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Research Article

Angiokeratoma corporis diffusum with glycopeptiduria is a recently recognized inborn error of glycoprotein catabolism resulting from the deficient activity of human alpha-N-acetylgalactosaminidase (E.C. 3.2.1.49; alpha-GalNAc). The first patient with this autosomal recessive disorder, a 46-yr-old consanguineous Japanese woman, presented with diffuse angiokeratoma, mild intellectual impairment, and peripheral neuroaxonal degeneration. Deficient alpha-GalNAc activity also has been reported in consanguineous brothers with an infantile-onset form of neuroaxonal dystrophy resulting from a missense mutation (designated E325K) in the alpha-GalNAc gene. To identify the mutation causing the phenotypically distinct adult-onset disorder, Southern and Northern hybridization analyses of DNA and RNA from the affected homozygote were performed which revealed a grossly normal alpha-GalNAc gene structure and normal transcript size and abundancy. Reverse transcription, amplification, and sequencing of the alpha-GalNAc transcript identified a single C to T transition at nucleotide (nt) 985 that predicted an arginine to tryptophan substitution in residue 329 (designated R329W) of the alpha-GalNAc polypeptide. This base substitution was confirmed by hybridization of PCR-amplified genomic DNA from family members with allele-specific oligonucleotides. Transient expression of an alpha-GalNAc construct containing the R329W mutation resulted in the expression of an immunoreactive polypeptide which had no detectable alpha-GalNAc activity. Comparison of the biosynthesis and stabilities of the transiently expressed and radiolabeled normal, E325K (infantile-onset) and R329W (adult-onset) alpha-GalNAc polypeptides in COS-1 cells indicated that both the […]

The Molecular Lesion in the α-*N*-Acetylgalactosaminidase Gene That Causes Angiokeratoma Corporis Diffusum with Glycopeptiduria

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Abstract

Angiokeratoma corporis diffusum with glycopeptiduria is a recently recognized inborn error of glycoprotein catabolism resulting from the deficient activity of human α-*N*-acetylgalactosaminidase (E.C.3.2.1.49; α-GalNAc). The first patient with this autosomal recessive disorder, a 46-yr-old consanguineous Japanese woman, presented with diffuse angiokeratoma, mild intellectual impairment, and peripheral neuroaxonal degeneration. Deficient α-GalNAc activity also has been reported in consanguineous brothers with an infantile-onset form of neuroaxonal dystrophy resulting from a missense mutation (designated E325K) in the α-GalNAc gene. To identify the mutation causing the phenotypically distinct adult-onset disorder, Southern and Northern hybridization analyses of DNA and RNA from the affected homozygote were performed which revealed a grossly normal α-GalNAc gene structure and normal transcript size and abundancy. Reverse transcription, amplification, and sequencing of the α-GalNAc transcript identified a single C to T transition at nucleotide (nt) 985 that predicted an arginine to tryptophan substitution in residue 329 (designated R329W) of the α-GalNAc polypeptide. This base substitution was confirmed by hybridization of PCR-amplified genomic DNA from family members with allele-specific oligonucleotides. Transient expression of an α-GalNAc construct containing the R329W mutation resulted in the expression of an immunoreactive polypeptide which had no detectable α-GalNAc activity. Comparison of the biosynthesis and stabilities of the transiently expressed and radiolabeled normal, E325K (infantile-onset) and R329W (adult-onset) α-GalNAc polypeptides in COS-1 cells indicated that both the mutant precursors were processed to the mature form; however, the E325K mutant polypeptide was more rapidly degraded than the R329W subunit, thereby providing a basis for the distinctly different infantile- and adult-onset phenotypes. (*J. Clin. Invest.* 1994. 94:839–845.) Key words: angiokeratoma • neuroaxonal dystrophy • lysosomal storage disease • α-*N*-acetylgalactosaminidase • mutation analysis • transient expression

Introduction

Angiokeratoma corporis diffusum with glycopeptiduria is a recently recognized inborn error of glycoprotein catabolism resulting from the deficient activity of the lysosomal glycohydrolase, α-*N*-acetylgalactosaminidase (E.C.3.2.1.49; α-GalNAc)[1] (1–3). The enzymatic defect, inherited as an autosomal recessive trait, leads to the tissue accumulation and increased urinary excretion of glycopeptides and oligosaccharides containing α-*N*-acetylgalactosaminyl moieties. The disorder was described in 1989 by Kanzaki et al. (1) in a 46-yr-old consanguineous Japanese female who had diffuse angiokeratoma, mild intellectual impairment, and peripheral neuroaxonal degeneration. The angiokeratoma, a disseminated petechiae-like eruption, first appeared on her lower torso at ~ 28 years of age and then spread slowly over her body in a distribution typical to that seen in hemizygotes with Fabry disease (4). Dilated, tortuous blood vessels were observed in her ocular conjunctiva and fundi. There were no other manifestations commonly found in other lysosomal storage diseases, including organomegaly, lymphadenopathy and skeletal abnormalities. The proband had a low intelligence (IQ = 70) and magnetic resonance imaging of the brain revealed a few small lacunar infarctions, but no gross parenchymal atrophy. Motor nerve conduction studies were normal, but the sensory nerves had a marked decrease in amplitude with normal velocity, indicating a peripheral neuroaxonal degeneration (3).

