino acids 1–24 of the β -galactosidase precursor protein, which contains seven potential N-linked glycosylation sites, as in the human protein. Overall sequence homology between feline and human β -galactosidase is 74% for the open reading frame and 82% for the amino acid sequence. After normal β -galactosidase was sequenced, the mutation responsible for feline G_{M1} gangliosidosis was defined as a G to C substitution at position 1448 of the open reading frame, resulting in an amino acid substitution at arginine 483, known to cause G_{M1} gangliosidosis in humans. Feline β -galactosidase messenger RNA levels were normal in cerebral cortex, as determined by quantitative RT-PCR assays. Although enzymatic activity is severely reduced by the mutation, a full-length feline β -galactosidase cDNA restored activity in transfected G_{M1} fibroblasts to 18-times normal. β -Galactosidase protein levels in G_{M1} tissues were normal on Western blots, but immunofluorescence analysis demonstrated that the majority of mutant β -galactosidase protein did not reach the lysosome. Additionally, G_{M1} cat fibroblasts demonstrated increased expression of glucose-related protein 78/BiP and protein disulfide isomerase, suggesting that the unfolded protein response plays a role in pathogenesis of feline G_{M1} gangliosidosis.

Keywords

G_{M1} gangliosidosis; Lysosomal storage disease; Animal model; Galactosidase; Feline

*Corresponding author. Fax: +1 334 844 5850. martidr@auburn.edu (D.R. Martin).

The lysosomal enzyme β -D-galactosidase (β gal, EC 3.2.1.23) cleaves terminal galactose residues from a variety of molecules, including gangliosides G_{A1} and G_{M1} . Deficiency of β gal is known to cause two lysosomal storage diseases: G_{M1} gangliosidosis (neuronopathic) and Morquio B Disease (mucopolysaccharidosis IVB, non-neuronopathic). G_{M1} gangliosidosis demonstrates varying degrees of clinical severity but is invariably fatal, and children with the most common and severe form of G_{M1} gangliosidosis usually die within 3 years of birth [1]. Although various experimental strategies have been contemplated or are under development for G_{M1} gangliosidosis, the blood–brain barrier seriously limits otherwise promising treatments such as enzyme replacement therapy by excluding circulating lysosomal enzymes from the brain.

GLB1, the gene responsible for production of β gal, contains 16 exons and maps to human chromosome 3 and cat chromosome B3 [2]. At least 59 mutations have been reported in *GLB1* (Human Gene Mutation Database, <http://www.hgmd.cf.ac.uk/ac/index.php>). Mutation of nucleotide 1445 (G > A) results in substitution of arginine with histidine at amino acid 482 (Arg482His). The first reported mutation in Caucasians, Arg482His is carried by 8.3% of the population in Pelendri, Cyprus [3] and also has been reported in people of Japanese [4–6] or Maltese [7] descent. Previous studies determined that the G1445A mutation results in normal size and amount of *GLB1* mRNA, although β gal protein may be slightly reduced on Western blots. When expressed singly in G_{M1} gangliosidosis fibroblasts or COS-1 cells, the Arg482His substitution produced a β gal protein with little to no residual activity using the 4-methylumbelliferyl- β -D-galactopyranoside synthetic substrate [3,4]. Although the *in vivo* biochemical effect of the Arg482His mutation often is difficult to discern because it occurs most frequently in compound heterozygotes, patients homozygous for the G1445A substitution present with the infantile (most severe) form of G_{M1} gangliosidosis [3,7,8]. A similar mutation, Arg482Cys, also produced no residual β gal activity after expression in G_{M1} gangliosidosis fibroblasts [4].

Feline G_{M1} gangliosidosis, first described in a Siamese cat in 1971 [9], models the juvenile form of the human disease. Onset of clinical neurological disease in affected cats occurs at approximately 3.5 months of age with a fine head or limb tremor. G_{M1} mutant cats have progressive dysmetria and ambulatory difficulties, with blindness and epileptiform seizures in the terminal disease stage at 9–10 months of age. In the current study, we identify an amino acid substitution analogous to Arg482His/Cys as the pathogenic mutation in feline G_{M1} gangliosidosis, which remains an important animal model for evaluation of translational therapeutic strategies. In addition to characterization of the mutation's effect on β gal transcript and protein, we present evidence to support a potential mechanistic basis for the enzymatic deficiency in cats and humans expressing this mutation.

Materials and methods

Nucleotide sequences

The feline β gal cDNA sequences generated by this study have been deposited in the GenBank database under Accession Nos. AF006749 (normal) and AF029974 (mutant).

