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Identification of the Base-Pair Substitution Responsible for a Human Acid Alpha Glucosidase Allele with Lower "Affinity" for Glycogen (GAA 2) and Transient Gene Expression in Deficient Cells

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Summary

The lysosomal enzyme termed acid alpha glucosidase (GAA), or acid maltase, is genetically polymorphic, with three alleles segregating in the normal population. The rarer GAA 2 allozyme has a lower affinity for glycogen and starch but not for lower-molecular-weight substrates. The GAA 2 allozyme can be detected by "affinity" electrophoresis in starch gel, since the lower affinity for the starch matrix results in a more rapid migration to the anode. Previously, we have isolated and sequenced the cDNA for GAA and transiently expressed the cDNA in deficient fibroblasts. In order to determine the molecular basis for the GAA 2 allozyme, we constructed a cDNA and a genomic DNA library from a GAA 2 cell line and determined the nucleotide sequence of the coding region. Only a single base-pair substitution of an A for a G at base-pair 271 was found, resulting in substitution of asparagine for aspartic acid at codon 91. This amino acid substitution is consistent with the more basic pI of the GAA 2 enzyme. The base-pair substitution also abolishes a *Taq*-I site, predicting the generation of a larger DNA fragment. This larger *Taq*-I fragment was also seen in two other individuals expressing the GAA 2 allozyme. A 5′ fragment containing the base-pair substitution was ligated to the remaining 3′ cDNA from a GAA 1 allele and cloned into an expression vector, and the hybrid cDNA was transiently expressed in SV40-transformed GAA-deficient fibroblasts. The enzyme activity exhibited the altered mobility of the GAA 2 allozyme, as demonstrated by electrophoresis in starch gel. The base-pair substitution is located in a 53-amino-acid fragment apparently cleaved during proteolytic processing to the mature enzyme. Therefore, these studies indicate that this area must be maintained in the mature enzymatically active protein, and previous conclusions from studies of processing and degradation must be reexamined.

Introduction

Human acid maltase, or acid alpha glucosidase (GAA), is a lysosomal enzyme which hydrolyzes glycogen and maltose to glucose (Hers 1963; Brown and Brown 1981). The GAA locus has been localized to human chromosome 17 (17q21-q23) Martiniuk et al. 1985*a*). Deficiency of GAA results in glycogen storage disease type II (Pompe 1932; Courtecuissf et al. 1965; Engel et al. 1973 Mehler and DiMauro 1977; Beratis et al. 1978, 1983; for review, see Hers et al. 1989). This disorder is both clinically and genetically heterogeneous. Clinically, the syndrome ranges from a fatal infantile (Pompe) disease to a late-onset form. At least 10 different mutations can be inferred by analysis of synthesis, processing, or turnover of the protein in related patients with the same and/or different phenotypes (Reuser et al. 1985, 1987). We have previously isolated and sequenced the cDNA for human GAA (Martiniuk et al. 1986, 1990*b;* Hoefsloot et al. 1988). The coding region contains 2,856 bp predicting 952 amino acids. More recent studies of the the gross structure of mRNA and DNA from affected patients have revealed extensive heterogeneity, with half of the infantile and adult patients having abnormali-

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ties (Hirschhorn et al. 1989; van der Ploeg et al. 1989; Martiniuk et al. 1990*a*). Recently, transient gene expression of the GAA cDNA and isolation of the promoter segment have been demonstrated (Tzall et al., in press).

GAA exhibits a genetic polymorphism, with three alleles (GAA 1, GAA 2, and GAA 4) segregating in a normal population (Swallow et al. 1975; Nickel and McAlpine 1982). The GAA 2 allozyme can be identified by starch-gel electrophoresis, since the enzyme has less "affinity" for the support-matrix starch and thus migrates more rapidly to the anode despite its basic pI (Swallow et al. 1975). The GAA 2 allozyme also has a higher K_m for glycogen, with normal values for maltose or the artificial substrate 4-methylumbelliferyl-alpha-D-glucoside (4-MU-Glc) (Swallow et al. 1975; Beratis et al. 1980). The GAA 1 allozyme has been purified to homogeneity by "affinity" chromatography utilizing the glucosidic matrix of Sephadex G100 and elution with maltose (deBarsy et al. 1972; Martiniuk et al. 1984). The GAA 2 enzyme does not bind substantially to the Sephadex G100 matrix, because of less affinity (Swallow et al. 1975). Immunoblot analysis of extracts from GAA 2 cell lines has not detected any gross alterations in processing or turnover, as compared with the GAA 1 allozyme (Swallow et al. 1989).

