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Deletion of exon 8 causes glycosylasparaginase deficiency in an African American aspartylglucosaminuria (AGU) patient

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We have indentified a GT-to-TT transversion at the splice donor site of intron 8 in the glycosylasparaginase gene from an African American aspartylglucosaminuria (AGU) patient. This mutation causes abnormal splicing of glycosylasparaginase pre-mRNA by joining exon 7 to 9 and excluding 134 bp exon 8. The effect of the mutation is compounded by a frame shift that occurs after the deletion site resulting in premature translational termination. The truncated AGU protein was neither catalytically active nor processed into mature α and β subunits. Both this and a previously characterized Finnish AGU mutation appear to affect folding of the single-chain precursor of glycosylasparaginase and thereby prevent transport of the enzyme to lysosomes.

Aspartylglucosaminuria; Glycosylasparaginase; Splicing defect

1. INTRODUCTION

Aspartylglucosaminuria (AGU) is an inherited disorder of glycoprotein catabolism caused by a deficiency of the amidase glycosylasparaginase (E.C. 3.5.1.26) [1]. The disease was first reported by Jenner and Pollitt in 1968 when they discovered large amounts of GlcNAc-Asn in the urine of two mentally retarded British siblings [2]. AGU is the third most prominent lysosomal storage disease with over 200 documented cases [5], however the majority of these are people of Finnish descent suggesting a founder effect.

Our laboratory recently isolated a cDNA encoding the entire protein coding sequence of human placenta glycosylasparaginase [3]. Subsequent to our report, Ikonen et al. [4] published a composite cDNA sequence of human liver glycosylasparaginase that showed complete sequence identity with our human placenta cDNA. In this latter study two missense point mutations were identified in 20 Finnish AGU patiets which were proposed to be responsible for causing glycosylasparaginase deficiency. These 'Finnish type' mutations were independently confirmed by Mononen et al. [5] and ourselves [6]. We also determined that only one of the two resulting amino acid substitutions, $Cys^{163} \rightarrow Ser$, abolishes glycosylasparaginase acitivity [6]. In addition, this mutation prevents post-translational cleavage of the precursor glycosylasparaginase protein into α and β subunits that constitute the mature enzyme [6]. The multiple effects of this $Cys^{163} \rightarrow Ser$ Finnish mutation strongly suggest that post-translational cleavage of the glycosylasparaginase protein is required for enzymatic activity.

In 1983 Hreidarssson et al. [7] diagnosed AGU in a 12-year-old black male. In this report we have characterized the mutation responsible for AGU in this African American patient (Patient TC79-842). The genetic cause, exon skipping, is distinct from the Finnish $Cys^{163} \rightarrow Ser$ mutation, and it is likely that a diversity of glycosylasparaginase mutations occur in humans. The gene sequence for this lysosomal amidase is reported in an accompanying paper [8], and this information will allow the various forms of AGU to be characterized at the molecular level.

2. METHODS AND MATERIALS

2.1. *PCR amplification of the glycosylasparaginase sequence from AGU patient TC79-842*

A primary fibroblast culture from AGU patient TC79-842 was generously provided by Dr George H. Thomas [7] (Kennedy Institute, Johns Hopkins University, Baltimore, MD, USA). Total RNA and genomic DNA were purified from cultured cells as described [6]. Three sets of oligonucleotide primers were used to amplify the glycosylasparaginase cDNA sequence from AGU patient TC79-842 [6]. The primers were designed such that they would generate three sequentially overlapping fragments that together would span the entire glycosylasparaginase protein coding region. Oligonucleotide primers for PCR amplification of genomic DNA were synthesized from intron sequence that flanks exon 8 [8] which was found to be deleted in patient TC79-842:

sense-strand primer, 5′ GAAGTAACCGACTCTTGT 3′

antisense-strand primer, 5′ GTCTTGAGATCATCAATG 3′

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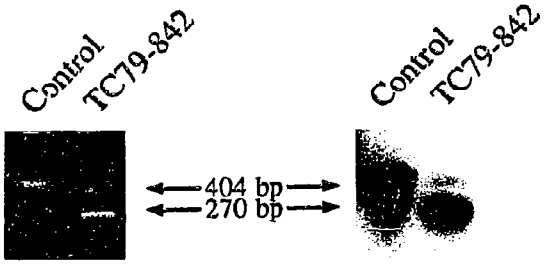

Fig. 1. PCR amplification of the carboxyl-terminal portion of glycosylasparaginase from AGU patient TC79-842. PCR products using primers previously described [6] were resolved on a 2.0% agarose gel and stained with ethidium bromide (left). The gel was Southern blotted on to a nylon membrane and probed with ^{32}P-labeled cDNA clone HPAsn.6 (right).