The affected homozygote had levels of α-GalNAc activity that were < 1% of normal in various sources as assayed with the recently synthesized substrate, 4-methylumbelliferyl-α-*N*-acetylgalactosaminide (4MU-α-GalNAc) (2). Her children had intermediate levels of activity consistent with being obligate heterozygotes for this autosomal recessive disorder. Using monospecific rabbit anti-human α-GalNAc antibodies, immunoblots of fibroblast extracts from the affected proband revealed no detectable immunoreactive enzyme protein, while extracts from her heterozygous son had two immunoreactive peptide species, the ~ 48-kD monomeric and ~ 117-kD dimeric forms of α-GalNAc observed in normal cultured fibroblasts and in preparations of the purified human enzyme. These findings sug-

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1. *Abbreviations used in this paper:* α-GalNAc, α-*N*-acetylgalactosaminidase; CRIM, cross-reacting immunological material; Endo H, endoglycosidase H; nt, nucleotide; PNGase F, peptide *N*-glycosidase F; 4-MU, 4-methylumbelliferyl.

gested that the α-GalNAc mutation in this family markedly altered the enzyme's activity and stability.

Previously, an infantile form of neuroaxonal dystrophy due to the deficient activity of lysosomal α-GalNAc was described in two consanguineous German boys (4–7). The course of this disorder was remarkably distinct from that of the adult-onset form, and was characterized by normal development until 8–12 mo of life, followed by a rapidly regressive course resulting in profound psychomotor retardation by three years of age. They had no apparent or histologic evidence of cutaneous involvement at 8 and 9 years of age. Morphologic analysis of cortical tissue revealed the characteristic "spheroids," which are the pathologic hallmark of the neuroaxonal dystrophies. The demonstration of accumulated *O*-linked sialoglycopeptides and the blood group A oligosaccharide in their urines led to the identification of deficient α-GalNAc activity as the primary enzymatic defect causing this form of infantile neuroaxonal dystrophy (5, 7, 8). The finding that the German brothers with infantile neuroaxonal dystrophy and the Japanese woman with diffuse angiokeratoma excreted the same sialoglycoproteins (9, 10), led to the discovery that the adult-onset proband also had deficient α-GalNAc activity (3). Thus, phenotypically distinct infantile- and adult-onset forms of α-GalNAc deficiency were delineated.

The isolation and expression of the full-length cDNA and genomic sequence encoding human α-GalNAc (11, 12) permitted investigation of the molecular lesions that cause the phenotypically distinct forms of α-GalNAc deficiency. The 12-kb α-GalNAc gene encodes a polypeptide of 411 amino acids including a signal peptide of 17 residues. Studies of human α-GalNAc biosynthesis indicated that the precursor polypeptide was cotranslationally glycosylated in the endoplasmic reticulum, and after carbohydrate modifications and phosphorylation of its high mannose-type oligosaccharide structures in the Golgi, the mature glycopeptide of 394 amino acids was trafficked to the lysosome via the mannose-6-phosphate receptor-mediated pathway (13). Presumably, the mature glycopeptide is dimerized to form the 117-kD active form in the Golgi. Recently, the molecular lesion in the consanguineous German brothers with the infantile-onset form was identified as a single G to A transition at nucleotide (nt) 973 which predicted a glutamic acid to lysine substitution at residue 325 (designated E325K) in the α-GalNAc polypeptide (14). Cultured fibroblasts from the infantile-onset patients, who were homoallelic for the E325K mutation, had < 1% normal α-GalNAc activity and no detectable immunoreactive enzyme protein. Studies of α-GalNAc biosynthesis in cultured fibroblasts from the infantile-onset probands suggested that the E325K precursor subunit was synthesized, but was so unstable that it was not processed to the mature lysosomal form (15).

In this communication, we report the specific missense mutation in the α-GalNAc gene (designated R329W), that causes adult-onset angiokeratoma corporis diffusum with glycopeptiduria in the consanguineous Japanese proband. This base substitution was confirmed by analysis of genomic DNA from family members and by transient expression studies. Of interest was the fact that the inactive enzyme protein expressed in COS-1 cells was immunologically detectable, whereas no cross-reacting immunological material (CRIM) was present in cultured fibroblasts from the adult proband. In addition, transient expression of α-GalNAc mutations E325K and R329W indicated that the R329W polypeptide was more stable than the E325K subunit, suggesting a molecular basis for these phenotypically distinct disorders.

Methods

Cell lines. Primary cultures of fibroblasts and lymphoblasts were established from skin biopsies and peripheral blood samples obtained with informed consent from members of the infantile- and adult-onset families and from normal controls. The COS-1 cells lines were purchased from the American Type Tissue Collection (Rockville, MD). The fibroblasts, lymphoblasts and COS-1 cell lines were grown in DME media supplemented with 10% fetal bovine serum, 1% penicillin, and 1 mg/ml streptomycin by standard procedures (16).

