Characterization of three alleles causing aspartylglycosaminuria: two from a British family and one from an American patient

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Aspartylglycosaminuria (AGU) is a lysosomal storage disease principally occurring in Finland that results from mutations in the structural gene for glycosylasparaginase (*AGU*). This work characterizes the inheritance of two previously reported *AGU* mutations in a British patient [Ikonen, Aula, Grön, Tollersrud, Halila, Manninen, Syvänen and Peltonen (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11222–11226]. Use of the PCR determined the glycosylasparaginase cDNA sequence from both parents of the British patient and his AGU-affected brother. The father of the British AGU-affected siblings was found to be a heterozygote carrier for a C → T point mutation which causes an Ala → Val amino-acid substitution, while the mother was heterozygous for a 7 bp deletion that results in premature translational termination. The brother of the previously studied patient was similarly shown to be a compound heterozygote. Expression in COS-1

cells revealed the paternal Ala → Val amino-acid substitution destroyed glycosylasparaginase catalytic activity, prevented transport of the mutant protein to the lysosome, and prevented maturation of the enzyme precursor to its native subunit structure. The Ala → Val mutation therefore affects glycosylasparaginase in a manner similar to the Finnish AGU Cys → Ser substitution, further supporting a linkage of glycosylasparaginase catalytic activity to its lysosomal transport and subunit processing [Fisher and Aronson (1991) *J. Biol. Chem.* **266**, 12105–12113]. In addition, a 5 bp deletion mutation from an American patient with AGU has been characterized. The deleted sequence occurs at the beginning of the glycosylasparaginase coding sequence, resulting in an extremely truncated polypeptide. The American 5 bp deletion and the British maternal 7 bp deletion possibly decrease mRNA stability.

INTRODUCTION

Aspartylglycosaminuria (aspartylglucosaminuria, AGU) is a rare inherited disorder of asparagine-linked glycoprotein catabolism (Jenner and Pollitt, 1967; Pollitt et al., 1968). Lysosomal glycosylasparaginase (EC 3.5.1.26), the deficient enzyme in AGU, is responsible for hydrolysing the amide bond of the GlcNAc-Asn carbohydrate-to-protein linkage (Aronson and Kuranda, 1989). Absence of glycosylasparaginase activity in AGU-affected cells allows aspartylglucosamine to accumulate as the major end-product of glycoprotein degradation within lysosomes. The large quantities of stored aspartylglucosamine and related minor fragments dramatically alter cell morphology and ultimately retard the physical and mental development of patients with AGU (Durand and O'Brien, 1982; Beaudet, 1983). Since the first report of AGU in two British siblings (Jenner and Pollitt, 1967; Pollitt et al., 1968), over 200 cases have been documented, with all but a few being individuals of Finnish descent (Palo and Mattsson, 1970; Autio, 1972; Autio et al., 1973). The estimated occurrence of AGU in Finland is 1:3600 with the carrier frequency predicted to be as high as 1:30 (Mononen et al., 1991a). Besides the original two British cases there have since been other reports of AGU in ethnically diverse patients outside Finland (Gehler et al., 1981; Hreidarsson et al., 1983); however, the current number is less than 15.

As a first step to studying the molecular genetics of AGU, the human glycosylasparaginase cDNA sequence was determined by our laboratory (Fisher et al., 1990). The cDNA encodes a 46 kDa precursor polypeptide that is post-translationally processed to a heavy (24 kDa) and light (18 kDa) subunit. (The 24 kDa subunit

of human glycosylasparaginase has most often appeared in the literature as α or heavy, while the 18 kDa subunit has been referred to as β or light. In an effort to establish consistency in the literature, we are adopting the usage of heavy and light to describe the subunits of glycosylasparaginase in this and subsequent publications.) We have also characterized the human glycosylasparaginase gene (*AGU*) and found it to be 13 kb with the open reading frame distributed among nine exons (Park et al., 1991). The most refined localization of the human gene to chromosome 4q32-q33 has been done recently by *in situ* hybridization (Morris et al., 1992). Using the collective cDNA and genomic *AGU* sequence information, the high frequency of AGU in Finland has been shown to be due to a single point mutation that causes a Cys-163 → Ser substitution in the heavy subunit (Ikonen et al., 1991a; Mononen et al., 1991b; Fisher and Aronson, 1991a). Expression studies in COS-1 cells indicate the loss of Cys-163 not only causes enzymic inactivation of glycosylasparaginase, but also prevents the precursor polypeptide from being both correctly transported to the lysosome and processed to its mature subunits (Fisher and Aronson, 1991a,b). The loss of lysosomal transport, subunit processing, and enzyme activity caused by the Finnish Cys-163 → Ser substitution led us to propose that the three events are interrelated, with subunit maturation being a required step for glycosylasparaginase activity (Fisher and Aronson, 1991a,b). Interestingly, Thr-206, which post-translationally becomes the N-terminal residue of the light subunit, has been implicated as an active-site residue since it forms a covalent linkage with an active-site-directed inhibitor of glycosylasparaginase (Kaartinen et al., 1991). Subunit processing possibly transfers Thr-206 from an internal location within the precursor polypeptide to an N-terminal position in the mature

Abbreviations used: AGU, aspartylglycosaminuria; *AGU*, glycosylasparaginase gene; ER, endoplasmic reticulum.