In order to determine the molecular basis of the GAA 2 allozyme, we have determined the nucleotide sequence of the cDNA for the GAA 2 allele and have identified the base-pair substitution responsible for the polymorphism. In addition, a hybrid cDNA was constructed by ligating a 5′ fragment containing the base-pair change to the remaining 3′ GAA 1 cDNA and by subcloning in the expression vector, pSV-2. Transient gene expression with this hybrid cDNA in SV40-transformed GAA-deficient fibroblasts changed the mobility of the GAA 1 allozyme to that of a GAA 2 allozyme.

Material and Methods

Cell Lines, DNA, and RNA Extraction

The GAA 2 cell lines, DC118 and H.M., were gifts of Dr. D. Swallow and were maintained in 20% FBS, Dulbecco's minimal essential medium with penicillin and streptomycin. DNA and RNA were extracted according to a method described elsewhere (Wigler et al. 1978; Martiniuk et al. 1985*b*). The GAA 2-1 cell line 3905 was a gift of Drs. G. LaBadie and K. Hirschhorn.

Construction and Screening of DNA and cDNA Libraries

A cDNA library from DC118 was synthesized using the Boehringer Mannheim cDNA construction kit (1013882). The previously isolated 1.9-kb GAA cDNA and more 5′ cDNA fragments (Martiniuk, et al. 1986, 1990*b*) were used to screen the cDNA library. The recombinant phage and DNA were isolated by standard procedures (Maniatis et al. 1982) and were subcloned into M13 and/or pUC19 for sequencing.

The 5′ region containing the initiation codon (ATG) was isolated utilizing a 5′ cDNA fragment (bp 24–427 to screen an *Eco*RI [9–25-kb] DC118 genomic DNA library constructed in lambda DASH [Stratagene, LaJolla]). *Sac*I fragments were subcloned into pUC19 and M13 for sequencing.

Sequencing

Sequencing of the coding and 3′ untranslated region was performed after subcloning various-sized cDNAs (and GAA genomic fragments) into M13mp19 and/or pUC19 and using the forward or reverse universal primer or GAA-specific primers by the Sanger dideoxy chain-termination method (Sanger et al. 1977) with Sequenase (U.S. Biochemicals).

Construction of Hybrid cDNA

The 5′ coding sequence containing the single base-pair change at 271 was initially digested at the *Nco*I site of the ATG (bp 1), followed by addition of *Hin*dIII linkers (Boehringer Mannheim 729906), which maintains the *Nco*I site after ligation of the linker. Digestion with *Sst*II at bp 350 releases the fragment for ligation to a GAA 1 cDNA digested at the same *Sst*II site (Martiniuk et al. 1990*b;* Tzall et al., in press). The full-length hybrid cDNA was ligated into the *Hin*dIII site of the expression vector pSV-2 (Orkin et al. 1985) and was sequenced through the ligation and polymorphic site.

Transient Gene Expression

SV40-transformed GAA-deficient fibroblasts were derived from GM4912 (NIH Human Genetic Mutant Cell Repository, Camden, NJ), a cell line with no mRNA or enzyme activity for human GAA. Transient gene expression was performed according to a method described elsewhere (Wigler et al. 1978; Martiniuk et al. 1985*b*), with 0.4×10^6 cells/100-mm petri dish 24 h before addition of 40 μg calcium phosphate–precipitated DNA from either the pSV-2 × hybrid cDNA or the pSV-2 × GAA 1 cDNA. Cells were harvested 48 h after addition of DNA, as described below.