Assays of α-GalNAc activity and protein. The α-GalNAc activity in cultured cell lines was determined with the synthetic fluorogenic substrate 4MU-α-GalNAc (17) as previously described (7). Protein concentration was determined by the fluorescamine assay (18). Immunoblot analyses of α-GalNAc enzyme protein in transfected COS-1 cells was performed using monospecific rabbit anti–human α-GalNAc antibodies as described (7, 11).

Southern and Northern hybridization analyses. Genomic DNA was isolated from at least 10^6 cultured lymphoblasts (19). DNA digested with various restriction endonucleases (e.g., BamHI, PstI, EcoRI, TaqI) (New England Biolabs, Beverly, MA), was electrophoresed in 1% agarose, transferred to BioTrace RP charge-modified nylon 66 binding matrix (Gelman Sciences, Inc., Ann Arbor, MI) (20) and hybridized with nick-translated α-GalNAc cDNA as the probe (21). To determine the relative sizes and amounts of the 2.2- and 3.6-kb α-GalNAc transcripts (11), total RNA was isolated from at least 10^8 cultured lymphoblasts from the adult-onset proband and normal individuals (22). RNA samples were electrophoresed in 1% agarose/formaldehyde denaturing gels (23), transferred to the BioTrace membranes described above, and analyzed with the radiolabeled α-GalNAc riboprobe synthesized with SP6 polymerase from the α-GalNAc 2.2-kb cDNA cloned into the pGEM 4Z vector (Promega, Madison, WI).

DNA amplification and sequencing of the mutant allele. Sense and anti-sense oligonucleotide primers designed to amplify the entire coding region of the α-GalNAc transcript in two overlapping fragments were synthesized on a model 380B DNA synthesizer (Applied Biosystems, Foster City, CA). The 5′ region of the α-GalNAc transcript (cDNA nucleotide [nt] −54 to 689 [11]) was amplified using the 32-mer sense primer, P1 (5′-AGTAGTGAATTCCTGATACACGCAGACCAGAT-3′), corresponding to α-GalNAc nt −34 to −53 with an additional 12 nt which included an EcoRI site, and a 32-mer antisense primer, P2 (5′-AGTAGTAAGCTTTTCAGGATGGAGAGCTCGCT-3′), corresponding to α-GalNAc cDNA nt 670–689 with a HindIII site for directional-subcloning. The 3′ region of the coding sequence (cDNA nt 595 to 1292) was amplified using a 32-mer sense primer, P3 (5′-AGTAGTGAATTCAGGGTGAACTACAGTCTGCT-3′), corresponding to nt 595–614 with an EcoRI site and a 32-mer antisense primer, P4 (5′-AGTAGTAAGCTTGCTCCATGGTCTAGGCTCAG-3′), corresponding to nt 1273–1292 and containing a HindIII site. Total RNA (10 μg) was reverse-transcribed to cDNA using the BRL cDNA Synthesis Kit (GIBCO-BRL, Gaithersburg, MD). One-fourth of the cDNA product was PCR-amplified (24) using the GeneAmp DNA Amplification Reagent Kit (Perkin Elmer Cetus, Norwalk, CT) and 1 μM of each primer. Each of the 30 PCR cycles consisted of denaturation at 94°C for 1 min; annealing at 37°C for 2 min; and extension at 60°C for 7 min. Each PCR product was phenol/chloroform extracted, ethanol precipitated, resuspended in 20 μl of H_2O, and then 2 μl was analyzed by electrophoresis in a 1.8% agarose gel. The remaining 18 μl was digested with EcoRI and HindIII and directionally subcloned into the M13mp18 and 19 vectors (25). Clones containing the PCR products were identified by plaque hybridization using the nick-translated α-GalNAc cDNA (21). Single-stranded template was isolated (25) from six separate M13 clones, and each was sequenced in both orientations by the dideoxy chain termination method (26) using universal and α-GalNAc-specific primers.

Oligonucleotide hybridization of PCR-amplified genomic DNA. Genomic DNAs (1 μg) from the affected homozygote, her obligate heterozygous son, her surviving siblings and normal individuals were PCR-amplified using primers that flanked the region of the mutation by the conditions described above. The 5′ sense primer was a 20-mer (5′-TGATGTCCACAGACCTGCGT-3′) corresponding to nt 842–861 and the 3′ antisense primer was a 20-mer (5′-CATATCGGTCCTGCAGC-TGA-3′) corresponding to nt 1005–1024 of the α-GalNAc full-length cDNA (10). Normal and mutation-specific oligonucleotide probes (21-mers) were synthesized with the sequences 5′-GTGTACATGCGGCCT-CTGTCC-3′ and 5′-GTGTACATGTGGCCTCTGTCC-3′, respectively. Dot blots were denatured, hybridized and washed as described (27).

