Characterization of the Mutation Responsible for Aspartylglucosaminuria in Three Finnish Patients

AMINO ACID SUBSTITUTION Cys<sup>163</sup> → Ser ABOLISHES THE ACTIVITY OF LYSOSOMAL GLYCOSYLASPARAGINASE AND ITS CONVERSION INTO SUBUNITS

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The mutation that causes a deficiency of the lysosomal amidase, glycosylasparaginase, has been characterized in fibroblasts from three Finnish patients diagnosed with aspartylglucosaminuria (AGU). The polymerase chain reaction was used to amplify the glycosylasparaginase protein coding sequence from the three AGU patients in order to compare them to the normal sequence from a full-length human placenta cDNA clone HPAsn.6 (Fisher, K. J., Trollersrud, O. K., and Aronson, N. N., Jr. (1990) FEBS Lett. 269, 440-444). Two base changes were found to be common to all three Finnish AGU patients, a G<sup>482</sup> → A transition that results in an Arg<sup>161</sup> → Gln substitution and a G<sup>498</sup> → C transversion that causes Cys<sup>162</sup> → Ser. Detection of both point mutations from PCR-amplified cDNA or genomic DNA was facilitated by their creation of newendonuclease restriction sites. Expression studies in COS-1 cells revealed only the Cys<sup>162</sup> → Ser mutation caused a deficiency of glycosylasparaginase activity. This same substitution also prevented the normal post-translational processing of the precursor glycosylasparaginase polypeptide into its α and β subunits. Cell-free expression of the single-chain glycosylasparaginase precursor did not produce an active enzyme, suggesting that post-translational generation of subunits may be required for catalytic activity.

Aspartylglucosaminuria (AGU) is an autosomal recessive inherited disorder of glycoprotein degradation caused by a deficiency of the lysosomal enzyme glycosylasparaginase (EC 3.5.1.26; N<sub>4</sub>-[β-N-acetylglucosaminyl]-L-asparaginase) (1). This hydrolase functions as an amidase during catabolism of asparagine-linked glycoproteins by cleaving the Asn-GlcNAc bond that joins oligosaccharide to protein (2). The depressed levels of glycosylasparaginase activity that are observed in AGU patients result in lysosomal storage of incomplete degradation products with aspartylglucosamine being the major residue (3). Although individuals afflicted with this condition appear to develop normally in their first few years of life, they subsequently undergo progressive physical and neurological deterioration.

Since the original report of AGU in two British siblings by Jenner and Polliot in 1967 (4, 5), over 200 cases of AGU have been reported, but almost all are people of Finnish descent. The occurrence of AGU in Finland is approximately 1:26,000 with the frequency of heterozygote carriers of the defective gene estimated to be as high as 1:40 in some areas of the country (6). Present methods used to diagnose AGU combine the detection of aspartylglucosamine excreted in urine with a direct enzymatic assay (7). Because individuals that are heterozygous for the AGU gene have been shown to have intermediate levels of glycosylasparaginase activity (8), relatives of AGU patients can be tested to identify carrier-carrier couples at risk of having AGU offspring. The enzymatic assay, however, may not be practical for screening the general population.

Our laboratory purified glycosylasparaginase from rat liver and found it to be a 49-kDa heterodimer having subunits of 24 (α) and 20 (β) kDa (9). The enzyme was also purified from human liver and was reported to have a molecular mass of 60 kDa consisting of three subunits 24, 18, and 17 kDa (10). Amino acid sequence obtained from the rat liver enzyme enabled us to recently isolate a cDNA clone (HPAsn.6) encoding the complete human glycosylasparaginase protein (11). The deduced amino acid sequence predicted a 54.6-kDa polypeptide that is post-translationally processed to generate two subunits of 19.5 (α) and 15 (β) kDa. We also demonstrated using antibodies specific for the α and β subunits of the rat enzyme that the 18- and 17-kDa human subunits reported by Bauman et al. (10) are derived from a common β subunit having variant carbohydrate structure. In this paper we have used the polymerase chain reaction to determine the glycosylasparaginase sequence from three unrelated Finnish AGU patients. All three were found to have two point mutations that result in two amino acid substitutions. Neither base change was noted in two American AGU patients (12). The effect of these substitutions on the function of glycosylasparaginase is addressed.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized by the Penn State Biotechnology Institute (University Park, PA). Primary fibroblast cultures from three unrelated Finnish AGU patients (GM05068, GM02056, GM02057) were obtained from the Human Genetic Mutant Cell Repository, Coriell Institute (Camden, NJ). Primary fibroblast cultures from two unrelated American AGU patients (12) (TC79–842 and TC79–886) were kindly provided by Dr. George H. Thomas.

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(Kennedy Institute, Johns Hopkins University, Baltimore, MD). Genomic DNA and RNA from Finnish controls were generously provided by Dr. David Valle (Johns Hopkins University, Baltimore, MD). A Lambda Fix II human placenta genomic library was purchased from Stratagene (La Jolla, CA). GeneAmp PCR reagent kit was from Perkin-Elmer Cetus Instruments. Sequenase version 2.0 DNA sequencing kit was purchased from United States Biochemical Corp. In vitro transcription kit and dog pancreatin microsomal membranes were purchased from Promega (Madison, WI). In vitro transcription kit was from Bethesda Research Laboratories. Restriction enzymes, protease K, and a random-primed labeling kit was obtained from Boehringer Mannheim Biochemicals. Endoexonuclease G and nuclease were purchased from Gencell Corporation (Boston, MA). The expression vector pSVL and \(a^{-32}\)P-labeled G DNA were procured from Pharmacia LKB Biotechnology Inc. Hybrid-N membranes were purchased from Amersham Corp. Immobilon polyvinylidene difluoride membranes were purchased from Millipore (Bedford, MA). Bio
tin-11-dUTP and oxidized glutathione were from Sigma. Deoxycadenosine 5'-[(\alpha-\sp {32}\)P]thiotriphosphate, deoxyctydine 5'-[\(\alpha\sp {32}\)P]triphosphate, and [\(\alpha\sp {32}\)S]methionine were obtained from Du Pont-New England Nuclear.