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enzyme that might optimally align the active site. A similar theme connecting enzyme activity, subunit processing, and lysosomal transport was also reported by our laboratory for the mutant glycosylasparaginase in an African-American AGU patient (Fisher and Aronson, 1991b). This patient has a GT → TT point mutation in the 5' splice site of intron 8 that results in both the deletion of exon 8 during pre-mRNA splicing and premature translational termination. The intron 8 splice-site mutation additionally affects the splicing of upstream introns resulting in at least two alternative glycosylasparaginase messages (Fisher and Aronson, 1991a).

At the time this manuscript was being prepared, Ikonen et al. (1991b) reported candidate mutations from a collection of non-Finnish AGU cell lines. Among them was a British patient (M.A.) who was proposed to be a compound heterozygote. Both AGU allele mutations occur in the region which encodes the heavy subunit, one having a 7 bp deletion and the second a point

mutation (Chomczynski and Sacchi, 1987). Genomic DNA was purified by the SDS/proteinase K/phenol/chloroform extraction method (Maniatis et al., 1982).

PCR amplification of cDNA and genomic DNA

Two sets of oligonucleotide primers (all primers were synthesized by the Pennsylvania State Biotechnology Institute, University Park, PA, U.S.A.), designated A and B, were used to amplify the glycosylasparaginase cDNA sequence encoded by AGU patients and carriers. The primers were designed such that they would generate overlapping fragments that together would span the entire glycosylasparaginase mature protein-coding region. The selected primer sequences are shown below with their 5' and 3'-ends numbered in parentheses according to the human placenta cDNA sequence where +1 is A of the translational initiation codon ATG (Fisher et al., 1990):

Set A sense-strand primer	5'-(10)AAGTCGAACCTGCTGTGCT(29)-3'
antisense-strand primer	5'-(523)GATCTGGTATAACATTCTC(504)-3'
Set B sense-strand primer	5'-(448)GCTTCTCAAGCTCTTCATT(467)-3'
antisense-strand primer	5'-(1065)ACAGATGTTGACAGTAAAGA(1046)-3'

mutation causing an Ala → Val amino-acid substitution. While the negative impact of the 7 bp deletion on glycosylasparaginase activity is predictable due to its effect on the open reading frame, the detriment caused by the Ala → Val mutation is less obvious. In the absence of expression studies the possibility of the Ala → Val substitution being a neutral polymorphism cannot be ruled

A third set of oligonucleotide primers, designated C, was designed to amplify the AGU genomic sequence surrounding a 5 bp deletion mutation that was discovered in exon 3 of patient TC79-886. The sense-strand primer was against sequence for exon 3 of the human AGU gene while the antisense-strand primer was against sequence from intron 3 (Park et al., 1991). The sequences of these primers are shown below:

Set C sense-strand primer (exon 3)	5'-(305)TAGGAGATCTCAGACGAATT(324)-3'
antisense-strand primer (intron3)	5'-AGCGAACTCCGTCTCAA-3'

out. Indeed, an Arg → Gln neutral polymorphism occurs along with the Cys → Ser AGU mutation in Finnish patients (Fisher and Aronson, 1991a). We now report the inheritance of the two mutated AGU alleles discovered in the British patient (M.A.) by examination of the glycosylasparaginase sequence from his birth mother (P.A.) and father (D.A.), as well as his AGU-afflicted brother (J.A.). Expression of the Ala → Val mutation in COS-1 cells has also been performed to determine the contribution of this allele in causing glycosylasparaginase deficiency. In addition to our further characterization of the mutations that have caused AGU in this British family, we also describe for the first time a 5 bp deletion in the AGU gene of an American patient with AGU. Clinical observations on the latter AGU case was reported previously by Hreidarsson et al. (1983).

EXPERIMENTAL

Cell culture and nucleic acid extraction

A primary fibroblast culture (TC79-886) from an American patient with AGU (Hreidarsson et al., 1983) was obtained from Dr. George H. Thomas (Kennedy Institute, Johns Hopkins University, Baltimore, MD, U.S.A.). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and maintained in a humidified atmosphere of 5% carbon dioxide. Total RNA was isolated from fibroblasts by the acid guanidium thiocyanate/phenol/chloroform extraction

method (Chomczynski and Sacchi, 1987). Genomic DNA was purified by the SDS/proteinase K/phenol/chloroform extraction method (Maniatis et al., 1982).