Feline G_{M1} gangliosidosis breeding colony

The clinical, biochemical and pathological characteristics of feline G_{M1} gangliosidosis have been characterized extensively [9–24]. For laboratory studies, the humane endpoint is defined by weight loss >15% of maximum and the inability to enter a litter box of 3 in. in height for 3 consecutive days. Affected cats in the G_{M1} gangliosidosis research colony are humanely euthanized at 7.7 ± 0.8 (standard deviation) months. β gal enzyme activity is substantially reduced in cultured fibroblasts and brain homogenate [9]. Obligate heterozygotes (parents of affected progeny) show no clinical signs of disease, but have reduced β gal enzyme activity ($\approx 50\%$ normal). All animal procedures are approved by the Auburn University Institutional Animal Care and Use Committee. Auburn University is accredited by the American Association for Assessment and Accreditation of Laboratory Animal Care.

Sequencing strategy

Consensus PCR primers were designed from homologous regions of the human and mouse β gal (*GLBI*) genes [25,26]. β gal was sequenced first from normal cats to establish the normal feline sequence, then from mutant cats to identify the mutation. Sequence determination was performed by amplifying four overlapping segments of the feline *GLBI* cDNA with Taq DNA polymerase (Applied Biosystems) using primers listed in Table 1. The 5' and 3' ends of the cDNA were sequenced using 5' RACE and 3' RACE systems (Invitrogen) with gene-specific primers 292n and 1252c, respectively. Once the mutation was defined from cDNA sequencing, it was confirmed by amplification and sequencing of genomic DNA with primers Ex14c and Int14n (Table 1), which amplify the mutation site in exon 14 of feline *GLBI* (exon designation based on comparison to human and mouse *GLBI* exon–intron boundaries) [27,28]. Because the mutation lies at the 3' end of exon 14, primer Int14n anneals in intron 14 and was designed after partial sequencing of intron 14. Subsequently, diagnostic assays to identify the genotype of all colony cats were performed on genomic DNA with primers Ex14c and Int14n.

For cDNA production, tissue or primary skin fibroblasts were frozen in liquid nitrogen immediately after harvest. RNA was harvested from frozen tissue (1–5 mg) or cells (2×10^7) in Trizol Reagent (Invitrogen/Life Technologies) as recommended by the manufacturer. Reverse transcription was performed with an oligo(dT) primer and Superscript II reverse transcriptase (Life Technologies) at 50 °C for 1 h.

Genomic DNA was isolated from snap-frozen tissue with the Stratagene DNA extraction kit or from whole blood with the InstaGene Whole Blood Kit (Bio-Rad) as recommended by the manufacturers.

Cloning of feline β gal

A full-length feline β gal cDNA was constructed with differing 5' untranslated regions by RT-PCR of cerebral cortex mRNA isolated from a genotypically normal cat. A common non-coding strand primer (fBgal stop) was paired with one of two coding strand primers containing the feline β gal initiation codon only (fBgal ATG, construct 1.11) or the initiation codon plus 12 base pairs of the native feline 5' untranslated region (fBgal 5' utr, construct

3.9) (Table 1). PCR reactions included 0.4 μ M of each primer, 2–10 μ l of cDNA, and 2.5 U of Pfu Turbo DNA Polymerase (Stratagene). Thirty cycles were performed of 95 °C for 30 s, 62 °C (fBgal ATG) or 58 °C (fBgal 5'utr) for 30 s, and 72 °C for 2 min. After gel extraction, A overhangs were added to the approximately 2.0 kb amplicons by a 30-min incubation with Taq DNA Polymerase at 72 °C. Amplicons were ligated by TA cloning into plasmid PCR3.1 (Invitrogen), which drives transgene expression with a cytomegalovirus promoter. Feline β gal clone sequences were verified by automated fluorescent DNA sequencing.

Cells and immortalization

Primary fibroblasts from normal or G_{M1} cats were isolated by incubating minced skin biopsies for 3 days in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1 $\times$ penicillin–streptomycin–amphotericin (D10 media, all components from Sigma) and 200 units/ml collagenase type Ia (Calbiochem). After plating the collagenase-digested skin pieces in D10 media alone, fibroblast colonies were trypsinized and replated. Primary fibroblasts were calcium-phosphate transfected with plasmid pSV3-DHFR (American Type Culture Collection), which contains the large T antigen of Simian Virus 40. Approximately 2 weeks after transfection, colonies of immortalized cells were evident in the tissue culture flasks and were trypsinized for further use [29].

Immortalized feline fibroblasts were calcium-phosphate transfected with plasmids 1.11 or 3.9 (described above), both of which contain a neomycin resistance gene. Transfected cells were selected by growth for 2 weeks in the neomycin analog, Geneticin (Invitrogen, 750 μ g/ml active). Non-transfected control cells died after selection in Geneticin for 1 week. Cell lysates were prepared in 0.1% Triton X-100, and fluorogenic enzyme assays were performed as previously described [60].