Northern Blot Analysis—Total RNA was isolated from fibroblasts by the acid guanidinium thiocyanate-phenol-chloroform extraction method (13). RNA samples (5–10 \(\mu\)g) were heated at 65 °C for 5 min, quick-cooled in an ice bath, and resolved on a 1.2% agarose gel, 2.2% formaldehyde gel. RNA was electrophoresed from the gel onto a Hydroblot membrane and incubated with antibodies against either the \(\alpha\) or \(\beta\) subunits of rat liver glycosylasparaginase.

PCR Amplification and DNA Sequencing—Three sets of oligonucleotides, A, B, and C, were synthesized using the nucleotide sequence from the human glycosylasparaginase cDNA HPAsn.6 (11). The oligonucleotides were designed so that when used in PCR synthesis of cDNA they would generate sequentially overlapping fragments (A, B, and C) that would span the entire glycosylasparaginase protein coding region (see Fig. 2).

Set A: sense-strand primer

5' → (23)GCTGGTTCCTCTTGGTGTAC (−4) 3'

antisense-strand primer

5' → (369)TGTTATTGTTGACTTATM (−338) 3'

Set B: sense-strand primer

5' → (305)TAGGAGATCTCAGACGAATT (−324) 3'

antisense-strand primer

5' → (748)TATCGTCAGCATAGGCTCCA (−729) 3'

Set C: sense-strand primer

5' → (682)GTACATCTCAAAATGTGATA (−681) 3'

antisense-strand primer

5' → (1065)ACAGATGTTGACAGTAAAGA (−1046) 3'

PCR reactions were performed using the geneAmp PCR Reagent Kit according to the manufacturer's instructions with the sense- and antisense-strand primers at 0.25 \(\mu\)M each. The PCR template was 1–2 \(\mu\)g of first strand cDNA prepared from random hexanucleotidereprimed RNA as described (14). Thermal cycling was done in an automated heating/cooling block (Perkin-Elmer Cetus) programmed for a temperature step cycle of 94 °C (1 min), 50 °C (1 min), and 72 °C (2 min). A total of 30 cycles were performed with the 72 °C step extended to 15 min in the final cycle. Amplification products were resolved on a 2% agarose gel, purified by gel electroelution, and sequenced by the DNA sequencing kit purchased from United States Biochemical Corp.

Genomic Cloning and PCR Amplification—A 440-bp BglII-PstI fragment from HPAsn.6 was random-primed-labeled with biotin-11
dUTP and used to screen a human placenta genomic library (16). Bacteriophage DNA was purified from isolated clones (17), digested with various restriction endonucleases, and resolved on a 1.2% agarose gel. Blotting, hybridization, and cDNA probe labeling procedures were essentially those described for Northern blot analysis with the exception that the labeled nucleotide for random-primed labeling was biotin-11 dUTP. DNA fragments containing exonic sequence were sequenced and analyzed as outlined for PCR amplification of cDNA.

Genomic DNA was purified from fibroblasts by the SDS-proteinase K/phenol-chloroform extraction method (17). Oligonucleotide primers were synthesized using the intron sequence flanking the exon that contains the two point mutations identified in Finnish-type AGU (as shown below).

Set A: sense-strand primer

5' → TGATGCTCCTAAGAGT 3'

antisense-strand primer

5' → CCAGTATCTGCTGTCCTG 3'

PCR reactions were the same as for amplification of cDNA except that the template was 0.5–1.0 \(\mu\)g of genomic DNA.

Southern Blot Analysis—An aliquot of the primer set B PCR reaction using a cDNA template from two controls, American AGU TC79–842 and Finnish AGU GM00568 were resolved on a 2.2% agarose gel, stained with ethidium bromide, and transferred to a nylon membrane. Genomic DNA (5–10 \(\mu\)g) from two Finnish controls (A. K. and E. C.) and three Finnish AGU patients was digested with EcoRI, resolved on a 0.8% agarose gel, and transferred to a nylon membrane. Hybridization conditions were the same as described for Northern blotting except that the hybridization and wash temperature was 65 °C.

Construction of AGU, AGU(G656) and AGU(A660) Sequences—Separation of the two Finnish AGU mutations was accomplished using oligonucleotide directed mutagenesis in combination with the polymerase chain reaction. This was possible because the two mutations were in close proximity of one another and both created new restriction sites. Complete full-length glycosylasparaginase sequences containing the separated mutations or both together were assembled by restriction fragment cassette replacement (see Fig. 2): 1) AGU contains both point mutations noted in Finnish-type AGU. The PCR product amplified from AGU cDNA using oligonucleotide primer set B was digested with BglII and PstI. This fragment contains both point mutations. The AGU BglII-PstI sequence was then used to replace the normal BglII-PstI sequence from HPAsn.6. 2) AGU(G656) contains only the G656 → A transition that maintains the G409 → C transversion (bold). The PCR product was digested with DdeI and PstI and used to replace the DdeI-PstI fragment from the AGU sequence. 3) AGU(A660) contains only the G660 → C transversion that changes Cys132 → Ser. This mutation creates an EcoRI site. An antisense-strand oligonucleotide (5' → (477)TGCTCAGATTTGCAGCCAA (496) 3') was synthesized such that it encompassed the sequence between the two point mutations but included only the DdeI site (italics). When used in combination with the antisense-strand primer from oligonucleotide set B, amplification of cDNA yielded a DNA fragment that maintains the G656 → A transition (underlined) but reverts the G660 → C transversion (bold). The PCR product was digested with DdeI and PstI and used to replace the DdeI-PstI fragment from the AGU sequence.