PCR reactions were performed as described previously using the GeneAmp PCR reagent kit from Perkin-Elmer Cetus Instruments (Fisher and Aronson, 1991a). The PCR template was either first-strand cDNA prepared from random hexanucleotide-primed RNA (primer sets A and B) or genomic DNA (primer set C). Amplified DNA fragments were resolved on an agarose gel, purified by electroelution into dialysis tubing, and sequenced either directly (Kusukawa et al., 1990) or subsequent to M13mp18/19 cloning. Sequence determination was done using a Sequenase Version 2.0 DNA sequencing kit as recommended by the manufacturer (United States Biochemical).

Northern-blot analysis

Approx. 20 µg of total RNA from AGU patient TC79-886 or control fibroblasts was resolved on a 1.2% (w/v) agarose/2.2% (w/v) formaldehyde gel. RNA was electroblotted from the gel on to a nylon membrane and the resulting blot hybridized with the full-length human placental glycosylasparaginase cDNA (Fisher et al., 1990) random-primed labelled with [5'-³²P]dCTP to a specific activity of approx. 10⁹ c.p.m./µg of cDNA. Pre-hybridization, hybridization, and wash conditions were as described previously (Fisher and Aronson, 1991a). An autoradiogram of the Northern blot was scanned with a densitometer to determine the relative amount of glycosylasparaginase mRNA expressed in AGU-affected TC79-886 fibroblasts compared with normal fibroblasts. The values for AGU and control samples were standardized to 28 S rRNA.

Expression in COS-1 cells

The full-length glycosylasparaginase cDNA sequence containing the British paternal *AGU* mutation (*AGU*^{Ala→Val}) was sub-cloned into the expression vector pSVL (Pharmacia LKB Biotechnology Inc.). The resulting construct, pSVL/*AGU*^{Ala→Val}, was transfected into COS-1 cells by the DEAE-dextran method (Cullen, 1987). A pSVL construct containing the normal human placental glycosylasparaginase cDNA, HPAsn.6 (pSVL/HPAsn.6), was also used in this study (Fisher and Aronson, 1991a). Three separate transfections of each vector were performed. Cell lysates of test or mock-transfected cells were prepared by sonication at 48–72 h post-transfection, assayed for glycosylasparaginase activity and total protein, and analysed by Western blotting as described elsewhere (Fisher and Aronson, 1991a). Immunofluorescent localization of transfected COS-1 cells was as described previously (Fisher and Aronson, 1991b).

RESULTS

American *AGU* patient TC79-886 contains a 5 bp deletion in the *AGU* open reading frame

Two glycosylasparaginase mRNAs of 1.5 kb and 2.3 kb have previously been shown to be expressed in human fibroblasts and are believed to arise from the utilization of alternative polyadenylation sites (Park et al., 1991; Fisher and Aronson, 1991a). When total RNA from American *AGU* patient TC79-886 fibroblasts was subjected to Northern-blot analysis, the 1.5 kb and 2.3 kb glycosylasparaginase messages were evident, but they exhibited a broadened banding pattern compared with an RNA sample from a normal fibroblast culture (Figure 1). In addition, the combined quantity of glycosylasparaginase mRNA expressed by *AGU* patient TC79-886 was found to be approx. 20% of normal after standardization against 28S rRNA. The smeared appearance of the glycosylasparaginase mRNAs from TC79-886 cells cannot be attributed to the quality of RNA that was isolated from this patient with *AGU* since a second preparation of RNA revealed similar results (results not shown).

DNA-sequence analysis of the PCR products from *AGU* patient TC79-886 generated by primer sets A and B revealed a 5 bp deletion (ACACA) spanning bp positions 367–371 of the human cDNA sequence (Figure 2a). The 5 bp deletion causes a shift in the *AGU* open reading frame, thus resulting in premature translational termination (Figure 2c). No additional discrepancies compared with the normal cDNA were observed. Genomic DNA was isolated from TC79-886 fibroblasts and used with oligonucleotide primer set C (see the Experimental section) to PCR amplify the region of exon 3 that contains the 5 bp deletion, as well as a portion of intron 3. Direct sequencing of the 300 bp genomic product again revealed the 5 bp deletion (Figure 2b). The entire population of genomic amplified material appeared to contain the 5 bp deletion since there was no evidence of overlapping normal sequence.