Enzyme Assay and Starch-Gel Electrophoresis

Fresh cells were harvested with a rubber policeman,

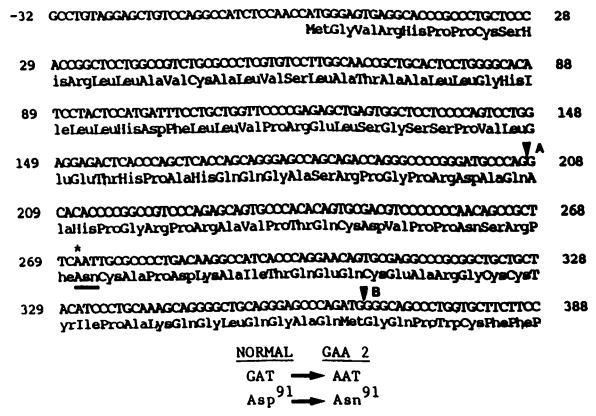

Figure 1 Location of base-pair change in the GAA 2 allozyme, relative to cleavage sites in the GAA protein: composite nucleotide sequence of the 5′ coding region obtained from the GAA 2 (DC118) cDNA and genomic DNA libraries. The A of the initiation codon (ATG) is designated as bp 1, and the amino acid sequence is indicated below. The G at base pair 271 was substituted by an A in the GAA 2 allele, as indicated by the asterisk over the A, resulting in a substitution of Asn for Asp at codon 91. Arrowheads indicate the sites of cleavage during processing as determined by sequencing of the N-terminal amino acids of the 110-kD protein (A) and 76-kD proteins (B) (codons 70 and 123, respectively) (Hoefsloot et al. 1988).

washed by centrifugation with PBS, lysed by sonication, and assayed with 4-MU-Glc according to a method reported elsewhere (Martiniuk and Hirschhorn 1981). Electrophoresis in starch gel and staining for GAA were according to a method described elsewhere (Martiniuk and Hirschhorn 1981).

Digestion of DNA and Southern Analysis

DNA was digested with restriction enzymes according to the manufacturer's directions. DNA was electrophoresed in 0.8% agarose gels, transferred to nitrocellulose by the method of Southern (1975), and hybridized with ^{32}P randomly primed probes by standard methods described elsewhere (Feinberg and Vogelstein 1983; Martiniuk et al. 1986).

Results

Nucleotide Sequence of the Coding Region

In order to determine the molecular basis of the GAA 2 polymorphism, we constructed a cDNA library from the GAA 2 dermoid cyst cell line DC118 in lambda gt11. Various-length cDNAs were obtained, subcloned, and sequenced. Almost all the coding region was sequenced with these cDNA fragments. The remaining areas were obtained by sequencing relevant areas from *Sac*I genomic fragments isolated from the GAA 2 genomic library. The resulting sequencing begins from −32 and proceeds to 3409 bp, with the ATG designated as bp 1. The sequence was identical to the previously reported GAA sequence (Martiniuk et al. 1990*b*), except at bp 271, which had an A substituting for the previously reported G (fig. 1). This base-pair substitution results in loss of a *Taq*I site and in a change in amino acid codon 91 from an aspartic acid to asparagine. In addition, this base-pair substitution was observed in independent clones from both the unamplified cDNA library and the genomic library, thereby eliminating the possibility of a cloning artifact. Finally, at all the base-pair polymorphisms we have defined (Martiniuk et al. 1990*b*), the GAA 2 allele had the most common nucleotide (data not shown).

Transient Gene Expression and Analysis by Starch-Gel Electrophoresis

To determine whether this base-pair substitution at 271 (a G to an A) was responsible for the GAA 2 polymorphism, we constructed a hybrid cDNA, by ligating to the 3′ end of a GAA 1 allele a 350-bp 5′ segment of the GAA 2 allele containing the G-to-A change at bp 271, and then subcloned the hybrid cDNA into the expression vector pSV-2. SV40-transformed GAA-deficient fibroblasts were transiently transfected with this plasmid, harvested 48 h later and assayed for GAA activity (table 1). Cells transfected with the hybrid cDNA showed 5.3% (0.12 vs. 2.6 U/g) of normal fibroblast enzyme activity, while extracts from untransfected cells had no detectable activity. Cells transfected with the GAA 1 allele expressed GAA activity almost identical to that expressed by the GAA 2 allele (0.10 vs. 0.12 U/g).