Reaction conditions and thermal cycling parameters for both cDNA and genomic DNA PCR amplifications were as described previously [6]. PCR fragments were sequenced directly [9] or subsequent to M13 mp18/19 cloning. DNA sequencing was done from both strands.

2.2. *Expression in COS-1 cells*

The glycosylasparaginase cDNA sequence from AGU patient TC79-842 ($AGU^{\Delta exon8}$) was subcloned into the expression vector pSVL to create the recombinant vector $pSVL/AGU^{\Delta exon8}$. Transfection of COS-1 cells was by the DEAE-dextran method using recombinant or native pSVL at 5 μg/100 mm culture dish [10]. Glycosylasparaginase enzyme assays and Western blot analyses were performed on transfected cell lysates 48 h post-transfection as described [6].

2.3. *Immunofluorescent cell staining*

COS-1 cells were grown in 35-mm culture dishes containing a sterile coverslip and transfected by the DEAE-dextran method with 1 μg/plate of either pSVL/HPAsn.6 (contains normal human placenta cDNA sequence HPAsn.6 [6]), $pSVL/AGU^{\Delta Fin}$ (contains the Finnish AGU glycosylasparaginase cDNA sequence [6]), $pSVL/AGU^{\Delta exon8}$, or pSVL/RLCB1 (contais cDNA sequence RLCB1 that encodes rat liver di-*N*-acetylchitobiase (unpublished)). At 48 h post-transfection cells were fixed in 3% paraformaldehyde/PBS, incubated with the appropriate rabbit primary antibody, and stained with a secondary goat anti-rabbit FITC-conjugated antibody as described [11].

3. RESULTS AND DISCUSSION

3.1. *Identification of the mutation responsible for glycosylasparaginase deficiency in American AGU patient TC79-842.*

Agarose gel electrophoresis of PCR products from AGU patient TC79-842 cDNA revealed the oligonucleotide primers that bracket the carboxyl-terminal portion of glycosylasparaginase (bp 660–1065) [3] amplified a fragment approx. 130 bp shorter in length than the expected 404 bp (Fig. 1). In addition to this primary product, a minor fragment of approx. 400 bp was also visible. Southern blot analysis showed both sequences were homologous to glycosylasparaginase (Fig. 1).

A large deletion of 134 bp spanning positions 807–940 of the protein-coding region of the glycosylasparaginase gene was confirmed by sequencing the 270 bp PCR product from patient TC79-842 (Fig. 2). Since the deletion does not maintain the open reading frame, premature translational termination would cause further loss of protein coding sequence. The collective effects of the 134 bp deletion would result in a truncated protein predicted to have an unglycosylated mass of 26.4 kDa after removal of the signal peptide compared to 34.6 kDa for the normal glycosylasparaginase sequence [3]. Alignment of the mutant cDNA sequence from patient TC79-842 with the intron-exon structure of the glycosylasparaginase gene determined by Park et al. [8] in our laboratory revealed the deleted 134 bp correspond exactly to the sequence encoded by exon 8 (Fig. 2).

To determine the genetic event that resulted in the loss of exon 8, oligonucleotide primers were designed from flanking intron sequence and used in PCR amplification of genomic DNA. Both TC79-842 and controls yielded a fragment of approx 430 bp in agree-