British *AGU* patients J.A. and M.A. are compound heterozygotes

Oligonucleotide primer sets A and B were also used to amplify first-strand cDNA prepared from RNA that was isolated from fibroblast cultures of the mother (P.A.) and father (D.A.) of two British *AGU*-affected male siblings, J.A. and M.A. DNA sequencing of M13 clones containing the primer-set-A PCR product from the parents P.A. or D.A. indicated that both are heterozygotic carriers for two different mutations (Figure 3a): a paternal C→T transition to bp position 302 (C-302→T)

Figure 1 Northern-blot analysis of RNA from fibroblasts of *AGU* patient TC79-886

Total RNA from a normal fibroblast cell line and fibroblasts from American *AGU* patient TC79-886 was resolved on a 1.2% (w/v) agarose/2.2% (w/v) formaldehyde gel, blotted on to a nylon membrane and hybridized with a human placental glycosylasparaginase cDNA (Fisher et al., 1990).

which causes an Ala-101→Val amino-acid substitution, and a maternal 7 bp deletion at the 5'-end of the heavy subunit, which causes a shift in reading frame and premature translational termination. The precise sequence that is deleted in the maternal allele is one of three possibilities due to the nature of the surrounding sequence. They are (99)TTGGCCC(105), (100)TGGCCCT(106), and (101)GGCCCTT(107), where the bp positions are given in parentheses (Figures 3a and 3c). No mutations were found upon sequencing the primer-set-B PCR product from either father or mother (results not shown), and therefore the C-302→T transition and the 7 bp deletion are the only changes in parental glycosylasparaginase mRNAs.

Direct sequencing of the PCR product generated with primer set A from D.A. demonstrated the paternal heterozygous genotype for the C-302→T allele (Figure 3b). The resulting sequencing gel revealed two populations of *AGU* sequences, one containing the normal cytosine nucleotide at bp position 302 and a second with the mutant thymine nucleotide. Direct sequencing was not performed with the primer-set-A PCR product from the mother P.A. It is of interest that the frequency of M13 clones that carried the maternal 7-bp-deletion allele was much lower than the normal allele. For instance, only one of 12 M13 clones of the primer-set-A product from P.A. that were sequenced contained the 7-bp-deletion mutation while the remaining 11 were normal. This unequal occurrence of mutant versus normal alleles was not noted for the paternal C-302→T base change.

Based on the results from P.A. and D.A., it was predicted that their two *AGU*-affected offspring J.A. and M.A. were compound heterozygotes for the 7 bp deletion and the C-302→T point mutation. Indeed, sequence analysis of the primer-set-A and primer-set-B PCR products from the two probands revealed the presence of both mutant alleles on different DNAs, which is thus consistent with Mendelian inheritance of genes (results not shown). The number of M13 clones that were sequenced from M.A. and J.A. which carried the *AGU*^{Δ7bp} allele was far less represented compared with the *AGU*^{C-302→T} allele, an observation which is similar to the low frequency of M13/*AGU*^{Δ7bp} clones that were sequenced from P.A. The heterozygous genotype of M.A. for the *AGU*^{C-302→T} allele was shown by direct sequencing of the primer-set-A PCR product (Figure 3b).

Expression of the paternal British C-302→T transition and immunofluorescent cell staining

To determine the consequence of the British paternal

Figure 2 Sequence analysis of a 5 bp deletion mutation found in the *AGU* sequence from AGU-affected TC79-886 fibroblasts

(a) The sequence from the normal human placental glycosylasparaginase cDNA HPA_{sn}.6 (Fisher et al., 1990) is shown on the left and the sequence from TC79-886 is shown on the right. The deleted 5 bp sequence in TC79-886 (ACACA) is boxed. (b) Direct sequencing of the primer-set-C (see the Experimental section) PCR product that was amplified from genomic DNA from AGU patient TC79-886. (c) Partial alignment of the normal and TC79-886 AGU sequences showing the shift in open reading frame and premature termination of translation caused by the *AGU* 5 bp deletion. The premature translational stop codon is indicated by asterisks.

AGU^{Ala → Val} allele, COS-1 cells were transfected with a pSVL expression vector construct containing either the normal human placental glycosylasparaginase cDNA (pSVL/HPA_{sn}.6) or the mutant glycosylasparaginase cDNA (pSVL/*AGU*^{Ala → Val}). Cells transfected with the normal glycosylasparaginase cDNA had a 15-fold increase in GlcNAc-Asn cleaving activity over that of mock-transfected cells (Figure 4). In addition, Western-blot analysis revealed that the expressed protein was correctly processed to the heavy and light subunits that are known to compose the mature active glycosylasparaginase enzyme (Figure 4) (Fisher et al., 1990; Fisher and Aronson, 1991a). The 27 kDa and 24 kDa bands that were detected with antibodies raised against the heavy subunit are different proteolytic forms (H. Park, unpublished work). In marked contrast with cells expressing normal *AGU*, COS-1 cells transfected with pSVL/*AGU*^{Ala → Val} displayed no increase in GlcNAc-Asn cleaving activity or the processed heavy and light subunits (Figure 4). Instead, the 46 kDa glycosylasparaginase precursor polypeptide was immunologically detected with both heavy- and light-subunit-specific antibodies (Figure 4). Only a minor amount of the 46 kDa uncleaved precursor was evident in cells transfected with pSVL/HPA_{sn}.6.