In order to prove that the GAA enzyme activity observed after transient gene expression with the hybrid cDNA was indeed due to the GAA 2 protein, we electrophoresed extracts from transfected cells in starch gel and stained them for enzyme activity with 4-MU-Glc (fig. 2). An extract from a GAA 2 (DC118) cell line (lane 3) showed a band of enzyme activity migrating rapidly to the anode because of less affinity for the starch matrix used for electrophoresis. An extract of cells from a GAA 1 cell line (lane 4) showed an enzyme band slowly migrating to the anode because of binding to the starch matrix. Extracts from the SV4O-transformed GM 4912 transiently transfected with the hybrid cDNA (lane 1) showed a fainter band migrating rapidly to the anode, comigrating with the GAA 2 extract in lane 3. An extract of cells from the SV4O-transformed GAA-deficient

Table 1

GAA Enzyme Activity

	Mean ± SD GAA Activity (μmol 4-MU-Glc hydrolyzed/min/g protein, at 37°C)	% of Normal
Normal fibroblast GM 08399	2.26 ± .25 ($n=4$)	100.0
SV40-transformed GM 4912	<.003	<.0
SV40-transformed GM 4912 transiently transfected with hybrid cDNA	.12 ± .021 ($n=5$)	5.3
SV40-transformed GM 4912 transiently transfected with GAA 1 cDNA	.10 ± .059 ($n=9$)	4.6

cell line (lane 2) showed no enzyme activity. Thus, the GAA 2 allozyme had the expected increased anodal mobility, as compared with the GAA 1 allozyme.

Analysis of DNA

The base-pair substitution also predicts the loss of a *Taq*I site. On the basis of sequencing of GAA genomic fragments (unpublished data), loss of the *Taq*I site should result in the appearance of an approximately 1.0-kb fragment from two smaller fragments, as detected by a probe from bp 23 to bp 425 (Martiniuk et al. 1990*b*). In order to determine whether other cell lines that express the GAA 2 allozyme contain the same base-pair change resulting in the loss of a *Taq*I site, we digested DNA with *Taq*I, electrophoresed and transferred it to nitrocellulose, and hybridized it with a 5′ cDNA probe. Two cell lines (DC118 and HM) phenotypically homozygous for the GAA 2 allozyme exhibited the larger *Taq*I fragment while a cell line heterozygous for the GAA 2 allozyme exhibited the larger, 1.0-kb fragment together with a smaller fragment (data not shown).

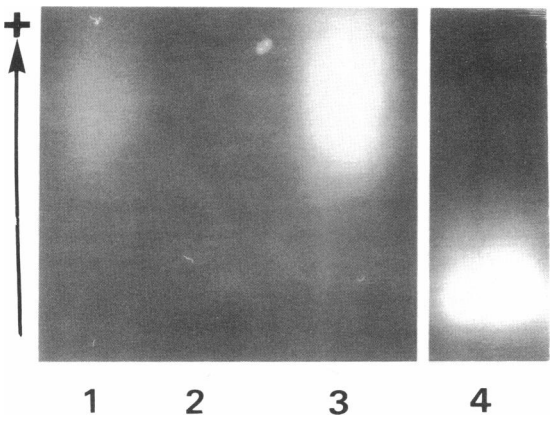

Figure 2 Composite photograph of starch-gel electrophoresis after staining for GAA with 4-MU-Glc. Lane 1, Extract of the SV40-transformed GAA-deficient cell line GM 4912 transiently transfected with the hybrid cDNA and showing a faster-migrating enzyme band, comigrating with the extract of the GAA 2 in lane 3. Lane 2, Extract of the SV40-transformed GAA-deficient cell line GM 4912 showing no enzyme activity. Lane 3, Extract of a GAA 2 cell line DC 118 showing a fluorescent band of enzyme activity rapidly migrating to the anode. Lane 4, Extract of the GAA 1 (GM7345) cell line showing a slowly migrating band of enzyme activity.