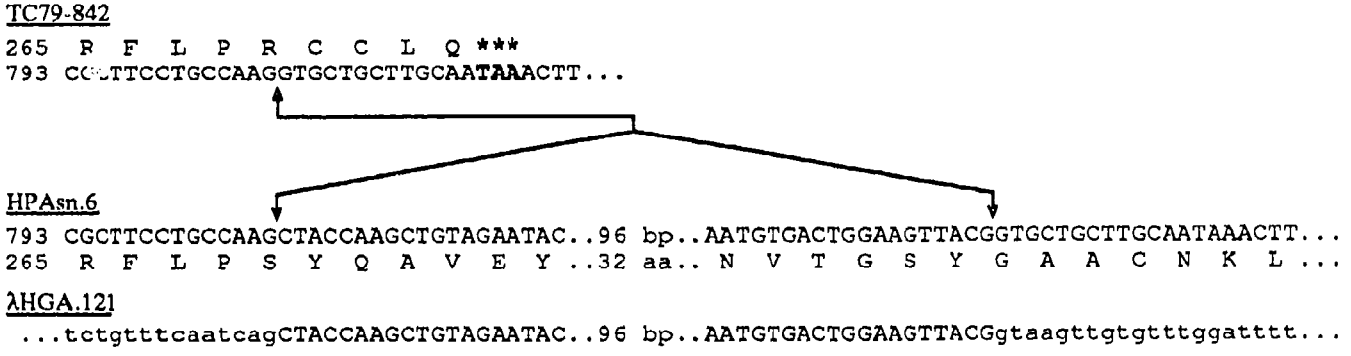

Fig. 2. Partial alignment of the glycosylasparaginase cDNA sequence from AGU patient TC79-842 and human placenta clone HPAsn.6 [3]. A 134-bp sequence from HPAsn.6 shown bracketed between arrows is deleted from the glycosylasparaginase sequence PCR amplified from TC79-842 RNA as indicated. A termination signal TAA indicated in bold print and by asterisks was brought in-frame as a result of the 134-bp deletion. Exon 8 and flanking intron sequence from human placenta clone λHGA.121 [8] is shown below the cDNA sequence of clone HPAsn.6. Intron sequence is given in lower-case lettering.

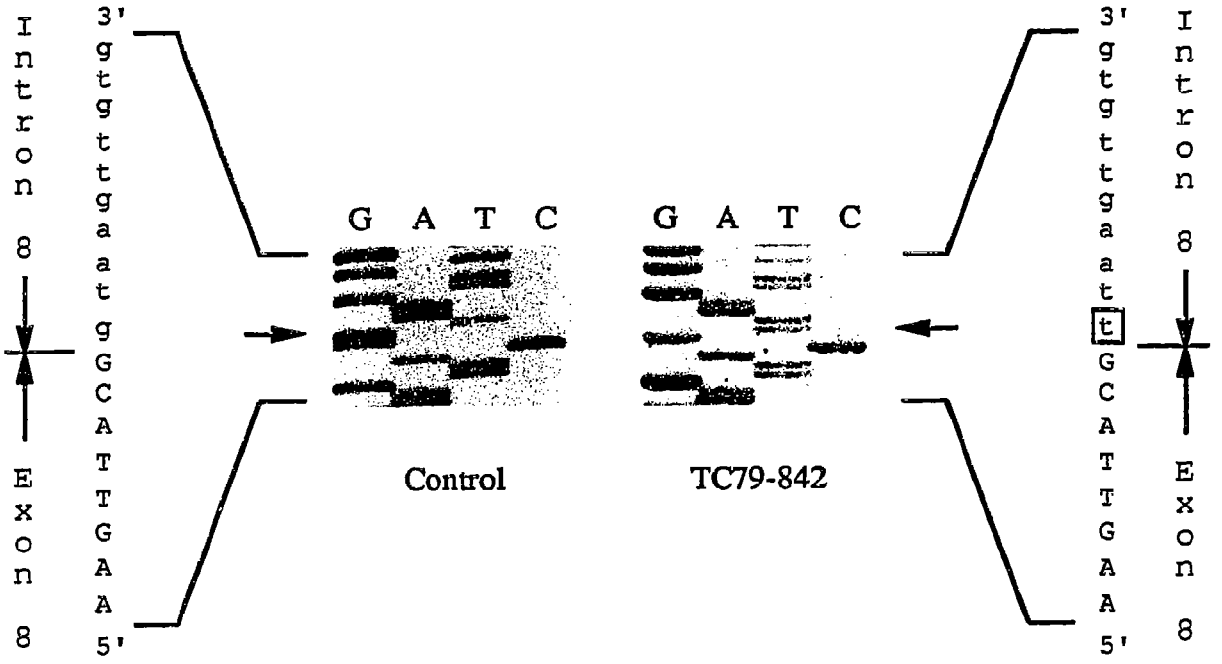

Fig. 3. Identification of a point mutation in the genomic PCR product from AGU patient TC79-842. The sequence on the left is from genomic clone λHGA.121 [8] and the sequence on the right was PCR amplified from TC79-842 genomic DNA. Dideoxynucleotide termination reactions appear on the gel in the order G, A, T, C. The DNA sequence is read 5′ to 3′ from bottom to top. The G→T point mutation discovered at the 5′ donor splice site of intron 8 is boxed. Arrows point to the exact location of the point mutation.