Human fibroblast cell lines were obtained from a control patient (Wt) and two patients with G_{M1} gangliosidosis: H1, a homozygous non-sense mutation (Arg351X/Arg351X) resulting in neither β gal enzyme activity nor cross reactive material by Western blot; and H2, a compound heterozygote (Arg148Ser/Asp332Asn) [30] with less than 1% residual β gal activity and low but detectable levels of immunoreactive β gal protein. The cell lines were maintained at 37 °C in α -MEM media (Wisent Inc.) supplemented with antibiotics (penicillin/streptomycin, Gibco BRL) and 10% (v/v) fetal bovine serum in a humidified atmosphere with 5% CO₂.

Quantitative RT-PCR

For analysis of β gal mRNA levels, six experiments were performed with a total of two age-matched, mutant–normal cat pairs. Data are expressed as means $\pm$ standard deviation (SD). Quantitative RT-PCR (qRT-PCR) assays for feline β gal mRNA were performed on an Applied Biosystems model 7700 sequence detector using primers 3a and 2b (Table 1). Because primer 3a anneals in exon 3, the qRT-PCR assay measures transcript levels of β gal only, not the elastin–laminin binding protein (a *GLBI* splice variant lacking exons 3, 4 and 6). Total RNA was harvested from frozen tissue and reverse transcribed with oligo(dT) as described above. Quadruplicate 25 μ l PCR reactions were performed with 1 $\times$ Sybr Green buffer, 3 mM MgCl₂, 0.2 mM dNTP's, 0.625 U Taq Gold, 0.25 U uracil-*N*-glycosylase (all

from Applied Biosystems), 0.4 μ M each primer and 0.5 μ l cDNA template per reaction. After initial incubations of 2 min at 50 °C and 10 min at 95 °C to activate uracil-*N*-glycosylase and Taq Gold, respectively, 40 cycles of amplification were performed as follows: 30 s at 95 °C, 30 s at 60 °C and 45 s at 72 °C. β gal copy numbers from experimental samples were extrapolated from a standard curve generated with the same amplification conditions as above, with cDNA replaced by varying copy numbers (10^2 – 10^8) of plasmid fBgal 3.9 (cloned in our laboratory), which contains the putative open reading frame of feline β gal. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12, GAPD) expression was analyzed as above with an annealing temperature of 58 °C for primers fGAPD 101c (5'-CCTTCATTGACCTCAACTACAT-3') and fGAPD 225n (5'-GAAGATGGTGATGGGCTTT-3'). A standard curve for GAPD amplification was generated with varying copy numbers (10^3 – 10^8) of plasmid fGAPD, which contains a partial feline GAPD cDNA cloned in our laboratory. To verify homogeneity of the amplicon generated from both β gal and GAPD PCR reactions, melting curve analysis of PCR product was performed on the Applied Biosystems model 7700 sequence detector with ABI Prism 7700 Dissociation Curve 1.0 software. PCR product was incubated for 15 s at 95 °C, 20 s at 60 °C, then 15 s at 95 °C. The ramp time between annealing (60 °C) and final denaturation (95 °C) was 19 min, 59 s. Additionally, automated fluorescent DNA sequencing was performed on selected samples of both β gal and GAPD PCR products to verify identity.

Monoclonal antibody to feline β gal

An immunogenic peptide for creation of a monoclonal antibody to feline lysosomal β gal was designed based on the published feline cDNA sequence (GenBank Accession No. AF006749). Peptide fBgal 148 (CGL RSS DPD YLA AVD K) includes amino acids 148–161 of feline β gal with CG at its amino terminus for conjugation of adjuvant. Peptide fBgal 148 is 100% homologous to human β gal and includes amino acids derived from the 3' end of exon 4 and the 5' end of exon 5. Peptide fBgal 148 bears no homology to the elastin–laminin binding protein, the alternatively spliced variant of *GLBI* resulting from removal of exons 3, 4 and 6 with a frame shift in exon 5 [31]. The peptide was synthesized at >95% purity, analyzed for net peptide content, and conjugated to keyhole limpet hemocyanin (KLH) by Global Peptide Services, LLC. Female Balb/c mice were immunized intramuscularly with the following emulsification: 100 μ g KLH-peptide, aluminum hydroxide gel adjuvant (Alhydrogel 85, Accurate Chemicals/Superfos Biosector, Denmark, equal volume of 0.4 mg/ml stock solution), 10 μ g CpG-1826 [32,33] (5'-TCC ATG ACG TTC CTG ACG TT-3', synthesized with a phosphorothioate backbone). Subsequent immunizations were performed as above but with 50 μ g KLH-peptide. The final immunization was performed with 25 μ g unconjugated peptide alone.

Isolation of spleen cells from immunized mice, fusion with myeloma cells and subsequent isolation of hybridoma clones were performed according to standard procedures by the Auburn University Hybridoma Facility. Hybridoma supernatants were screened by Western blotting for selection of the best antibody clone.