Expression in COS Cells—HPAsn.6, AGU, AGU(G656) and AGU(A660) were independently subcloned into the expression vector pSVL. Transfection into COS-1 cells was by the DEAE-dextran method using 5 \(\mu\)g/100-mm culture dish of either recombinant or native pSVL (18). Three independent transfections were performed for each sequence. Cells from each 100-mm plate were harvested 48 h posttransfection, suspended in 200 \(\mu\)l PBS, 0.1% (v/v) Triton X-100, and solubilized. Glycosylasparaginase activity in a final volume of 50 \(\mu\)l containing 40 mM phosphate buffer (pH 7.0) and 5 mM aspartylglucosamine. Assays were conducted at 37 °C for 3 h. Quantitation of released GlcNAc was by the Morgan-Eison reaction according to Reissig et al. (19). Protein samples (5–10 \(\mu\)g) for immunoblot analysis were electrophoresed on a 15% SDS, polyacrylamide gel, electrotablotted onto a polyvinylidene difluoride membrane, and incubated with antibodies against either the \(\alpha\) or \(\beta\) subunits of rat liver glycosylasparaginase.
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In Vitro Transcription—The human glycosylasparaginase cDNA HPAsn.6 was subcloned into the XhoI site of pSP64. In vitro transcription reactions were performed in a 100-μl volume containing 10 μg of linearized DNA template, 500 nM mG(5')ppp(5')G, and 80 units of SP6 RNA polymerase according to the kit manufacturer. After incubation at 37 °C for 1 h, a second 80 units of polymerase was added and the 37 °C incubation continued for 1 h. At the end of the reaction, template DNA was destroyed by incubating with 5 units of RQ1 DNase at 37 °C for 15 min. Reactions were extracted with phenol/chloroform and unincorporated ribonucleotides removed by successive ethanol precipitations.

In Vitro Translation—Cell-free translation of the glycosylasparaginase transcript was carried out at 30 °C for 1 h in a 30-μl reaction containing 0.5 μg of mRNA, 10 μl rabbit reticulocyte lysate, 40 units of RNasin, and 20 μCi \(^{35}S\)methionine. The reactions were supplemented with 1.5 mM dithiothreitol to a final reaction concentration of 2.0 mM. Nuclease-treated dog pancreatic microsomal membranes (3.6 equivalents) and oxidized glutathione (1-3 mM) were added to reactions as indicated. Reactions tested for glycosylasparaginase activity were diluted 2-fold with PBS, 0.2% SDS. A 15-μl aliquot of the diluted reaction was incubated with 5 mM aspartylglucosamin in 40 mM phosphate buffer (pH 7.0) (final volume 50 μl) at 37 °C for 20 h. Quantitation of released GlcNAc was by the Morgan-Elson reaction according to Reissig et al. (19). Protease protection assays and endoglycosidase H treatment were as described (20). Colorimetric detection of the antigen-antibody complex was as described (20).

RESULTS

Northern Blot Analysis of AGU Fibroblasts—Initial characterization of the mutation responsible for AGU in three Finnish patients involved RNA hybridization with a 440-bp BglII-PstI fragment from HPAsn.6. Two bands of approximately 1.5 and 2.3 kb were detected in RNA from both a normal and three Finnish AGU fibroblast cell lines (Fig. 1). The relative amounts of both messages appeared to be equivalent between control and AGU samples. These results indicate that the mutation responsible for the AGU condition in the three Finnish patients does not cause any noticeable change in size, stability, or expression of the glycosylasparaginase message.

Identification of Finnish-type AGU Mutations by PCR—Three sets of oligonucleotides were used to PCR amplify the glycosylasparaginase protein coding sequence from AGU cDNA (Fig. 2). Sequence analysis of PCR products A, B, and C revealed the 444-bp fragment amplified from all three Finnish AGU cell lines by primer set B (305-748 of the open reading frame) contained a G → A transition at base pair position 482 and a G → C transversion at base pair position 488 (Fig. 3). Both mutations result in amino acid substitutions; G\(^{482}\) → A changes Arg\(^{161}\) → Gln and G\(^{488}\) → C converts Cys\(^{163}\) → Ser. In addition to DNA sequencing, the two AGU

![Fig. 1. Northern blot of RNA from Finnish AGU fibroblast cell lines. Total RNA from a control fibroblast cell line (lane 1) and three Finnish AGU fibroblast cell lines (GM00568, lane 2; GM20566, lane 3; GM02057, lane 4) were resolved on a 1.2% agarose, 2.2% formaldehyde gel, blotted onto a nylon membrane, and probed with a BglII-PstI fragment from HPAsn.6.](attachment:image1)