Discussion

In order to determine the molecular basis for the GAA 2 polymorphism, we sequenced the complete coding region from a GAA 2 cell line. Only a single base-pair difference could be identified from the previously reported consensus sequence (Martiniuk et al. 1990*b*), with an A substituting for a G at bp 271. This substitution changes the amino acid codon from an aspartic acid to an asparagine. In addition, we demonstrated, by construction of a hybrid cDNA with the GAA 1 cDNA, that this base-pair substitution was responsible for the GAA 2 allozyme. This hybrid cDNA transiently expressed in SV40-transformed GAA-deficient fibroblasts and had the characteristics of a GAA 2 allozyme, as demonstrated by starch-gel electrophoresis. The substitution of an A for a G occurs at a CpG dimer which has been shown to be a "hot spot" for mutations (Barker et al. 1984; Cooper and Youssoufian 1988). This substitution also resulted in loss of a *Taq*I site and thus in the appearance of a larger restriction fragment. It is possible that other amino acid substitutions could give rise to the phenotype of the GAA 2 allozyme. However, the loss of a *Taq*I site and the generation of a larger fragment in other cell lines expressing the GAA 2 allozyme suggest that the GAA 2 allozyme is genetically homogeneous. To the best of our knowledge this is the

first identification, by an RFLP, of a classical biochemical protein polymorphism.

The location of the base-pair substitution responsible for the GAA 2 allozyme was somewhat unexpected, since this area is proteolytically cleaved during processing. The enzyme is synthesized as an approximately 105-kD protein that is glycosylated, phosphorylated, and proteolytically processed. Pulse-chase experiments have indicated that approximately 110-kD glycosylated species are cleaved to approximately 76- and 70-kD proteins and smaller species (25–20 kD) (Hasilik and Neufeld 1980; Kornfeld 1986; Reuser et al. 1985). Enzymatically active, purified GAA exhibits all the fully processed/degraded forms even in the absence of reducing agents, with the 76- and 70-kD proteins as the most abundant species (Martiniuk et al. 1984). Amino acid sequencing of the 110-, 76-, and 70-kD species has shown that the N-termini are located at codons 70, 123, and 204, respectively (Hoefsloot et al. 1988). Since the amino acid change found in the GAA 2 allozyme is located in codon 91, this 5′ N-terminal fragment must remain attached to the mature enzymatically active protein. The fragment predicted to contain the GAA 2 base-pair change spans codons 70–122 (53 amino acids) and has a predicted size of 6.677 kD. This fragment contains five cysteines, and thus it is possible that it remains attached by disulfide bonds—as is the case for beta-hexosaminidase (Quon et al. 1989)—but might not have been detected by SDS-PAGE (tube gels) (Martiniuk et al. 1984). The change in amino acid from an aspartate to an asparagine at codon 91 could result in a larger protein, because of a block in processing or degradation. However, Swallow et al. (1989) have shown by immunoblot analysis that processing of the enzyme in this GAA 2 cell line is identical to that of the GAA 1 allozyme in that the mature proteins are not grossly different in size from each other. On the basis of the current molecular analysis of the GAA 2 allozyme, we now suggest that the N-terminal fragment encompassing codon 91 (either the previously identified 20–25-kD fragment or a smaller, previously undetected peptide) remains associated, in the native state, with the larger processed forms and is an integral component of the active enzyme.

Recently, Barnes and Wynn (1988) have made predictions, on the basis of comparison with human/rabbit isomaltase, of two potential binding sites for glycogen for GAA. While one of these predictions was based on incorrect sequencing (Hoefsloot et al. 1988; Martiniuk et al. 1990*b*), the base-pair substitution found for the GAA 2 allele is not located in the remaining predicted area. Alternatively, the amino acid substitution from the charged aspartate to the polar asparagine may not be located at the actual binding site but could result in folding alterations which could ultimately decrease the affinity for glycogen. Although asparagine is also a potential site for the transfer of the N-linked oligosaccharide in glycosylation of the protein, the asparagine is not in the required triplet sequence of Asn-X-Ser/Thr (Sabatini and Adesnick 1989). Further studies of protein synthesis, degradation, and turnover, combined with molecular and biochemical analysis, may lead to a better understanding of the structure-function of GAA and other lysosomal proteins.

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