Western blotting

Feline β gal—Western blots were performed with 25 μ g of protein on a 10% SDS–polyacrylamide gel according to the method of Laemmli [34]. Protein was transferred to nitrocellulose membranes (BioRad or Pierce), which were blocked in 5.0–7.5% non-fat dry milk and probed with hybridoma supernatant or a 1:6000 dilution (264 ng/ml) of mouse anti-rabbit GAPD (Chemicon). Secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG/M) was diluted 1:120,000–1:180,000 and visualized with Supersignal West Dura chemiluminescent substrate (Pierce).

For quantification of protein levels, blots were scanned with a HP ScanJet 4570c flatbed scanner and digitized with Un-Scan-It gel Version 5.1 (Silk Scientific). The pixel density of each fBgal 148 band was normalized to the corresponding pixel density of GAPD. Normalized band intensities then were compared between normal and G_{M1} mutant animals. Tissue protein from two age-matched, mutant–normal pairs of cats was used for Western blotting. Four experiments were performed for kidney protein, and five experiments were performed for cerebral cortex.

BiP/PDI—Human and cat fibroblast cell lysates were obtained by repeated freezing and thawing in the presence of 0.1% sodium deoxy-taurocholate (w/w) followed by a 20 min centrifugation at 16,000g in a benchtop centrifuge at 4 °C. The reduced and denatured total proteins (40 μ g) from fibroblast lysates were separated by SDS–PAGE as described above. Pre-stained high molecular weight protein standard was purchased from Invitrogen. Separated proteins were transferred to nitrocellulose (Whatman) for Western blot analysis using an ECL system (Amersham) as previously described [35]. Primary antibodies were a goat polyclonal IgG anti-human GRP78 (BiP) (1:200, Santa Cruz Biotechnology Inc.) and a mouse monoclonal IgG₁ anti-Protein Disulfide Isomerase (PDI) (1:1400, Stressgen). Membranes were stripped using a commercial solution (Pierce) between BiP and PDI labeling. Secondary antibodies were HRP-conjugated donkey anti-goat and donkey anti-mouse, respectively (1:5000, Jackson). GRP78/BiP and PDI run at apparent molecular masses of ~78 and ~58 kDa, respectively.

Indirect immunofluorescence and confocal microscopy imaging

Indirect immunolabelling was performed using a protocol previously described [36] with small modifications. In short, cells were seeded onto 18 mm diameter coverslips for 16–20 h, then washed and fixed with 4% paraformaldehyde (EMS) in PBS, pH 7.2, for 20 min at 37 °C. Blocking and permeabilization was performed for 1 h at room temperature with SS–PBS (0.2% saponin (Sigma) and 10% either goat or horse normal serum (Wisent Inc.) in PBS). Coverslips were overlaid with primary and secondary antibodies (diluted in SS–PBS) for 1 h each at room temperature (in the dark for secondary antibodies). Nuclear staining was done with DAPI (Molecular Probes) at 1:50,000 in PBS, and coverslips were mounted onto glass slides with fluorescent mounting medium (DakoCytomation). Primary antibodies were as follows: rabbit polyclonal IgG anti-human β gal [37]; mouse monoclonal IgG₁ anti-human LAMP-1 and LAMP-2 (Developmental Studies Hybridoma Bank, University of Iowa); mouse monoclonal IgG₁ anti-Protein Disulfide Isomerase (Stressgen). Anti-human LAMP-2 was used for immunofluorescence with cat cells because the anti-human LAMP-1

antibody did not cross-react. Secondary antibodies were chicken anti-rabbit Alexa fluor 488 for β gal and either chicken anti-goat or anti-mouse Alexa fluor 594 (Molecular Probes) at a 1:200 dilution in SS–PBS solution. Samples were analyzed using a Zeiss Axiovert confocal laser microscope equipped with a 63×1.4 numerical aperture Apochromat objective (Zeiss) and LSM 510 software. DAPI-stained nuclei were detected on the same system with a Chameleon two-photon laser. Confocal images were imported and contrast/brightness adjusted using Volocity 4 program (Improvision Inc.).

Statistical analysis

Statistical significance was analyzed using a one sample, two-tailed t -test [38] with the null hypothesis $H_0: \mu = 1$, where μ is the quotient defined by G_{M1} mutant β gal/normal β gal after both values were normalized to GAPD levels in the experimental samples. Therefore, if no difference in β gal levels exists between G_{M1} mutant and normal samples, then $\mu = 1$.

Results

The feline β gal cDNA was sequenced from normal cats and found to be very similar to human and mouse β gal sequences [25–28,39]. The feline open reading frame is 2010 base pairs (bp), compared to 2031 bp and 1941 bp for human and mouse, respectively. The overall sequence homology is 74% for cat versus human sequences and 72% for cat versus mouse sequences. (The human β gal open reading frame is 70% homologous to mouse.) Predicted protein length is 669 amino acids for feline β gal, compared to 677 amino acids for human β gal and 647 amino acids for mouse β gal, with an overall homology of 82% for cat versus human protein and 76% for cat versus mouse protein. (Human β gal protein is 80% homologous to mouse protein.) Based on sequence homology, the putative feline signal peptide (which is cleaved from the protein after facilitating translocation of the nascent protein into the endoplasmic reticulum) consists of amino acids 1–24, compared to amino acids 1–23 and 1–24 in human and mouse, respectively. Seven potential asparagine (N)-linked glycosylation sites occur at amino acids 27, 248, 429, 465, 499, 547 and 557, with six of seven sites found also in the human protein and five of seven sites found in the mouse protein.