Computer-assisted analyses. Local secondary structure for the normal and both mutant enzyme subunits were predicted by the algorithms of Chou and Fasman (28) and Garnier et al. (29) using the University of Wisconsin Computer Group software (30). A region of 20 amino acids corresponding to residues 310–330 which included the E325K and R329W substitutions were used to predict the local secondary structures.

Construction of the mutant cDNA and transient expression in COS-1 cells. The BamHI–KpnI fragment from a M13 clone containing the nt 985 C to T substitution, was used as a cassette to replace the corresponding region in the full-length α-GalNAc cDNA (11). The sequence was confirmed by dideoxy sequencing, and then the mutant cDNA was subcloned into the eukaryotic expression vector p91023(B) (31) kindly provided by Dr. R. J. Kaufman (Genetics Institute, Cambridge, MA). This expression construct was designated p91-R329W, and the previously constructed expression vectors for the normal and E325K alleles (14) were designated p91-AGB and p91-E325K, respectively. For transient expression studies, each construct was transfected into COS-1 cells (32), harvested at 72 h after transfection, and then assayed for activity and immunoreactive protein as previously described (7).

To compare the biosynthesis and stabilities of the normal and mutant proteins, the p91-AGB, p91-E325K and p91-R329W expression constructs (20 μg DNA each) were individually transfected into 5×10^6 COS-1 cells by electroporation as previously described (Biorad Gene Pulser, Richmond, CA). The transfected COS-1 cells were radiolabeled for 15, 20, 30, or 60 min with 100 μCi of [^{35}S]methionine (Dupont New England Nuclear, Boston, MA) per 100 mM dish containing 1×10^6 cells at 72 h after transfection. Cells were harvested at various time points after labeling and the normal, R329W and E325K α-GalNAc polypeptides were immunoprecipitated using *Staphylococcus aureus* cells (Pansorbin, Calbiochem, San Diego, CA). Equal amounts of immunoprecipitated protein were electrophoresed on a standard tricine sodium dodecyl sulfate 10% polyacrylamide (SDS-PAGE) gel as previously described (14, 33). Autoradiograms were scanned and analyzed using the "Image Processing and Analysis" version 1.41 software kindly provided by Wayne Rasband (National Institutes of Health).

To investigate the glycosylation of the expressed normal α-GalNAc protein, COS-1 cells were transfected with the p91-AGB construct, and after 72 h were labeled for 1 h, were harvested immediately, and immunoprecipitated as described above. The immunoprecipitated enzyme was digested with peptide *N*-glycosidase F (PNGase F) and endoglycosidase H (Endo H) (Boehringer Mannheim, Indianapolis, IN) as described (34). The digested proteins were desalted using microcentricons (Amicon, Beverly, MA) and electrophoresed as described above.

Results

Identification of the α-GalNAc mutation in the adult-onset proband. To characterize the nature of the α-GalNAc mutation in the adult-onset proband with angiokeratoma corporis diffusum and glycopeptiduria, Southern and Northern hybridization analyses were performed with four restriction endonucleases on lymphoblast genomic DNA and total RNA from the affected homozygote, her obligate heterozygous children and unrelated normal individuals. No gene rearrangements were observed, the family was not polymorphic for the BamHI and TaqI restriction length fragment polymorphisms (11), and the ~ 2.2- and ~ 3.6-kb α-GalNAc transcripts were of normal size and abundancy (data not shown). Therefore, to identify a probable point mutation or small insertion or deletion in the coding region, total RNA isolated from cultured lymphoblasts derived from the proband was reverse transcribed, and the coding region was PCR-amplified and sequenced in both orientations. Sequence analysis of all 12 subclones revealed a C to T transition at nt 985 which predicted an arginine to tryptophan substitution at residue 329 (Fig. 1). This substitution, designated R325W, was confirmed by hybridization of allele-specific oligonucleotides with PCR-amplified genomic DNA from the affected homozygote, her obligate heterozygous son, her sibs and normal individuals. As shown in Fig. 2, the amplified genomic sequence from the affected homozygote hybridized only with the mutation-specific oligonucleotide. In support of this finding, the genomic PCR product from each of her children hybridized with both the normal and mutation-specific oligonucleotides. Furthermore, the heterozygous status of three of her four sibs as determined by α-GalNAc enzyme activity (3) was confirmed by allele-specific hybridization (data not shown).

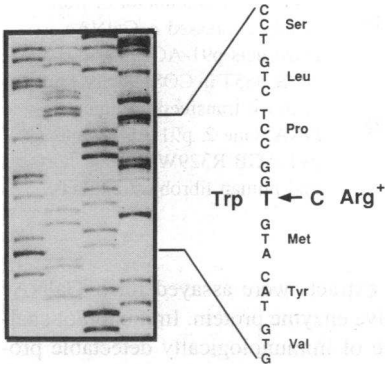

Figure 1. Partial DNA sequence of the reverse-transcribed and PCR-amplified α-GalNAc transcript from the adult-onset patient. Compared to the normal cDNA sequence (*left*), a C to T transition at nt 985 which predicted an arginine to tryptophan substitution at polypeptide residue 329, was identified in all subclones containing the α-GalNAc PCR product from the affected homozygote. See text for details.