Immunofluorescent staining of COS-1 cells expressing the normal recombinant glycosylasparaginase protein revealed a punctate staining pattern that is typical for lysosomal localization (Figure 5a) (Van Dongen et al., 1984; Hoefsloot et al., 1990). This unique distribution of label was not evident in cells expressing the *AGU*^{Ala → Val} mutant glycosylasparaginase protein. Staining in these cells was intensely perinuclear with diffusion into the cytoplasm, a pattern which has been shown to be associated with endoplasmic reticulum (ER) localization (Van Dongen et al., 1984; Hoefsloot et al., 1990). Collectively, these results strongly suggest that the British paternal Ala → Val substitution abolishes glycosylasparaginase activity, prevents preteolytic

processing of the precursor protein to mature heavy and light subunits, and prevents lysosomal targeting.

DISCUSSION

Patient TC79-886 is the third reported AGU case in the U.S.A. (Hreidarsson et al., 1983). We have identified the molecular defect that causes AGU in patient TC79-886 to be a 5 bp deletion within the glycosylasparaginase-protein-coding region (Figure 2). The primary effect of this mutation is a shift in reading frame that results in premature termination of translation. The predicted glycosylasparaginase product from TC79-886 is a truncated polypeptide of 118 amino-acid residues (13 kDa), after removal of the signal peptide, with the C-terminal 19 residues encoding an out-of-frame non-glycosylasparaginase protein segment (Figure 2c). In terms of the native mature glycosylasparaginase structure, this mutant protein comprises only 67% of the heavy subunit and lacks a light subunit. We expressed the protein product of *AGU*^{Δ7bp} from patient TC79-886 in COS-1 cells and although a 13 kDa peptide was evident by Western blotting with heavy-subunit-specific antibodies, the level of expression was extremely low (results not shown). This might reflect increased susceptibility of the mutant protein to proteolytic degradation *in vivo* or a reduced antigenicity towards the heavy-subunit antibodies. Enzyme assays conducted on the transfected COS-1 cell extract indicated that the truncated 13 kDa protein was not catalytically active (results not shown) as would be expected, since there is a loss of the entire light subunit whose N-terminal Thr-206 has been proposed to be an important active-site residue (Kaartinen et al., 1991).

A possible secondary outcome of the American *AGU*^{Δ7bp} mutation is that it reduces the stability of the transcribed mRNA. A Northern blot of RNA from patient TC79-886 revealed glycosylasparaginase mRNAs with a broad, smeared appearance

Figure 3 Inheritance of two alleles causing AGU in a British family

(a) The glycosylasparaginase cDNA sequence from the mother (P.A.) and father (D.A.) of two AGU-afflicted males (J.A. and M.A.) was determined. P.A. was found to be a heterozygous carrier of a 7 bp deletion (left) while the father was found to be heterozygous for a C → T mis-sense mutation (right). The exact 7 bp sequence that is deleted in the maternal *AGU* allele is one of three possibilities as indicated by the brackets. Both normal and mutant sequences shown here were obtained by sequencing M13 clones containing PCR fragments from the mother and father. The family pedigree is shown below. (b) Direct sequencing of PCR fragments amplified from the first-strand cDNA template of the father D.A. and one of the siblings M.A. The sequence that is shown corresponds to the region where the paternal C → T mis-sense mutation was discovered. The presence of both a C and a T nucleotide in the PCR-amplified product from D.A. and M.A. is indicated with an asterisk. The deduced amino-acid sequence in single-letter designation is presented on the right along with the nucleotide sequence. (c) Partial alignment of the glycosylasparaginase sequences encoded by the maternal normal and AGU-causing alleles is shown together with the shift in open reading frame and premature termination of translation caused by the 7 bp deletion. The premature translational stop codon is marked with asterisks.

(Figure 1) suggesting that the mRNA is partially degraded. The relative quantity of glycosylasparaginase message determined in patient TC79-886 by PCR, using 28S rRNA as a standard, was also decreased compared with normal levels, and previously we found it necessary to increase by 5-fold the amount of cDNA template in order to achieve minimal ethidium bromide detection of the correct PCR product from the mid-region of TC79-886 (Fisher and Aronson, 1991a). Further experiments are necessary to verify these suggestions that glycosylasparaginase mRNA expressed by American AGU-affected patient TC79-886 is less stable.