![Fig. 2. Restriction map of human placenta glycosylasparaginase clone HPAsn.6 showing strategy used for PCR amplification of cDNA. The hatched box indicates protein coding sequence. Bars under the restriction map labeled A, B, and C show the size and spatial distribution of the three fragments that were amplified from AGU and control cDNA. The arrowshead shows the site where the two missense point mutations were identified in PCR fragment B from the three Finnish AGU patients. Bars labeled AGU-Q161 and AGU-S163 show the PCR fragments that were amplified from AGU cDNA and used for construction of sequences containing only the Arg\(^{161}\) → Gln or Cys\(^{163}\) → Ser mutation, respectively. The hollow box at the ends of each bar represent the PCR primers. Endonuclease sites used for restriction analysis and construction of full-length AGU, AGU-Q161, and AGU-S163 sequences are given. Restriction sites in bold print indicate the two sites produced as a result of the Finnish AGU mutations.](attachment:image2)

![Fig. 3. Two point mutations identified in the glycosylasparaginase sequence from Finnish AGU fibroblasts. The sequence on the left is from glycosylasparaginase cDNA clone HPAsn.6, and the sequence on the right is PCR fragment B from Finnish AGU patient GM00668. Dideoxynucleotide termination reactions appear on the gel in the order G, A, T, C. The DNA sequence is read 5' to 3' from bottom to top. Nucleotides labeled with an asterisk show the G\(^{482}\) → A transition and the G\(^{488}\) → C transversion discovered in the glycosylasparaginase sequence from all three Finnish AGU patients. The amino acid substitutions that result from the two point mutations are shown in bold print. Arrows point to the exact location where the point mutations are located on the gel.](attachment:image3)
mutations were documented by restriction analysis. This was possible because of a DdeI site created by G→A (CTCAG) and an EcoRI site created by G→C (GAATTC). Endonuclease digestion with DdeI cleaved PCR fragment B (444 bp) twice into bands of 270, 166, and 8 bp, the latter of which is not visible by agarose gel electrophoresis (Fig. 4). The second DdeI site is in the 5′ PCR primer (Fig. 2). Digestion with EcoRI resulted in two bands of 266 and 178 bp. PCR product B amplified from Finnish and non-Finnish control cDNA remained uncleaved when digested with EcoRI and released only the 8-bp fragment when digested with DdeI. Only one of 10 Finnish controls is shown in Fig. 4. Absence of both mutations in the Finnish controls examined thus far suggests they are not polymorphisms common to the Finnish population.

PCR fragment B from two American AGU patients did not exhibit the DdeI or EcoRI sites created by the Finnish AGU point mutations, thereby revealing heterogeneity in the molecular genetics of AGU (Fig. 4). The original amplification of cDNA from American patient TC79–886 using template amounts identical to the other samples did not generate a discernable product on an ethidium bromide stained gel. It was necessary to increase the quantity of cDNA template 5-fold to produce barely detectable products (Fig. 4). Consistent with this low amplification was the very reduced levels of glycosylasparaginase message detected on a Northern blot containing RNA from TC79–886 (data not shown). Preliminary DNA sequencing of the PCR products from the second American patient TC79–842 revealed a 134-bp deletion in the open reading frame of the glycosylasparaginase cDNA (data not presented).

Southern blot analysis of the primer set B PCR product from Finnish or American AGU and control cDNA revealed three minor glycosylasparaginase-homologous fragments (414, 368, and 320 bp) that were consistently amplified along with the major 444-bp fragment (Fig. 5). The 368-bp fragment is also evident in Fig. 4; however, the 414-bp fragment was not resolved from the 444-bp product and the 320-bp fragment is not visible. The 414- and 368-bp products were sequenced and found to be identical to the 444-bp sequence with the exception of a 30-bp deletion (base pairs 677–706) in the 414-bp fragment and a 76-bp deletion (base pairs 623–698) in the 368-bp fragment (Fig. 6). Besides the loss of coding sequence, the −76-bp sequence shifts the reading frame such that a translation stop signal appears immediately after the deletion site. The resulting translation product would be a truncated protein of approximately 19 kDa that essentially encodes only the α subunit of glycosylasparaginase. The −30-bp sequence does not alter the reading frame that follows the deleted region. It is of interest that both the −76- and −30-bp sequences are present in greater amounts at the apparent expense of the complete 444-bp fragment in the American AGU patient TC79–842 (Fig. 5).

**PCR Amplification and Southern Blot Analysis of Genomic DNA**—To confirm the mutations in the genomic sequence from the three Finnish AGU patients using PCR, we needed both information on the intron-exon structure of the glycosylasparaginase gene and sequence from the introns that border the exon where the mutations were identified. Six clones were isolated and taken to purification after screening approximately 10^6 plaque-forming units of a human placenta genomic library. Southern blot mapping of the bacteriophage DNA from one of the six clones, GHPAsn.1, revealed a 3.2-kb EcoRI fragment that contains the exon with the Finnish AGU mutations. DNA sequencing showed this “AGU exon” to be 113 bp in length with the 5′ and 3′ borders at positions 395 and 507 of the cDNA sequence, respectively (Fig. 7). Oligonucleotide primers designed from the flanking intron sequence were used to PCR amplify the AGU exon (Fig. 7). Genomic DNA from both AGU and control fibroblasts produced the expected 430-bp fragment. When digested with DdeI, the PCR product from control samples yielded two bands of 282 and 148 bp due to a restriction site in the 3′ intron (Fig. 8). Only one of five Finnish controls that were examined is shown in Fig. 8. Although Finnish AGU samples also produced the 148-bp band, the 282-bp band seen in controls was cleaved into two fragments of 191 and 91 bp as a result of the DdeI site created by the G→A mutation. The two American AGU samples yielded DdeI digest banding patterns identical to controls. EcoRI digestion of the 430-bp genomic PCR product from controls and American AGU patients had no effect, whereas the PCR product from Finnish AGU genomic DNA was cleaved into the expected 235- and 195-bp fragments due to the EcoRI site created by the G→C mutation (Fig. 8). The complete digestion by DdeI or EcoRI of the genomic PCR fragment from the three Finnish patients verifies that both mutations are encoded by the defective glycosylasparaginase gene and suggests that all three patients are homozygous for the AGU allele.