Expression of the R329W adult-onset mutation in COS-1 cells. To further characterize this mutation, the BamHI–KpnI fragment (nt 921–1193) from a M13 subclone containing the R329W mutation was introduced as a cassette into the full-length α-GalNAc cDNA (11). The mutated cDNA (designated pAGB-R329W) was subcloned into the eukaryotic expression vector p91023(B) (designated p91-R329W), electrotransfected into 5×10^6 COS-1 cells, and after incubation for 72 h, the

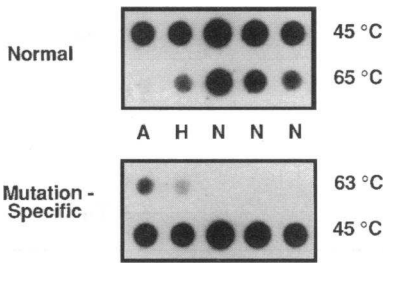

Figure 2. Allele specific oligonucleotide hybridization of PCR-amplified genomic DNA isolated from lymphoblasts of the affected homozygote (*A*), her obligate heterozygous son (*H*), and three unrelated normal individuals (*N*). Hybridization was carried out initially at 45°C and the filters were washed at the indicated temperatures for allele specific binding. See text for details.

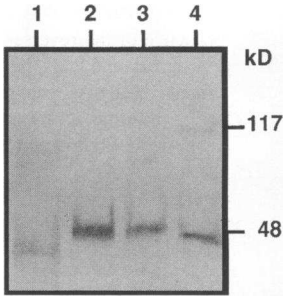

Figure 3. Immunoblot of transiently expressed α-GalNAc constructs p91-AGB and p91-AGB-985T in COS-1 cells. Lane *1,* mock transfection minus DNA; lane *2,* p91-AGB; lane *3,* p91-AGB-R329W; lane *4,* normal human fibroblast extracts.

cells were harvested and extracts were assayed for α-GalNAc activity and immunoreactive enzyme protein. Immunoblot analysis revealed the presence of immunologically detectable protein with a subunit molecular weight of ~ 48–49 kD (Fig. 3). However, the 4MU-α-GalNAc activity in the COS-1 cells was not increased above endogenous levels, whereas transfection with the normal construct, p91-AGB, resulted in immunodetectable protein which had an α-GalNAc activity that was about 30-fold higher than the mean endogenous level or the mean level in mock-transfected COS-1 cells (Table I).

Secondary structure analyses. Computer-assisted regional secondary structural analysis of residues 310 to 330 predicted differences in the normal R329W and E325K structures (22, 23). As illustrated in Fig. 4, the arginine to tryptophan substitution in R329W extended a random coil region in and around the area of the mutation. For comparison, the E325K mutation replaced the random coil region with α-helical structure.

Transient expression and comparison of the biosynthesis and relative stabilities of the expressed infantile- and adult-onset polypeptides. The biosynthesis and relative stabilities of the normal, E325K and R329W α-GalNAc polypeptides were determined by transient expression and pulse-chase labeling of the respective constructs in COS-1 cells. These experiments were conducted at 72 h after electroporation with radiolabeling for 15-, 20-, 30-, or 60-min pulses. As shown in Fig. 5, a 60-min pulse revealed six radiolabeled species for the normal and mutant enzymes with estimated molecular weights of ~ 39, 42, 44, 46, 49, and 53 kD, with the 49- or 53-kD species being

Table 1. Transient Expression of the Normal and R329W α-GalNAc cDNAs

Transfected construct	α-GalNAc activity*
	U/mg protein
p91-AGB	
Mean	168
Range (n = 4)	127–261
p91-AGB-R329W	
Mean	5.8
Range (n = 4)	4.7–7.5
Mock (No DNA)	
Mean	5.3
Range (n = 4)	3.2–7.4

*Enzymatic activity is expressed as the mean and range of four independent transfections. The reaction mixture contained 0.8 mM 4MU-α-GalNAc as substrate.