When β gal cDNA from mutant cats was compared to the normal sequence, a guanine to cytosine substitution was detected at base 1448 of the open reading frame (G1448C), causing an arginine to proline substitution at amino acid 483 of the feline β gal protein (Arg483Pro). Human G_{M1} gangliosidosis is known to be caused by similar mutations, Arg482His [3,6,8] and Arg482Cys [4]. The feline mutation resides in exon 14 of *GLB1*, as determined by sequence homology with the known exon–intron boundaries in human and mouse *GLB1* [27,28].

To analyze a large number of cat samples for verification that the G1448C mutation is the pathogenic mutation in feline G_{M1} gangliosidosis, amplification of exon 14 was performed as described in Materials and methods section. By automated fluorescent DNA sequencing of the exon 14 amplicon from genomic DNA, 377 cats from this colony have been genotyped. To date, 136 phenotypically normal cats (36.1%) possess a G at position 1448, while an additional 180 phenotypically normal cats (47.7%) exhibit overlapping G and C

peaks at position 1448, identifying them as heterozygotes for the mutant allele. The mutant allele has been identified in a homozygous state in 61 cats (16.2%), all of which developed stereotypical clinical signs of G_{M1} gangliosidosis. The homozygous G1448C mutation has never been detected in a phenotypically normal cat.

A full-length feline β gal cDNA was cloned from a genotypically normal cat into mammalian expression vector pCR3.1 (Invitrogen). As described in Materials and methods section, two separate full-length cDNAs were generated containing (1) the ATG translation initiation codon only or (2) 12 base pairs of the feline β gal 5' untranslated region followed by the initiation codon. Transfection of the two feline β gal cDNAs into immortalized fibroblasts from a G_{M1} gangliosidosis cat restored enzymatic activity to levels approximately 18-fold higher than fibroblasts from a normal cat and 200-fold higher than untransfected G_{M1} cells (Table 2). The construct that included the feline β gal 5' untranslated region (3.9) produced the highest levels of enzymatic activity in G_{M1} fibroblasts (18-fold above normal), but the construct including only the initiation codon (1.11) still was able to generate normal levels of β gal activity.

To analyze the molecular consequences of the G1448C mutation in feline G_{M1} gangliosidosis, quantitative RT-PCR assays were performed with primers 3a and 2b using cDNA isolated from cerebral cortex of normal and G_{M1} mutant cats. After normalization of data to GAPD, feline β gal mRNA levels in G_{M1} gangliosidosis cortex were not statistically different from normal ($115.9 \pm 15.7\%$, $P > 0.05$). Melting temperature analysis of the qRT-PCR product revealed a single dissociation peak, strongly suggesting amplification of a single PCR product. In addition, single-band PCR product was observed during gel purification of the qRT-PCR product, which demonstrated no contamination of the primary PCR product upon automated fluorescent DNA sequence analysis (data not shown).

To discern if cats with G_{M1} gangliosidosis produce steady-state levels of β gal protein, Western blots were performed with monoclonal antibody fBgal 148, which recognizes β gal from cat and other species such as human and cow. Anti-fBgal 148 does not recognize the elastin-laminin binding protein, an alternatively spliced variant of *GLB1*, due to lack of homology in the peptide sequence (see Materials and methods). In kidney, a 76 kDa band was evident at equal levels in normal and G_{M1} affected cats (Fig. 1). When normalized to GAPD protein levels, β gal in mutant kidney was $115.1 \pm 29.2\%$ of normal (mean $\pm$ SD), not a statistically significant difference ($P > 0.20$, $n = 4$). Similarly in cerebral cortex, cats exhibit a 63 kDa band of approximately equal intensity between normal and G_{M1} affected individuals. After normalization to GAPD, β gal in mutant cerebral cortex was $93.9 \pm 17.4\%$ of normal ($P > 0.20$, $n = 5$). Molecular weight differences in bands from cortex and kidney samples likely represent precursor versus mature forms of β gal.