The other two *AGU* mutations that we have focused on in this study were identified in a British family with two AGU-afflicted male siblings J. A. and M. A. The original diagnosis was based on the presence of large quantities of aspartylglucosamine excreted in the urine of both probands. Enzyme assays were conducted on fibroblasts from the two probands revealing both J. A. and M. A. contained 1.1–3.8 μ -units/mg of protein of GlcNAc–Asn cleaving activity compared with controls ($n = 12$) having a mean of 108 μ -units/mg of protein. Fibroblasts from the mother of J. A. and M.A., as well as cells from her brother and sister, were also tested and found to contain 52.1, 72.5, and

Figure 4 Western-blot analysis of normal and AGU-causing glycosylasparaginase expressed in COS-1 cells

Lysates from COS-1 cells transfected with either pSVL, pSVL/HPAsn.6 (Fisher et al., 1990; Fisher and Aronson, 1991a), or pSVL/AGU^{Ala → Val} were resolved on a 15% (w/v) polyacrylamide gel and electrophoretically transferred to a membrane support. Lanes labelled H were incubated with heavy-subunit-specific antibodies, whereas lanes labelled L were incubated with light-subunit-specific antibodies. Detection of the antibody-antigen complex was with a secondary alkaline phosphatase conjugate. An aliquot of the same lysates that were used for immunoblotting was also assayed for glycosylasparaginase activity and total protein. Enzyme assays were performed only once for each set of transfected cells, and the specific activity of each lysate is given in m-unit/mg of protein (nmol of GlcNAc released/min at 37 °C per mg of protein). The sizes of immunologically detected proteins are given at the right.

Figure 5 Immunofluorescent localization of glycosylasparaginase containing the British paternal Ala → Val substitution

COS-1 cells transfected with either the normal human placental cDNA HPAsn.6 (a) (Fisher et al., 1990; Fisher and Aronson, 1991b) or the British paternal AGU-afflicted glycosylasparaginase sequence (b) were fixed on cover slides and incubated with glycosylasparaginase heavy- and light-subunit-specific antibodies. Detection was with a secondary fluorescein conjugate.

108 μ -units/mg of protein of glycosylasparaginase activity respectively (A. H. Fensom, unpublished work). The intermediate levels of glycosylasparaginase activity measured in the fibroblasts of the mother P.A., and possibly her brother, suggest an AGU

heterozygous genotype, as has been shown for other carriers of an AGU allele (Aula et al., 1973, 1974).

Ikonen et al. (1991b) recently reported that one of the British siblings (M.A.) was a compound heterozygote containing a 7 bp deletion and a C → T point mutation. Our results support their findings. The two brothers J.A. and M.A. have the same two mutations with the AGU^{Δ7bp} allele contributed by the mother P.A. and the AGU^{Ala → Val} allele by the father D.A. (Figure 3). The maternal 7 bp deletion occurs near the 5'-end of the AGU coding region, causing a shift in the reading frame and premature translational termination. The impact of this mutation is similar to the American AGU^{Δ5bp} allele discussed earlier, yet results in an even more truncated glycosylasparaginase protein: a 21-amino-acid polypeptide whose C-terminal 11 residues are out-of-frame. Such a shortened protein sequence would be unlikely to be stable *in vivo* or enzymically active. Also similar to the American AGU^{Δ5bp} mutation were results that suggested the British AGU^{Δ7bp} allele causes a decrease in mRNA stability. Specifically, the frequency of M13 clones that were sequenced and found to contain the 7 bp deletion was significantly lower compared with M13 clones carrying either the normal allele, in the case of the mother P.A., or the AGU^{Ala → Val} allele, in the case of the probands J.A. and M.A. While this result could be due to peculiarities with the biology of M13 bacteriophage, it might also reflect a lower copy number of mRNA encoding the AGU^{Δ7bp} mutation resulting from mRNA instability.

The British paternal C → T point mutation causes a substitution of Ala-101 by Val in the heavy subunit of glycosylasparaginase. The two additional methyl groups in valine could interfere with protein folding; for example, Daopin et al. (1991) in studying the bacteriophage T4 lysozyme found an Ala → Val mutation decreased the stability of the enzyme. This change was proposed to force apart residues that form an α helix, thus weakening many van der Waals interactions and hydrogen bonds that stabilize the wild-type structure. The mutant T4 enzyme still folds, which implies that structural changes are occurring to alleviate overlaps between the introduced methyl groups and the atoms in the surrounding residues. It is possible that potential helix-forming amino-acid residues about the substituted valine in the heavy subunit of glycosylasparaginase might not be able to accommodate the additional methyl groups, thereby preventing proper folding of the mutant enzyme. Indeed, immunofluorescent cell staining of COS-1 cells transfected with the paternal AGU^{Ala → Val} cDNA revealed the expressed protein was not transported to the lysosome (Figure 5), but instead exhibited a staining pattern that is consistent with ER localization (Van Dongen et al., 1984; Hoefsloot et al., 1990). The failure to release secreted or organelle-bound proteins from the ER or other early compartments has been shown to be a consequence of the inability of the protein to achieve its correct conformational state (Yamamoto et al., 1986). We have reported that the major Finnish AGU Cys-163 → Ser mutation similarly produces a protein that is not transported to the lysosome (Fisher and Aronson, 1991a), presumably due to improper folding caused by the loss of a disulphide bridge.