ment with the predicted 428 bp. Sequence analysis of the product from TC79-842 indicated that deletion of exon 8 is likely the result of a splicing defect caused by a G→T transversion (Fig. 3). This point mutation destroys the GT consensus dinucleotide at the 5′ donor splice site of intron 8. Northern blotting revealed the two glycosylasparaginase messages that normally result from utilization of different polyadenylation signals [6,8] are approx. 150 bp shorter in patient TC79-842 relative to controls (not shown). Interestingly the G→T point mutation may not completely eliminate splicing at the exon 8/intron 8 border as evidenced by the minor 400 bp cDNA PCR product seen in Fig. 1. We have not determined whether the mutant donor splice site or a nearby cryptic one is being utilized. The deletion of an entire exon due to a mutation similar to that presented here has been described previously [12,13]. Among lysosomal storage diseases a patient with late infantile Tay-Sachs had a G→A mutation at the last nucleotide of exon 5 that produced *N*-acetyl β-D-hexosaminidase α mRNA in which exon 5 was skipped [14]. This patient also produced approx. 3% normally spliced message. Another prevalent infantile form of Tay-Sachs has a mutation at a consensus splice site position equivalent to that seen in AGU patient TC79-842 that is caused by a GT→CT transversion at the 5′ donor splice site of intron 12 [15,16]. The defective nature of mRNA in this particular Tay-Sachs group is not a skipped exon as observed here, but instead is a set of abnormally spliced and unstable messages that can include intron 12 [17].

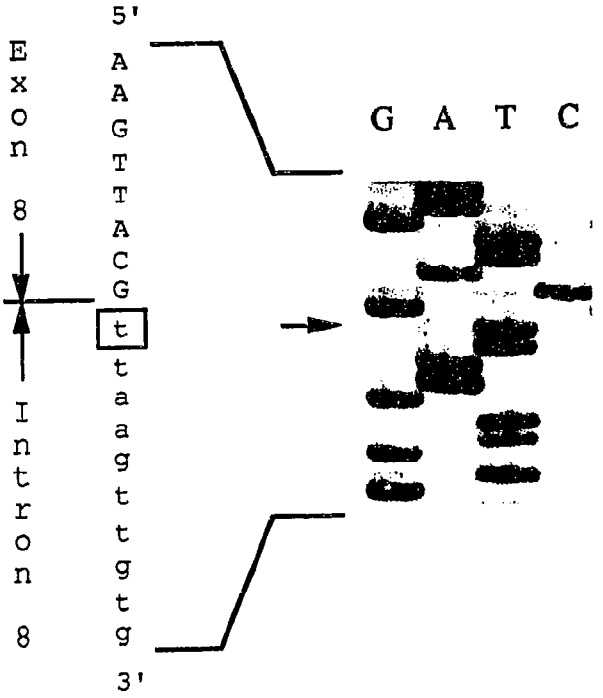

Fig. 4. Direct sequencing of genomic PCR product from AGU patient TC79-842. The 428 bp genomic PCR product for exon 8 was gel purified and sequenced directly using the sense or anti-sense strand primers described in section 2. The sequence that is shown used the anti-sense strand primer. Reactions appear on the gel in the order G, A, T, C. The sequence is read 5′ to 3′ from top to bottom. The G→T point mutation is boxed and indicated with an arrow.

Direct sequencing of the genomic PCR fragment from patient TC79-842 revealed the G→T point mutation was present in the entire population of amplified sequences (Fig. 4). This strongly suggests that patient TC79-842 is homozygous for the 5′ donor splice site AGU mutation. The possibility of compound heterozygosity involving a gene rearrangement not detected with the PCR primers used in this study is not likely based on Southern blotting experiments of genomic DNA from patient TC79-842 (not shown).