To further demonstrate the AGU genotype of the three Finnish patients, genomic DNA isolated from the three patients was digested with EcoRI and analyzed by Southern blotting. This approach was not applied to the G→A mutation because of the high frequency with which the DdeI site
controls (Fig. 9). Whereas all three Finnish AGU patients contains samples from a non-Finnish control P. P. The 0.8-kb fragment that is released from the 3.2-kb fragment also displayed the 3.3- and 1.2-kb bands, the 3.2-kb EcoRI fragment seen in controls was replaced by a 2.4-kb fragment. The 76-bp deletion shifts the open reading frame, thereby resulting in a premature stop signal (TAG) indicated by an asterisk. The charge-enriched sequence enclosed within a bracket is the region where normal post-translational cleavage occurs to generate the α and β subunits of glycosylasparaginase (11).

Fig. 6. Glycosylasparaginase-related sequences PCR amplified from AGU and control cDNA. A section of the sequence from glycosylasparaginase cDNA HPAsn.6 (11) is shown positioned between sequence from two glycosylasparaginase-related PCR products amplified from both AGU and control cDNA using primer set B. The sequence found to be missing from the −30-bp sequence (top) spans base pairs 677–706 of HPAsn.6 and is indicated between the downward vertical arrows. The deleted 30 bp terminates with a GT (underlined) at the 5’ end and an AG (underlined) at the 3’ end. Sequence that was found to be deleted from the −76-bp sequence (bottom) corresponds to base pairs 623–698 of HPAsn.6 and is indicated between the upward vertical arrows. Genomic cloning revealed an intron flanks both the 5’ and 3’ ends of the 76-bp deleted sequence at the gene level. The 76-bp deletion shifts the open reading frame, thereby resulting in a premature stop signal (TAG) indicated by an asterisk. The charge-enriched sequence enclosed within a bracket is the region where normal post-translational cleavage occurs to generate the α and β subunits of glycosylasparaginase (11).

FIG. 7. Partial sequence from a 3.2-kb EcoRI fragment isolated from genomic clone GHPAsn.1. The exon that encodes the sequence where the two Finnish point mutations were identified is shown in capital letters and flanking intron sequence is in lowercase letters. The 5’ border of the exon is base pair 395, and the 3’ border is base pair 507. The single-letter amino acid designation is given above the exon sequence. Oligonucleotide primers were synthesized based on the indicated intron sequence and used to PCR amplify the Finnish AGU exon. The nucleotides in bold print show the G4R → A transition and G488 → C transversion. The new restriction sites that are created as a result of the mutations are also shown.

FIG. 8. Restriction analysis of PCR products amplified from AGU and control genomic DNA. PCR products were digested with either DdeI (D) or EcoRI (E) and resolved on a 2% agarose gel alongside an aliquot of the reaction that was not digested (−). The gel contains samples from a non-Finnish control (NF), a Finnish control P. P. (FC), three Finnish AGU (00568, 02056, 02057), and two American AGU (79–842, 79–886).

Restriction site occurs in the mammalian genome. The BglII-PstI fragment from HPAsn.6 that was used to probe the blot hybridized to three bands of 3.3, 3.2, and 1.2 kb in the two controls (Fig. 9). Whereas all three Finnish AGU patients also displayed the 3.3- and 1.2-kb bands, the 3.2-kb EcoRI fragment seen in controls was replaced by a 2.4-kb fragment. The 0.8-kb fragment that is released from the 3.2-kb fragment as a result of the G488 → C mutation is not detectable with the BglII-PstI probe, because it contains virtually all intron sequence (Fig. 7). These results are consistent with our genomic PCR findings and therefore strongly support the conclusion that all three Finnish AGU patients are homozygous for the G488 → C mutation.

COS Cell Transfections—To determine the effects Arg481 → Gln and Cys482 → Ser have on the function of glycosylasparaginase, expression studies were conducted in COS-1 cells using the vector pSVL. The four cDNA sequences that were tested for their ability to generate active glycosylasparaginase included HPAsn.6, AGU, AGU481, and AGU482. Lysates from cells transfected with HPAsn.6 or AGU481 expressed levels of glycosylasparaginase that were 24- and 20-fold greater than control cells, respectively (Fig. 10). When samples of these lysates were resolved on a polyacrylamide gel and blotted for immunostaining, the results from the two sequences were identical. The α subunit-specific antibody detected a major band of 24 kDa that corresponds in size to the α subunit of glycosylasparaginase and a minor band of 27 kDa, neither of which was evident in the lysate from cells transfected with the control pSVL vector (Fig. 10). The higher molecular weight minor band is likely the α subunit with the signal peptide intact (predicted from HPAsn.6 to be 2.6 kDa), suggesting the high levels of expression overloaded the signal peptidase pathway. This is supported by the absence of the 27-kDa band when levels of expression were increased only 10-fold (data not shown). We have observed a similar signal peptide-containing product using the pSVL/COS-1 system.
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Fig. 9. Southern blot analysis of genomic DNA from Finnish AGU patients. Samples of genomic DNA (5–10 μg) from two Finnish controls (A. K. and E. C.) and three Finnish AGU patients (GM00656, GM02056, GM02057) were digested with EcoRI, resolved on a 0.8% agarose gel, blotted onto a nylon membrane, and probed with a $^{32}$P-labeled BglII-PstI fragment from HPAsn.6. The first two control samples shown in the figure are the same as the second two except the exposure time to x-ray film was reduced to distinguish the 3.5- and 3.2-kb bands.