In addition to being trapped in an extra-lysosomal structure, the British paternal AGU^{Ala → Val} protein is neither catalytically active nor post-translationally processed to the heavy and light subunits that constitute the mature enzyme (Figure 4). Interestingly, the biochemical phenotype caused by the British Ala-101 → Val amino-acid substitution is identical to that of the Finnish AGU Cys-163 → Ser substitution (Fisher and Aronson, 1991a,b). An African-American GT^{intron8} → TT splice-site point mutation also produces a protein, albeit truncated at the C-terminus, that is inactive and unprocessed (Fisher and Aronson,

1991b). We have now identified three different *AGU* mutations that affect lysosomal transport, subunit processing and catalytic activity, strongly suggesting that the three events are interrelated. It is very likely that for each mutation the failure to achieve the correct heavy/light subunit structure is the result of the mutant proteins not being transported from the ER to the lysosome. Post-translational processing is common among lysosomal enzymes and is believed to occur either en route to the lysosome or in the hydrolytic lysosomal environment. While these proteolytic modifications often appear to have no impact on enzyme activity, cathepsin D (Yonezawa et al., 1988) and the protective protein of β -galactosidase and neuraminidase (Galjart et al., 1988) have evolved where cleavage of a specific peptide bond is an obligatory activation step. Thus, cathepsin D and the protective protein are true zymogens that become functional only after they are delivered to the lysosome. The same logic can be applied to glycosylasparaginase, to explain the loss of enzyme activity caused by the British and Finnish amino-acid substitutions in terms of an absence of subunit processing. In the case of the truncated African-American *AGU* protein, the loss of 33 amino-acid residues from the C-terminus is more likely to be the governing force.

Each of the three *AGU* mutations that we have addressed in this paper were found to be surrounded by sequences that suggest possible mechanisms to account for how the genetic alterations arose. The 5 bp deletion of American patient TC79-886 (ACACA) occurs in a region where a dinucleotide AC is repeated five times in tandem (TACAACACACACCTTTT) (Figure 2). As a result of the repetition, this region shows motifs of overlapping direct repeats and symmetrical elements, that can induce short gene deletions by slipped mispairing (Krawczak and Cooper, 1991). The British maternal *AGU* allele involves a 7 bp sequence that is deleted from a site (CTTGGCCCTTTAAG) which contains a CTT direct repeat and a region of partial self-complementarity (CTT/AAG) (Figure 3). The gene deletion could be caused by local primary and secondary DNA structure and the partial self-complementarity can permit sequencers to form misaligned secondary structure. The non-complementary portions of these structures then provide templates for frameshifts and short deletions through the removal of the unpaired bases by endonucleases. This event is followed by repair DNA synthesis (Krawczak and Cooper, 1991). The British paternal C $\rightarrow$ T mutation is surrounded by a direct repeat of GTAGGA(C-302 $\rightarrow$ T)AGTAGGA which could increase the frequency of this particular transitional mutation (Krawczak and Cooper, 1991). The transitional mutation is in agreement with the slipped mispairing mechanism between two direct repeats; however, it is not predicted if the repeat copies are aligned. Other factors such as local dNTP concentrations could be important (D. N. Cooper, personal communication).

In this study we have identified a new *AGU* allele which causes *AGU* from an American patient TC79-886 (*AGU* ^{Δ 5bp}) and demonstrated the inheritance of two previously reported *AGU* alleles from a British patient (*AGU* ^{Δ 7bp} and *AGU*^{Ala $\rightarrow$ Val}). While the majority of known *AGU* cases afflict people of Finnish descent, due to a founder effect, the occurrence of two different mutations in a single British family with unrelated parents suggests *AGU* alleles giving rise to *AGU* may be more prevalent in the non-Finnish gene pool than presumed. Interestingly, the British paternal mutation has also been reported in a patient of Italian origin (Ikonen et al., 1991b). Of the different variety of *AGU* mutations that have been characterized, those that create

amino-acid substitutions (*AGU*^{Cys $\rightarrow$ Ser} and *AGU*^{Ala $\rightarrow$ Val}) have provided great insight into the biochemistry of glycosylasparaginase. Both appear to affect folding of the precursor glycosylasparaginase protein based on the inability of the defective proteins to be transported from the ER or related compartments to the lysosome. This consequently prevents subunit maturation, which we have proposed to be necessary for catalytic activity (Fisher and Aronson, 1991a,b). Therefore, the Finnish *AGU*^{Cys $\rightarrow$ Ser} and British *AGU*^{Ala $\rightarrow$ Val} amino-acid substitutions might cause glycosylasparaginase deficiency from two vantages, lysosomal targeting and/or catalytic activation.

This work was supported by United States Public Health Service Grant DK-33314 from the National Institute of Diabetes and Digestive and Kidney Diseases.