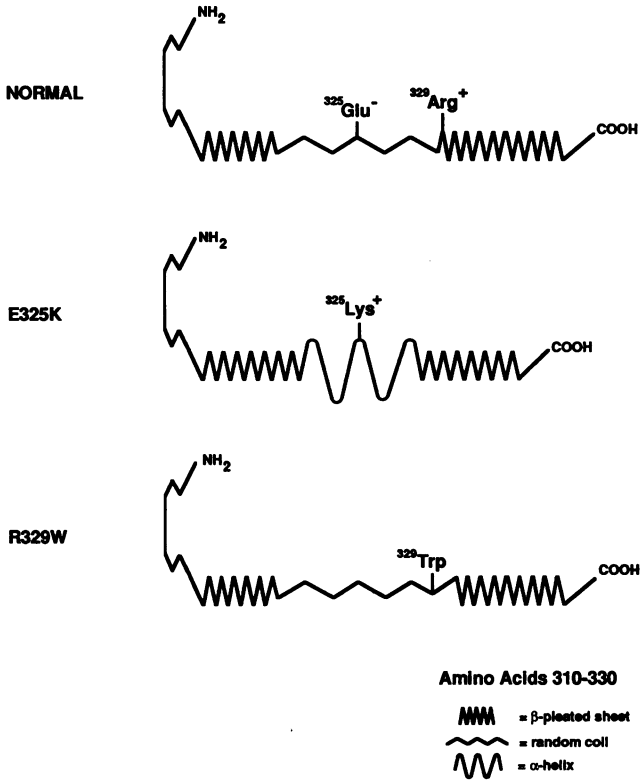

Figure 4. Computer-assisted secondary structure predicted for normal, E325K and R329W α-GalNAc residues 310–330. Note that the E325K mutation replaced a random coil region with an α-helical structure, whereas the R329W mutation extended the random coil region in the area of the mutation. See text for details.

most prominent. These species formed a ladder with 2–3-kD increments. Immediately after the 1 h pulse-labeling, the normal ~ 49 and ~ 53 kD forms were present in approximately equal amounts (Fig. 5 *A,* 0 h lane); after a 12–24 h chase, the 53-kD species appeared to be processed to a diffuse band(s) with a slightly lower molecular weight (~48–49 kD). Similarly, approximately equal amounts of the ~ 49- and 53-kD species of the E325K and R329W subunits also were observed immediately following radiolabeling; after a 12–24-h chase, only the mature subunit (~48–49 kD) was predominantly detected (Figs. 5, *B* and *C,* respectively).

Shorter pulses of 15, 20 (not shown), and 30 min were performed to determine if the 49- or 53-kD species was the enzyme precursor subunit. In these experiments, both the 49- and 53-kD species were detected (Fig. 6); shorter pulses did not label sufficient enzyme for detection. The nature of the oligosaccharide chains on the normal polypeptide was investigated by subjecting the immunoprecipitated enzyme to digestion with PNGase F and Endo H before electrophoresis. As shown in Fig. 7, PNGase F digestion reduced all the enzyme species to a single broad band of ~ 39 kD; similarly, the Endo H digestion converted the enzyme bands to as a single broad band with an estimated molecular weight of ~ 42 kD.

The relative quantities of the radiolabeled immunoreactive proteins expressed by the normal and mutant E325K and R329W constructs at 0, 6, 12, 24, and 48 h after labeling were determined by densitometry. Since there was variability in sig-

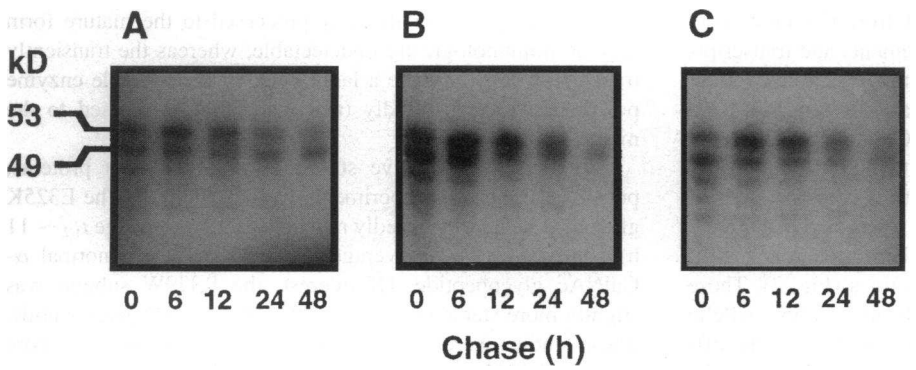

Figure 5. Biosynthesis of (*A*) normal, (*B*) E325K, and (*C*) R329W α-GalNAc transiently transfected COS-1 cells. At 72 h after transfection, α-GalNAc was labeled for 1 h by incorporation of [^{35}S]methionine and immunoprecipitated either directly (0 h of chase) or after 6, 12, 24, 48 h of chase from the cell homogenate. Note that at 0 h of chase, the ~ 49- and ~ 53-kD enzyme subunits were detected for the normal and both mutant glycopeptides. The lighter bands ranging from 39 to 46 kD represent partially glycosylated forms of the α-GalNAc polypeptide.

nal strength for the normal enzyme subunit due to transfection efficiency and for the mutant subunits primarily due to their inherent instability, the results are reported as the means and ranges for three independent experiments. The approximate half-life of the normal enzyme in COS-1 cells was about 37 h with a range of 20–68 h in independent experiments. In contrast, the R329W and E325K α-GalNAc polypeptides had estimated half-lives of 19 h (range, 18–20 h) and 11 h (range, 8–12 h), respectively.