Fig. 10. Expression of glycosylasparaginase cDNA HPAsn.6 and three AGU sequences in COS-1 cells. Lysates from cells transfected with one of five sequences, pSVL (no cDNA sequence), HPAsn.6, AGU (contains both Q161 and S163 substitutions), AGU$^{Q161}$ (contains only Q161 substitution), or AGU$^{S163}$ (contains only S163 substitution) were resolved on a 15% SDS-polyacrylamide gel and electrophoretically transferred to an Immobilon membrane. Lanes labeled α were incubated with an α subunit-specific antibody, whereas lanes labeled β were incubated with a β subunit-specific antibody. Aliquots of the same lysates used for immunoblot analysis were also assayed for glycosylasparaginase activity. The specific activity of each lysate is given in milliunits/mg (nanomoles of GlcNAc released/min at 37 °C/mg of protein).

for the overexpression of another lysosomal enzyme di-N-acetyllactosaminidase.$^2$ Immunoblot analysis of the HPAsn.6 and AGU$^{Q161}$ lysates with the β subunit-specific antibody uniquely detected a major band of 18 kDa that corresponds in size to the β subunit of glycosylasparaginase (Fig. 10). The minor low molecular weight bands also noted in the two lysates were again probably a consequence of the high levels of expression combined with variation in the structure of attached oligosaccharides (9, 11). These minor bands were not observed when levels of expression were limited to 10-fold (not shown). At these lower levels of expression only the 17- and 18-kDa carbohydrate variant forms of the β subunit were evident.

The other two cDNA sequences that were expressed in COS-1 cells, AGU and AGU$^{S163}$, showed no increase in glycosylasparaginase activity (Fig. 10). Immunostaining with the α and β subunit-specific antibodies revealed both sequences were being expressed; however, instead of generating the normal α/β subunit structure, they produced a single 43-kDa polypeptide that was not present in the control lysate (Fig. 10). The molecular mass of this protein is in accordance with that of the glycosylasparaginase protein predicted from HPAsn.6 before it is cleaved into its two subunits (11). Results from these transfection studies suggest that of the two mutations noted in the three Finnish AGU cell lines, only that which causes Cys$^{163}$ → Ser leads to a loss of catalytic activity. In addition, the Cys$^{163}$ → Ser substitution also prevents the post-translational proteolytic cleavage of glycosylasparaginase into its α and β subunits.

Cell-free Expression—Our finding that the Cys$^{163}$ → Ser mutation inhibited both catalytic activity and production of the α/β subunits of glycosylasparaginase raised questions regarding the role of proteolytic processing in activation of this amidase. To test for any relationship between the two events, we conducted in vitro translation experiments using mRNA generated from HPAsn.6 with the goal of assaying the uncleaved precursor form of glycosylasparaginase for activity. Cell-free translation of HPAsn.6 mRNA in the absence of dog pancreatic microsomal membranes produced a polypeptide of approximately 41 kDa that was immunoprecipitable with glycosylasparaginase α and β subunit-specific antibodies (Fig. 11). This primary translation product was susceptible to proteinase K digestion in the absence of Triton X-100. On addition of microsomal membranes to the translation reaction, the 41-kDa polypeptide was replaced by two bands of 43 and 39 kDa, both of which were resistant to proteinase K degradation in the absence of Triton X-100. Solubilization of the membranes with detergent rendered the 43- and 39-kDa bands susceptible to proteolysis. These results suggest that the latter two polypeptides are processed forms of the 41-kDa primary translation product resulting from its translocation across the microsomal membrane. The difference in molecular weight between the two processed polypeptides is due to carbohydrate, since treatment with endoglycosidase H or N-glycanase converted both to a single band of 37 kDa. Removal of the 2.6-kDa signal peptide predicted from the deduced amino acid sequence from HPAsn.6 would approximately account for the size difference between the deglycosylated 37-kDa protein and the 41-kDa primary translation product synthesized in the absence of microsomal membranes.

Oxidized glutathione was next added to cell-free translation reactions containing microsomal membranes in an effort to direct glycosylasparaginase molecules into their native post-translational conformation (23). An aliquot of each reaction was tested for glycosylasparaginase activity while a second aliquot was analyzed on an SDS-polyacrylamide gel in the presence or absence of a reductant as a measure of specific activity (versus random protein conformations (23). When products were analyzed under reducing conditions, oxidized glutathione at 2 mM was found to cause stimulation in the synthesis of the 43-kDa band (Fig. 11B). Electrophoresis of reaction products under nonreducing conditions revealed the 39- and 43-kDa proteins remained as distinguishable bands only when oxidized glutathione was present at 1 or 2 mM (not shown). Without oxidized glutathione or at concentrations above 2 mM, the 39- and 43-kDa products were no longer resolved, but instead migrated as a continuous band (not shown). Enzymatic assays for glycosylasparaginase activity were conducted on all of the in vitro translation reactions, but no hydrolysis of Asn-GlcNAc was detected.