REFERENCES

- Aronson, Jr, N. N. and Kuranda, M. J. (1989) *FASEB J.* **3**, 2615–2622
- Aula, P., Nanto, V., Laipio, M.-L. and Autio, S. (1973) *Clin. Genet.* **4**, 297–300
- Aula, P., Autio, S., Raivio, K. and Nanto, V. (1974) *Humangenetik* **25**, 307–314
- Autio, S. (1972) *J. Ment. Defic. Res. Monograph. Series I*, 1–39
- Autio, S., Visakorpi, J. K. and Jarvinen, H. (1973) *Ann. Clin. Res.* **5**, 149–155
- Beaudet, A. L. (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., Frederickson, D. S., Goldstein, J. L. and Brown, M. S., eds.), pp. 788–802, McGraw-Hill, New York
- Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Cullen, B. R. (1987) *Methods Enzymol.* **152**, 684–704
- Daopin, S., Alber, T., Baase, W. A., Wozniak, J. A. and Matthews, B. W. (1991) *J. Mol. Biol.* **221**, 647–667
- Durand, P. and O'Brien, J. S. (1982) in *Genetic Errors of Glycoprotein Metabolism* (Durand, P. and O'Brien, J. S., eds.), pp. 123–152, Edi. Ernes, Milan
- Fisher, K. J. and Aronson, Jr, N. N. (1991a) *J. Biol. Chem.* **266**, 12105–12113
- Fisher, K. J. and Aronson, Jr, N. N. (1991b) *FEBS Lett.* **288**, 173–178
- Fisher, K. J., Tollersrud, O. K. and Aronson, Jr, N. N. (1990) *FEBS Lett.* **269**, 440–444
- Galjart, N. J., Gillemans, N., Harris, A., van der Horst, G. T. J., Verheijen, F. W., Galjaard, H. and d'Azzo, A. (1988) *Cell* **54**, 755–764
- Gehler, J., Sewell, A. C., Becker, C., Hartmann, J. and Spranger, J. (1981) *Helv. Paediat. Acta* **36**, 179–189
- Hoefstloot, L. H., Willemsen, R., Kroos, M. A., Hoogveen-Westerveld, M., Hermans, M. M. P., Van der Ploeg, A. T., Oostra, B. A. and Reuser, A. J. J. (1990) *Biochem. J.* **272**, 485–492
- Hreidarsson, S., Thomas, G. H., Valle, D. L., Stevenson, R. E., Taylor, H., McCarty, J., Coker, S. B. and Green, W. R. (1983) *Clin. Genet.* **23**, 427–435
- Ikonen, E., Baumann, M., Gron, K., Syvanen, A.-C., Enomaa, N., Halila, R., Aula, P. and Peltonen, L. (1991a) *EMBO J.* **10**, 51–58
- Ikonen, E., Aula, P., Gron, K., Tollersrud, O., Halila, R., Manninen, T., Syvanen, A. C. and Peltonen, L. (1991b) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11222–11226
- Jenner, F. A. and Pollitt, R. J. (1967) *Biochem. J.* **103**, 48–49
- Kaartinen, V., Williams, J. C., Tomich, J., Yates, J. R., Hood, L. E. and Mononen, I. (1991) *J. Biol. Chem.* **266**, 5860–5869
- Krawczak, M. and Cooper, D. N. (1991) *Hum. Genet.* **86**, 425–441
- Kusukawa, N., Uemori, T., Asada, K. and Kato, I. (1990) *BioTechniques* **9**, 66–72
- Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Mononen, I., Heisterkamp, N., Kaartinen, V., Williams, J. C., Yates, J. R., Griffin, P. R., Hood, L. E. and Groffen, J. (1991a) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2941–2945
- Mononen, T., Mononen, I., Matilainen, R. and Airaksinen, E. (1991b) *Hum. Genet.* **87**, 266–268
- Morris, C., Heisterkamp, N., Groffen, J., Williams, J. C. and Mononen, I. (1992) *Hum. Genet.* **88**, 295–297
- Palo, J. and Mattsson, K. (1970) *J. Ment. Defic. Res.* **14**, 168–173
- Park, H., Fisher, K. J. and Aronson, Jr, N. N. (1991) *FEBS Lett.* **288**, 168–172
- Pollitt, R. J., Jenner, F. A. and Merksey, H. (1968) *Lancet* **2**, 253–255
- Van Dongen, J. M., Barneveld, R. A., Geuze, H. J. and Galjaard, H. (1984) *Histochem. J.* **16**, 941–954
- Yamamoto, T., Bishop, R. W., Brown, M. S., Goldstein, J. L. and Russell, D. W. (1986) *Science* **232**, 1230–1237
- Yonezawa, S., Takahashi, T., Wang, X.-J., Wong, R. N. S., Hartsuck, J. A. and Tang, J. (1988) *J. Biol. Chem.* **263**, 16504–16511