Discussion

Adult-onset angiokeratoma corporis diffusum with glycopeptiduria was first described in 1989 by Kanzaki et al. (1). The pattern of urinary glycopeptide and oligosaccharide excretion in the adult proband was identical to that in patients with infantile neuroaxonal dystrophy due to deficient α-GalNAc activity (10), suggesting that α-GalNAc activity also might be deficient in the milder, adult-onset disorder. Subsequently, markedly deficient α-GalNAc activity was demonstrated in various sources from the adult-onset proband (2). Of note, cultured fibroblasts from both the infantile- and adult-onset probands had little, if any, residual α-GalNAc activity and no immunoreactive enzyme protein (2, 7). Thus, the milder adult-onset phenotype could not be explained by the presence of residual activity, as has been the case for the adult-onset forms of many other lysosomal storage diseases (e.g., Tay-Sachs, Gaucher and Niemann-Pick diseases, 35–37).

Recently, the missense mutation E325K was identified as the molecular lesion causing the infantile-onset form of α-GalNAc deficiency. Transient expression of the E325K cDNA in COS-1 cells produced the immunoreactive ~ 48–49-kD α-GalNAc enzyme glycopeptide which had no detectable enzymatic activity (14), indicating that the mutant enzyme polypeptide was synthesized and processed to the mature form, but was very unstable since it was not detectable in cultured fibroblasts from these probands. Subsequently, Hu et al. (15) investigated the biosynthesis of the E325K enzyme in cultured fibroblasts and reported that a 52-kD immunoreactive enzyme precursor was synthesized, but was not phosphorylated or processed to a detectable ~ 49-kD mature form. Since they could not detect the enzyme precursor in the culture media following NH_4Cl treatment of the mutant fibroblasts (in contrast to the secretion of the ~ 52-kD α-GalNAc precursor from normal fibroblasts), it was concluded that the mutant α-GalNAc precursor polypeptide was rapidly degraded. Their results differed from the previously reported finding that the transiently expressed E325K polypeptide was processed to its mature form in COS-1 cells (14). Thus, further studies are required to determine if the mature form of the E325K precursor glycopeptide is processed to the mature lysosomal form, or if the mutant precursor is degraded, precluding its delivery to the lysosome.

To compare the nature of the α-GalNAc lesions causing the infantile- and adult-onset phenotypes, efforts were first undertaken to identify the mutation in the family with angiokeratoma corporis diffusum with glycopeptiduria. Southern and Northern

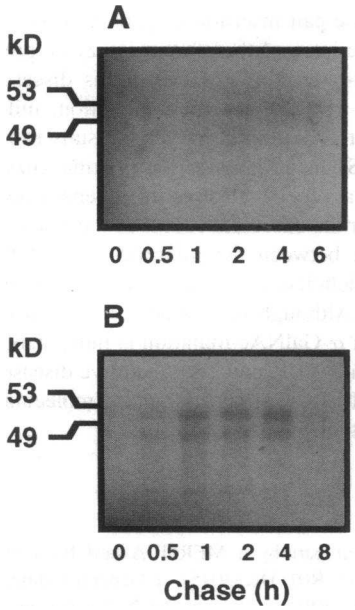

Figure 6. Biosynthesis of normal α-GalNAc transiently expressed in COS-1 cells. α-GalNAc was labeled at 72 h post-transfection by incorporation of [^{35}S]methionine for (*A*) 15 min or (*B*) 30 min. Cells were chased for the indicated time periods. The intensity of the signal varied due to the amount of recovered enzyme in the immunoprecipitate.

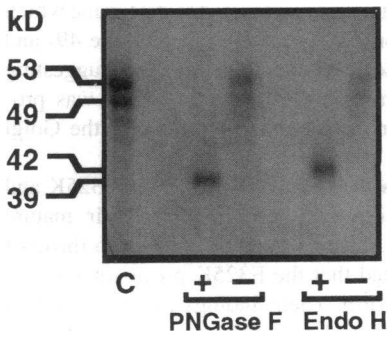

Figure 7. Endoglycosidase digestion of α-GalNAc transiently expressed in COS-1 cells. At 72 h after transfection, α-GalNAc was labeled for 1 h with [^{35}S]-methionine and immunoprecipitated. *C* represents untreated α-GalNAc enzyme; the other samples were treated with either PNGase F or Endo H.

hybridization analyses of DNA and RNA from the adult-onset proband revealed no gross gene rearrangements and transcripts of normal size and abundancy. Subsequent reverse transcription, amplification, and sequencing of the α-GalNAc transcript from the adult-onset patient revealed a single C to T transition at nt 985 in the coding region (Fig. 1). This transition, which predicted an arginine to tryptophan substitution at residue 329 of the α-GalNAc polypeptide (R329W), was confirmed in genomic DNA from the affected homozygote and her obligate heterozygous son using allele-specific oligonucleotides (Fig. 2). These results indicated that the adult-onset proband was homoallelic for this mutation, consistent with the fact that she was the offspring of a first cousin marriage. Transient expression of the mutant R329W cDNA in COS-1 cells resulted in the production of an immunoreactive ~ 48–49-kD polypeptide, although no immunoreactive enzyme protein was previously detected in cultured fibroblasts from the affected proband. The finding of immunoreactive enzyme in the COS-1 cells reflects the "pulsed" synthesis of the catalytically impaired and unstable R329W enzyme polypeptide precursor, which was then processed to the mature lysosomal form. Thus, the classification of a mutation as CRIM-negative based on studies of the patient's cultured cells (or tissues) does not imply that the enzyme was not synthesized.