$^2$ K. J. Fisher, manuscript in preparation.
**DISCUSSION**

The recent isolation by our laboratory of a full-length cDNA encoding glycosylasparaginase has enabled us to characterize the molecular genetics of AGU in three Finnish patients. Fibroblasts from the three patients were first examined for the presence of an RNA message for the deficient enzyme. Northern blot analysis revealed AGU and control fibroblasts to be indistinguishable with respect to the size and relative amounts of two glycosylasparaginase-related messages that were found (Fig. 1). In our original report of the cDNA sequence from HPAsn.6 (11), we identified a single 2.1-kb message in human placenta mRNA when probed with a rat glycosylasparaginase PCR fragment that corresponds to base pairs 628-848 of the human sequence. Here we have detected a second message of 1.5 kb in both AGU and control fibroblasts using a BglII-PstI fragment from HPAsn.6 that extends 318 bp farther 5' (310-753) than the previously used rat probe. This second message was also found in human placenta (data not shown). It is possible that the 1.5-kb message resulted from alternative splicing of the primary glycosylasparaginase message and did not hybridize with the rat probe, because the putative spliced-out portion overlaps the rat probe sequence. Alternative splicing involving loss of coding sequence has been reported for other lysosomal enzymes including β-D-galactosidase (24), β-D-glucuronidase (25), and di-N-acetylchitobiase.

The possibility of an alternative splicing mechanism for the glycosylasparaginase primary transcript is supported by the PCR amplification of at least two minor glycosylasparaginase-related sequences in both AGU and control cDNA (Fig. 5). We have been able to demonstrate through sequence analysis of glycosylasparaginase genomic clones not presented in this paper that the deleted 76-bp sequence encompasses an entire exon (Fig. 6). Interestingly, the frameshift caused by the 76-bp deletion would result in a truncated protein predicted to encode virtually only the α subunit. Examination of the −30- and −76-bp sequence revealed the dinucleotide sequence at the 5' and 3' ends of the deleted sequence (GT/AG) conforms to the consensus sequence for intron splice sites (Fig. 6). This suggests an alternative splicing event where the normal 5' and 3' splice sites that define the intron located at position 698-699 are bypassed in favor of cryptic splice sites that reside within the open reading frame. Alternative splicing mechanisms involving the cassette-style loss of an entire exon and utilization of cryptic splice sites have been described previously (26). Although the results presented here suggest the two alternative splicing events that result in the −30- and −76-bp sequences are normal, but minor, aspect of glycosylasparaginase primary transcript splicing, the factors that determine their abundance in the mRNA pool appear to be altered in the American AGU patient TC79-842 (Fig. 5). These two messages are present in this patient at levels that approach the normal sequence. Preliminary results from our analysis of the mutation that causes AGU in patient TC79-842 show a deletion of 134 bp that is likely a splicing defect. This mutation therefore appears to have a secondary effect on splicing by stimulating an increase in the production of the alternative −30- and −76-bp sequences.

The two point mutations that were identified by PCR of cDNA in the glycosylasparaginase sequence from the three Finnish AGU patients involve a G→A transition and a G→C transversion (Fig. 3). Both mutations result in new endonuclease restriction sites thereby making it possible to use these sites as markers to establish their presence at the genomic level. Although PCR amplification of genomic DNA suggests the three patients are homozygous for the Finnish AGU allele (Fig. 8), it is possible that one or all are actually compound heterozygotes where the second allele is not detectable by PCR amplification of genomic DNA. Such a result might arise due to a gene rearrangement that destroys one or both of the PCR primer sequences. Southern blot analysis of EcoRI-digested genomic DNA, however, revealed the only difference between control and Finnish AGU patients was the expected conversion of a 3.2-kb fragment in the former to a 2.4-kb fragment in the latter due to the G→C mutation (Fig. 9). In the absence of family studies or more extensive Southern blot analyses, the data presented here can be considered strongly suggestive, but not yet definitive, for the Finnish AGU allele being homozygous in these patients.

Because both Finnish point mutations result in amino acid substitutions, it was necessary to separate them and express each independent of one another to determine their individual contribution in causing glycosylasparaginase deficiency. Expression studies in COS-1 cells revealed only one of the substitutions, Cys→Ser, caused inactivation of glycosylasparaginase activity (Fig. 10). The Arg→Gln substitution had no significant effect on activity of the enzyme, and like the normal HPAsn.6 sequence, the protein was post-transla-
proper folding, but in this instance the cleavage site of a paraginase protein by its involvement in a disulfide bridge. This results in the Cys$^{163}$ → Ser substitution on glycosylasparaginase subunits which is essential for folding of the glycosylasparaginase precursor polypeptide. The close proximity of the two Finnish AGU mutations is suggestive of a possible relationship between them. Visual examination of the sequence about the mutated region did not reveal any obvious mechanism whereby one mutation could have resulted in the other. However, the $G^{460} \rightarrow A$ transition involves a CpG site, a dinucleotide sequence known to be a “hot spot” for mutations via methylation of C followed by deamination to T (27, 28). Whether this commonly observed mutation was the primary event that subsequently led to the $G^{460} \rightarrow C$ transversion that eliminates glycosylasparaginase activity, or vice versa, is unknown. An alternative theory is that the two mutations are not directly related but instead the Arg$^{163} \rightarrow$ Gln substitution is a neutral polymorphism that occurred by chance in the Finnish AGU founder. As a result this polymorphism is now linked to the Cys$^{163}$ → Ser AGU mutation.