To evaluate whether mutant β gal protein is correctly targeted to the lysosome, immunofluorescence was performed with a previously characterized polyclonal antibody to human β gal (anti-P-Gal) [37]. In normal cat fibroblasts, β gal was correctly targeted to the lysosomes, as demonstrated by co-localization of β gal primarily with lysosome associated membrane protein (LAMP2). Very little co-localization of β gal and PDI was observed. However, mutant β gal in G_{M1} cat fibroblasts did not co-localize with LAMP2, indicating a

non-lysosomal address for the mutant protein (Fig. 2). Based on cytological evidence (perinuclear staining with anti-P-Gal antibody) and faint co-localization with PDI, mutant β gal appears to be localized to the endoplasmic reticulum (ER). Similar subcellular addresses for β gal were documented in human fibroblasts, with the majority of wild-type β gal found in the lysosomes of normal cells and the majority of mutant β gal found in the ER of the H2 cell line, derived from a G_{M1} patient who was a compound heterozygote (Arg148Ser/Asp332Asn). The human H1 cell line, homozygous for a premature termination codon mutation (Arg351X), was included as a control and did not generate β gal cross-reactive material.

Subcellular localization of β gal also was studied in G_{M1} cat cells transfected with plasmids expressing normal (1.11) or supranormal (3.9) levels of wild-type β gal (Fig. 2). In 1.11 cells expressing normal β gal specific activity, the targeting defect was corrected, as demonstrated by substantial increases in co-localization of β gal and LAMP2. Interestingly, in 3.9 cells expressing supranormal levels of β gal specific activity, the majority of β gal protein (wild-type) co-localized with PDI, demonstrating an ER address.

Localization of a mutant lysosomal protein such as β gal to the ER suggests the possibility of ER stress, which could trigger the unfolded protein response (UPR). For an initial evaluation of the UPR in human and cat G_{M1} gangliosidosis, Western blots for GRP78/BiP or PDI were performed on fibroblast lysate from normal and affected cells (Fig. 3). Strong up-regulation of both GRP78/BiP and PDI was detected in G_{M1} gangliosidosis fibroblasts from human H2 cells (Arg148Ser/Asp332Asn) and from feline G_{M1} cells (Arg483Pro/Arg483Pro). Moderate increases in GRP78/BiP were documented in the human H1 cell line (Arg351X/Arg351X), although PDI levels were not increased.

Discussion

G_{M1} gangliosidosis is a fatal, neurodegenerative lysosomal storage disease that, in its most common and severe form, causes the demise of human infants by 3 years of age [1]. G_{M1} gangliosidosis also occurs in cats [9], dogs [40,41], sheep [42,43] and knockout mice [44–46], and these animal models are very valuable for disease characterization and therapeutic development.

In the current study, the genetic mutation for the well-characterized feline model of G_{M1} gangliosidosis was defined as G1448C, resulting in an Arg483Pro substitution. Messenger RNA levels of the β gal transcript were normal in the cerebral cortex of affected animals ($115.9 \pm 15.7\%$, $P > 0.05$). Because the upstream primer (3a) for the qRT-PCR assay annealed in exon 3, results reflect transcript levels for β gal only. No contribution is expected from the alternatively spliced variant of *GLBI*, the elastin–laminin binding protein, which lacks exons 3, 4 and 6 [31].

When 377 members of the feline G_{M1} breeding colony were tested for the mutant allele by a PCR-based diagnostic assay (see above), 36.1% were homozygous for the normal allele while only 16.2% were homozygous for the mutant allele, suggesting a detrimental effect of the G1448C mutation on fecundity. Similar observations have been reported in mouse

models of lysosomal storage disease. For example, statistically significant abnormalities were detected in the sperm of mice heterozygous for knockout (KO) of the acid sphingomyelinase (ASM) gene. Although they produced normal numbers of sperm, heterozygotes demonstrated abnormal sperm morphology, mitochondrial membrane potential and ability to undergo capacitation or the acrosome reaction. Strikingly, when sperm from heterozygous ASMKO mice was used for *in vitro* fertilization of heterozygous oocytes followed by implantation into pseudopregnant wild-type mice, 37.8% of the resulting offspring were normal and 15.6% of the offspring were ASMKO affected mice [47]. Also, mice heterozygous for the β gal knockout mutation produced offspring of which 18% were affected by G_{M1} gangliosidosis [44], and sperm abnormalities have been reported in the mouse models of Niemann-Pick Disease type C [48] and globoid cell leukodystrophy (Twitcher mouse) [49].