Of interest was the finding that the E325K and R329W missense mutations causing the infantile- and adult-onset forms of this disease resulted in amino acid substitutions that were separated by only three residues. Based on these and previous studies (2, 14, 15), it was hypothesized that both the E325K and R329W mutations encoded unstable and catalytically impaired enzyme precursor glycopeptides that were normally processed, the R329W glycopeptide being slightly more stable, such that it retained sufficient residual activity (~ 1% or less) to modify the disease phenotype to the milder adult-onset form. To test this hypothesis, the normal and mutant E325K and R329W glycopeptides were transiently expressed in COS-1 cells and their biosynthesis and relative stabilities were determined. Of note, six enzyme forms (ranging from an estimated 39 to 53 kD) were detected for the normal and mutant enzymes after a 1-h pulse (Fig. 5). These forms presumably represent the unglycosylated polypeptide subunit and the sequentially glycosylated forms, as five of the six putative *N*-glycosylation sites are occupied (M. Hodgson and R. J. Desnick, unpublished results). Thus, human α-GalNAc was translated so slowly that the sequential glycosylation of the enzyme was revealed in these biosynthesis studies. Consistent with this explanation, digestion of the enzyme glycoforms with PNGase F or Endo H resulted in single species of the expected molecular weights for the deglycosylated enzymes (Fig. 7).

Since the 49- and 53-kD bands had the greatest intensity, shorter pulses of 15–30 min were performed to determine which was the enzyme precursor (Fig. 6). However, both the 49- and 53-kD glycoforms were detected in similar amounts, suggesting that the 53-kD glycoform was the precursor which was processed to a slightly lower molecular weight form in the Golgi and/or lysosomes.

Biosynthesis studies also revealed that both the E325K and R329W enzyme precursors were processed to their mature forms (Figs. 5, *B* and *C*). These results differed from those of Hu et al. (15) who reported that the E325K precursor was not processed to the mature form. Their findings can be explained by the fact that cultured fibroblasts produce such low levels of the unstable precursor that any processed to the mature form may be immunologically undetectable, whereas the transiently transfected cells produce a large pulse of the unstable enzyme precursor which is rapidly (and detectably) processed to the mature form.

To assess the relative stabilities of the mutant proteins, pulse-chase labeling experiments were performed. The E325K glycopeptide had a markedly reduced stability (average $t_{1/2} \sim 11$ h) compared to that (average $t_{1/2} \sim 37$ h) of the normal α-GalNAc glycopeptide. Of interest, the R329W subunit was slightly more stable ($t_{1/2} \sim 19$ h) than the E325K glycopeptide. These studies were consistent with the concept that the more unstable E325K mutation resulted in little, if any, active enzyme, and the more severe neuroaxonal dystrophy phenotype, while the slightly more stable R329W mutation, which presumably retained some residual activity, caused the more mild phenotype.

Further investigation of the molecular basis of these remarkably distinct phenotypes will require the stable, high-level expression of the normal and mutant constructs to produce sufficient quantities of the mutant enzyme proteins for more accurate comparison of their stabilities as well as their physical and kinetic properties. Such studies may definitively determine if the E325K or R329W mutations alter enzyme processing, subunit association, the interaction with different substrates, and/or other factors in the catalytic complex.

Among the lysosomal storage diseases, the occurrence of different allelic mutations that result in severe infantile-onset and milder, latter or adult-onset forms is common. For example, patients with infantile Tay-Sachs, Gaucher type 2 or Niemann-Pick type A disease experience a severe neurodegenerative course with death by five years of age, whereas patients with the milder, later-onset forms survive into adulthood and have mild (Tay-Sachs) or no (Gaucher type 1 and Niemann-Pick type B) neurologic involvement. In each of these disorders, the infantile-onset neurodegenerative phenotypes result from mutations in the respective gene that produce no enzyme or inactive enzyme polypeptides, whereas the mutations causing the later-onset, milder forms retain a few percent or less of normal activity. In infantile Tay-Sachs disease, the most common mutations include a substitution in a consensus splicing sequence (38) and a four base pair insertion (39), both of which produce little, if any, transcript, and therefore no enzyme protein. In contrast, the adult-onset form of Tay-Sachs disease, which is characterized by a muscle wasting condition, mild intellectual loss, and a relatively normal lifespan, results from a missense mutation (G269S) that retains < 5% of normal enzymatic activity (40). Thus, a very small amount of enzymatic activity can completely alter the onset and course of the disease. Presumably, the difference between the infantile- and adult-onset forms of α-GalNAc deficiency are analogous to those of other lysosomal disorders. Although the studies reported here indicate that the adult-onset α-GalNAc mutation is more stable than that causing the infantile-onset neurodegenerative disease, further studies are required to delineate the precise molecular pathologies of these phenotypically diverse forms.

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