Understanding the process that converts normal glycosylasparaginase into its $\alpha/\beta$ subunits may help explain how the Cys$^{163}$ → Ser mutation causes AGU. Several lysosomal hydrolases have been shown to undergo a variety of proteolytic modifications in the lysosome. Examples of this include removal of the so-called “pro-sequence” from the $\alpha$ and $\beta$ subunits of hexosaminidase and the further processing of the $\beta$ subunit into fragments (29–31). If formation of the $\alpha$ and $\beta$ subunits of glycosylasparaginase also occurs in the proteolytic environment of the lysosome, the Cys$^{163}$ → Ser mutation may prevent cleavage indirectly by inhibiting the transport of glycosylasparaginase to the lysosome from an early compartment such as the endoplasmic reticulum or Golgi. In this regard Cys$^{163}$ may be important for folding of the glycosylasparaginase protein by its involvement in a disulfide bridge. Disruption of this disulfide could result in an improperly folded protein that consequently might become trapped in the endoplasmic reticulum because of a conformational effect or an interaction of the freed sulfhydryl with a component in the endoplasmic reticulum (32). Loss of a disulfide has been previously implicated to impair lysosomal transport of a mutant hydrolase (33). Another explanation to account for loss of the proposed lysosomal cleavage may again involve improper folding, but in this instance the cleavage site of a correctly transported glycosylasparaginase precursor is not exposed in a suitable conformation for proteolysis.

A critical question that remains is whether the loss of activity and cleavage inhibition are independent or interrelated events caused by the Cys$^{163}$ → Ser mutation. It is possible that Cys$^{163}$ may be involved at the glycosylasparaginase active site and substitution to Ser$^{163}$ abolishes the catalytic mechanism. However it would be extraordinary that a single amino acid substitution both directly inhibited enzymatic activity at the active site and prevented post-translational cleavage into subunits. We consider a more plausible explanation to be that the two inhibitory events are related in either of two ways. First, the proposed disulfide with which Cys$^{163}$ might be involved is of critical importance to the structure of glycosylasparaginase such that its loss causes a sufficient change in conformation to inhibit both activity and cleavage. The second possible relationship is that the mutation inhibits generation of the $\alpha/\beta$ subunit structure which is essential for catalytic activity. Our laboratory has studied the structure of glycosylasparaginase from six different species and have found all maintain the $\alpha/\beta$ subunit formula. Such conserved formation of two subunits from a single polypeptide through evolution suggests that this is a vital aspect of the enzyme. One may envision that cleavage of the polypeptide allows the two subunits to orient themselves in a manner that is not possible when both are part of a common polypeptide. Lysosomal cathepsin D and the “protective protein” for $\beta$-d-galactosidase and $N$-acetyl-$\alpha$-neuraminidase have also been shown to require an activation step (34, 35).

To test the hypothesis that the single-chain uncleaved form of glycosylasparaginase is not catalytically active, we expressed the normal glycosylasparaginase cDNA sequence HPAsn.6 in a cell-free system. Sonderfeld-Fresko and Proia (21) used this procedure to generate enzymatically active $\beta$-hexosaminidase and reported that there was a requirement for oxidized glutathione. Although we performed the cell-free translation of glycosylasparaginase mRNA under conditions designed to achieve native protein folding (21, 29), none of the resulting single-chain products showed Asn-GlcNAc chains that are diagnostic of enzyme activity. It is difficult to conclude with certainty whether the lack of catalytic expression was indeed due to the inactivity of an uncleaved form of glycosylasparaginase or to improper folding of the enzyme. To distinguish these two possibilities, we analyzed the translation products on an SDS-polyacrylamide gel in the absence of a reductant where electrophoretic migration was used to assess the degree of specific disulfide bond formation (23). In the presence of 1 or 2 mM oxidized glutathione, the cell-free translated glycosylasparaginase products migrated as relatively discrete bands which would imply that these proteins were properly folded and therefore enzymatically competent. Since no glycosylasparaginase activity was detected, we favor the concept that the uncleaved glycosylasparaginase protein is not catalytically active, and the cleavage event that results in the $\alpha$ and $\beta$ subunits is an activation step.

In this report we have identified an amino acid substitution (Cys$^{163}$ → Ser) in three Finnish AGU patients that inhibits both the enzymatic activity and post-translational cleavage of the glycosylasparaginase subunits. Two of these AGU patients (TC79–842 and TC79–866) were found not to contain the Finnish AGU mutation revealing the existence of other gene alterations that cause a deficiency of glycosylasparaginase (Figs. 4 and 8). With regard to the Finnish population, however, it is likely that Cys$^{163}$ → Ser will be responsible for the majority of the known cases. This has been shown to be the case for other Finnish enriched genetic disorders (36). The AGU$^{163}$ mutation has established itself in the Finnish population and will undoubtedly cause future medical/social problems as long as heterozygous carriers remain unidentified. We have demonstrated the ability to detect the Finnish AGU $G^{460} \rightarrow C$ transversion by PCR amplification of cDNA or genomic DNA, followed by EcoRI digestion of the product and agarose gel electrophoresis (Figs. 4 and 8). This simple and rapid procedure offers the ability to accurately test and counsel potential carrier-carrier risk couples, as well as to screen the general population for the defective allele.

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4 O. K. Tollersrud, manuscript in preparation.
Addendum—Finnish scientists have recently reported the same two missense mutations to be present in the glycosylasparaginase sequence from all 20 AGU patients they have so far examined in their country (37). This number of patients represents approximately 10% of the known cases in Finland. Their observation both supports our results presented here and indicates that the high incidence of the Cys→Ser AGU mutation in Finland can be explained by a founder effect.

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