Although the analogous human mutation often occurs in G_{M1} patients who are compound heterozygotes, the Arg482His/Cys mutation in homozygosity results in a severe, infantile-onset disease phenotype [3,7,8]. Fibroblasts and leukocytes from patients homozygous for the Arg482His mutation express <1% of normal β gal activity, as with the mutant protein expressed in immortalized G_{M1} fibroblasts [4] or COS-1 cells [3]. However, cats homozygous for the Arg483Pro mutation more closely resemble juvenile-onset G_{M1} gangliosidosis, with less severe clinical disease and no hepatosplenomegaly [9]. Clinical disease onset in affected cats begins at approximately 3.5 months, and the humane endpoint is reached at 7.7 months. Less severe clinical disease is explained by substantial residual enzymatic activity in affected cats, as demonstrated in skin fibroblasts (9.2%, Table 2) and cerebral cortex (5%, data not shown). It is possible that the Arg483Pro mutation allows retention of residual enzymatic activity compared to the histidine or cysteine substitutions in mutant human β gal. This could be due to residual catalytic activity toward G_{M1} ganglioside and/or partial trafficking of mutant β gal to the lysosome (as suggested by faint co-localization of β gal and LAMP2 antibodies in Fig. 2). When a wild-type feline β gal cDNA is expressed at normal levels in G_{M1} cat fibroblasts, lysosomal localization of β gal is achieved. However, when wild-type feline β gal is expressed at high levels (18-fold above normal), the majority of the β gal protein is detected in the ER, probably *en route* to secretion from the cell. Because the β gal precursor is enzymatically active toward the synthetic 4MU substrate [50], specific activity assays of cell lysates measure total β gal activity (including β gal in the ER or secretory vesicles), not solely lysosomal β gal. Therefore, the current data does not permit determination of the amount of β gal that reaches the lysosome in overexpressing 3.9 cells (Fig. 2) or whether it would be sufficient to remove G_{M1} storage.

The UPR has been implicated in many disease processes [51–53], including lysosomal storage diseases such as Gaucher [54] and Fabry Disease [55,56], infantile neuronal ceroid lipofuscinosis (INCL) [57–59] and G_{M1} gangliosidosis [46]. Specifically, in a knockout mouse model of G_{M1} gangliosidosis, GRP78/BiP was shown to be upregulated along with a variety of other markers for UPR activation. Although calcium dysregulation resulting from G_{M1} ganglioside accumulation was shown to induce the UPR in knockout mice, similar results were obtained from cultured feline fibroblasts (Fig. 2), in which G_{M1} synthesis is undetectable. This suggests that the UPR is induced by the presence of mutant β gal protein in the ER. Therefore, it is possible that at least two triggers for induction of the UPR exist in

GM1 cats: (1) calcium dysregulation resulting from GM1 accumulation in the neuronal ER, and (2) accumulation of mutant β gal protein, which is not present in GM1 knockout mice. Of the two potential UPR triggers listed above, GM1-induced calcium dysregulation may be the most devastating, should it cause generalized protein misfolding and ER-associated degradation of otherwise normal proteins. However triggered, prolonged ER stress is known to induce UPR-mediated apoptotic pathways that effect neuronal death in neurodegenerative lysosomal diseases. For example, in the mouse model of INCL, brain elevations of several UPR markers accompanied increases in the apoptotic mediators, caspases 9, 12, 3 and 4 [57–59]. We hypothesize that a similar mechanism of neuronal apoptosis occurs in feline GM1 gangliosidosis, although a more precise elucidation of mechanistic pathways will require further investigation.

In summary, this report identifies the disease-causing mutation in feline GM1 gangliosidosis as Arg483Pro, similar to known pathogenic mutations of Arg482 in humans. Cerebral cortex and kidney tissues from GM1 cats produce normal amounts of β gal, as evidenced by Western blotting. However, the mutant β gal protein in feline and human GM1 cells is not effectively targeted to the lysosome but is retained in the ER. Continued definition of pathogenic pathways in the well-established feline disease model further enhances its utility for therapeutic research and is expected to stimulate innovative new therapies for GM1 gangliosidosis.

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Fig. 1.

Western blot of β gal from normal and G_{M1} mutant tissues. (Upper panel) A monoclonal antibody (fBgal 148) to feline β gal was used to probe blotted protein (25 μ g/lane) from G_{M1} mutant (lanes 1 and 3) and normal (lanes 2 and 4) tissues. G_{M1} mutant cats exhibited approximately normal levels of β gal protein in cerebral cortex (lanes 1 and 2) and kidney (lanes 3 and 4). Semi-purified β gal from bovine liver (lane 5, Sigma-Aldrich) provided a positive control for antibody specificity. Bands of different molecular weights represent differentially processed precursor or mature forms of β gal. (Lower panel) To verify equal loading of the lanes, an antibody to GAPD (Chemicon) was used. When normalized to GAPD, β gal levels in mutant tissues were not statistically different from normal: cerebral cortex, $93.9 \pm 17.4\%$ of normal ($P > 0.20$, $n = 5$); kidney, $115.1 \pm 29.2\%$ of normal ($P > 0.20$, $n = 4$). Note that no band was visualized after semi-purified β gal (lane 5) was probed with anti-GAPD.

Fig. 2.

Immunofluorescence localization of β gal in normal and G_{M1} gangliosidosis fibroblasts. All cells were probed with an antibody to β gal (green). Antibodies for subcellular localization (red) included LAMP-1/2 (left panel) and PDI (right panel) to identify lysosomes and the ER, respectively. Merged images demonstrated co-localization of β gal with LAMP-1/2 or PDI (yellow–orange). Human cell lines were derived from skin biopsies of wild-type (Wt) or G_{M1} gangliosidosis patients (H1, H2). Wild-type cells demonstrated lysosomal localization of β gal. Patient H1 was homozygous for a premature termination codon (Arg351X) and did not produce β gal cross-reactive material. Patient H2 was a compound heterozygote

(Arg148Ser/Asp332Asn) and produced β gal cross-reactive material that localized primarily to the ER. Cat cells included those from wild-type and G_{M1} gangliosidosis (GM1) individuals. Also shown are cat G_{M1} cells transfected with plasmids that express normal (1.11) or supranormal (3.9) levels of β gal. While wild-type cat β gal clearly localizes to the lysosome, G_{M1} cat β gal demonstrates faint localization to both the lysosome and ER. G_{M1} cells restored to normal levels of enzymatic activity (1.11) demonstrate lysosomal localization of β gal, while G_{M1} cells expressing above-normal levels of enzymatic activity (3.9) demonstrate primarily ER localization of β gal.

Fig. 3.

G_{M1} gangliosidosis fibroblasts demonstrate increased levels of BiP and PDI, markers for the induction of the unfolded protein response. Western blot using anti-GRP78/BiP (top panel), anti-PDI (middle panel) or anti-actin (bottom panel) IgG of total cell protein (40 µg) from: wild-type human fibroblasts, Wt/H; human G_{M1} gangliosidosis-patient fibroblasts, Arg351X/Arg351X (CRM-negative for βgal), GM1/H1; human G_{M1} gangliosidosis-patient fibroblasts, Arg148Ser/Asp332Asn (CRM-positive for βgal), GM1/H2; wild-type feline fibroblasts, Wt/Cat; feline G_{M1} gangliosidosis fibroblasts, Arg483Pro/Arg483Pro (CRM-positive for βgal), GM1/Cat.

Table 1Feline β gal primers

Category	Fragment	Primer	Sequence (5′→3′)	Location ^a
Amplification	1	5′ RACE		
		292n	CTG GGG CTC GTG AAA GTT C	301–283
	2	273c	TGA ACG CCA TCC AGA C	255–270
		1465n	GAG TGA TCA CAT AAC TTC G	1412–1394
	3	1169c	GCC CAA GTT TGC ATA TG	1132–1148
		5b	CTG GAT AAA GGT GTC CTG	1744–1727
	4	1252c	CTC TGA ACG TTC TGT GTC CTG	1188–1208
		3′ RACE		
	Sequencing	361c	GCT GGC TCA TGA GCT GGG	352–369
		437n	CAC TCT GCA CAG ATG TAG G	414–396
541c		TGC CCA AGA TGA AGC CTC TC	522–541	
702n		CGG TGG TGA ACA GAA GCA C	686–668	
775c		GCA TCT ACG CCA CCG TG	729–745	
E8c		GGG GCC AAC CTC ACT CCA GA	855–874	
1028n		GTA GCT GGT GGG CTG TG	1002–1018	
1288n		CTG GAT AAA CGT CAA CGG ATA AAG	1249–1226	
1377c		AAC CCC ACA CCC CTG TC	1307–1323	
1568c		GAC CCT CGG TTC CAG TGT C	1522–1540	
Diagnostic	Ex14c	GGA GTC CTG GAG CGA AG	1382–1398	
	Int14n	GAG AAG AAT GTA TGT GCG AGA GT	Intron 14	
qRT-PCR	3a	GCC CTA CAT CTG TGC AGA GT	394–413	
	2b	CTT CAT CTT GGG CAG AAG GA	535–516	
Cloning	fBgal ATG	<u>ATG</u> GAC TTC CCC GGG GCT GCT	23–43	
	fBgal 5′ utr	AGA GGC TGG AGG <u>ATG</u> GAC TT	11–30	
	fBgal stop	CCA TCA GAC ACG GTC CCA TC	2035–2016	

^aNucleotide numbering based on GenBank sequence AF006749 (normal feline β gal). Initial primer names and sequences were based on homology between human and mouse β gal cDNAs and may not be 100% homologous to the feline sequence. Translation initiation codon is underlined.

Table 2Specific activity in feline GM₁ gangliosidosis fibroblasts^a

Sample	β -Galactosidase	Hexosaminidase	Mannosidase
Normal	77.3	913.6	26.4
GM ₁	7.1	1628.0	49.2
fBgal ATG	77.2	2308.8	78.2
fBgal 5'utr	1394.5	1416.1	70.8

^aGM₁ gangliosidosis fibroblasts were assayed untransfected (GM₁) or transfected with two separate constructs described in Materials and methods section: fBgal ATG and fBgal 5'utr. Specific activity values are expressed as nmol 4 MU/mg protein/h. Controls included untransfected GM₁ and normal cat fibroblasts as well as substrates for the lysosomal enzymes β -N-acetylhexosaminidase and α -mannosidase. Total hexosaminidase activity was measured with 4MU-N-acetyl- β -D-glucosaminide (4MUG) while mannosidase activity was measured with 4MU- α -D-mannopyranoside. Enzyme assays were performed as previously described [60].