3.2. *Expression of the AGU sequence from TC79-842*

The mutant glycosylasparaginase cDNA sequence from TC79-842 ($AGU^{\Delta exon8}$) was expressed in COS-1 cells to determine its catalytic potential. Lysates from cells transfected with the normal glycosylasparaginase cDNA HPAsn.6 expressed 20-fold greater levels of Asn-GlcNAc cleaving activity compared to cells transfected with the pSVL vector alone (Fig. 5). This increase in activity was concomitant with the detection of the processed α subunit on a Western blot (Fig. 5). A minor amount of the 41 kDa precursor glycosylasparaginase polypeptide was also evident due to the high levels of expression [6]. Cells transfected with the mutant $AGU^{\Delta exon8}$ sequence did not show an increase in glycosylasparaginase activity or the processed α subunit (Fig. 5). However, an immunoreactive 30 kDa polypeptide that corresponds to the predicted size of the truncated $AGU^{\Delta exon8}$ protein was detected. This peptide was also weakly antigenic towards β subunit-specific antibody (not shown). These data show that the aberrant glycosylasparaginase protein is made by patient TC79-842, but it is not cleaved post-translationally or active.

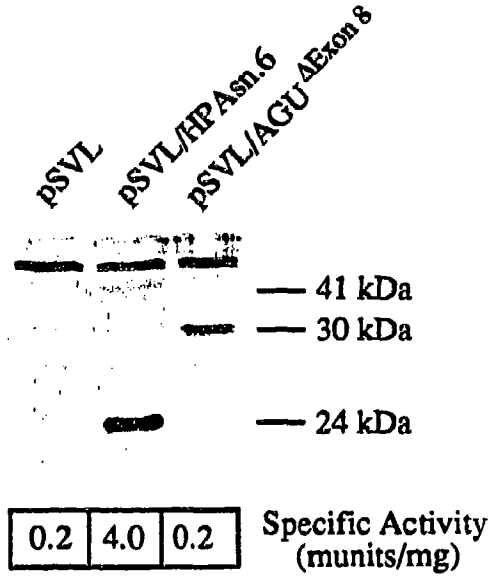

Fig. 5. COS-1 cell expression of transfected glycosylasparaginase sequence from cDNAs HPAsn.6 and American AGU patient TC79-842. Lysates from cells transfected with pSVL, pSVL/HPAsn.6 or pSVL/$AGU^{\Delta exon8}$ were resolved on a 15% SDS-polyacrylamide gel and electrophoretically transferred to an Immobilon membrane (Millipore). Specific α subunit antibodies were used to detect the α subunit and unprocessed glycosylasparaginase. Aliquots of the same lysates used for Western blotting were also assayed for glycosylasparaginase activity.

3.3. *Localization of the mutant AGU protein*

In a previous study we proposed that the Cys^{163}→Ser mutation common to three Finnish AGU patients resulted in the loss of a disulfide bridge that consequently had a pronounced effect on conformational folding of the precursor glycosylasparaginase polypeptide [6]. We further suggested that this inhibited post-translational processing to the α and β subunits by one of two mechanisms. Either the incorrectly folded protein was trapped in the ER, thus preventing transport to the lysosome where the post-translational clippage event is thought to occur, or the polypeptide was successfully transported, but was not in the proper conformation for its cleavage into subunits. This same hypothesis can be applied to the mutant glycosylasparaginase protein from the American AGU patient TC79-842, since it was also found not to be processed to the α/β subunit structure. Such a large loss of protein coding sequence caused by the 134 bp deletion could have a significant effect on the folding of the precursor polypeptide.

Immunofluorescent cell staining of transfected COS-1 cells was performed to examine cellular localization of both the Finnish and American AGU proteins. To first establish the lysosomal staining pattern, COS-1 cells expressing the rat liver lysosomal exoglycosidase di-*N*-acetylchitobiase [18] were stained and found to exhibit a punctate distribution of fluorescence typical for lysosomal labeling [19–21] (Fig. 6a). Cells expressing the normal glycosylasparaginase cDNA HPAsn.6 [6] also produced the same punctate labeling pattern (Fig. 6b). Examination of cells expressing either the $AGU^{\Delta Fin}$ or $AGU^{\Delta exon8}$ sequences revealed a different pattern. In both instances the fluorescent label was more evenly dispersed extending from the nucleus into the cytoplasm (Fig. 6c,d). This same diffuse type of labeling has been shown to occur for a mutant form of α-D-glucosidase that is not transported to the lysosome, but instead is found localized in the ER and Golgi [21].

The results presented here strongly suggest that mutations in both Finnish AGU and this new American form prevent the aberrant proteins from successfully entering the lysosomes due to their entrapment in the ER or other early compartments. In addition to the enzyme being incorrectly localized, loss of lysosomal targeting would have the indirect effect of preventing post-translational processing of the precursor polypeptide to α and β subunits, a step we propose to be necessary for glycosylasparaginase to become active [6].

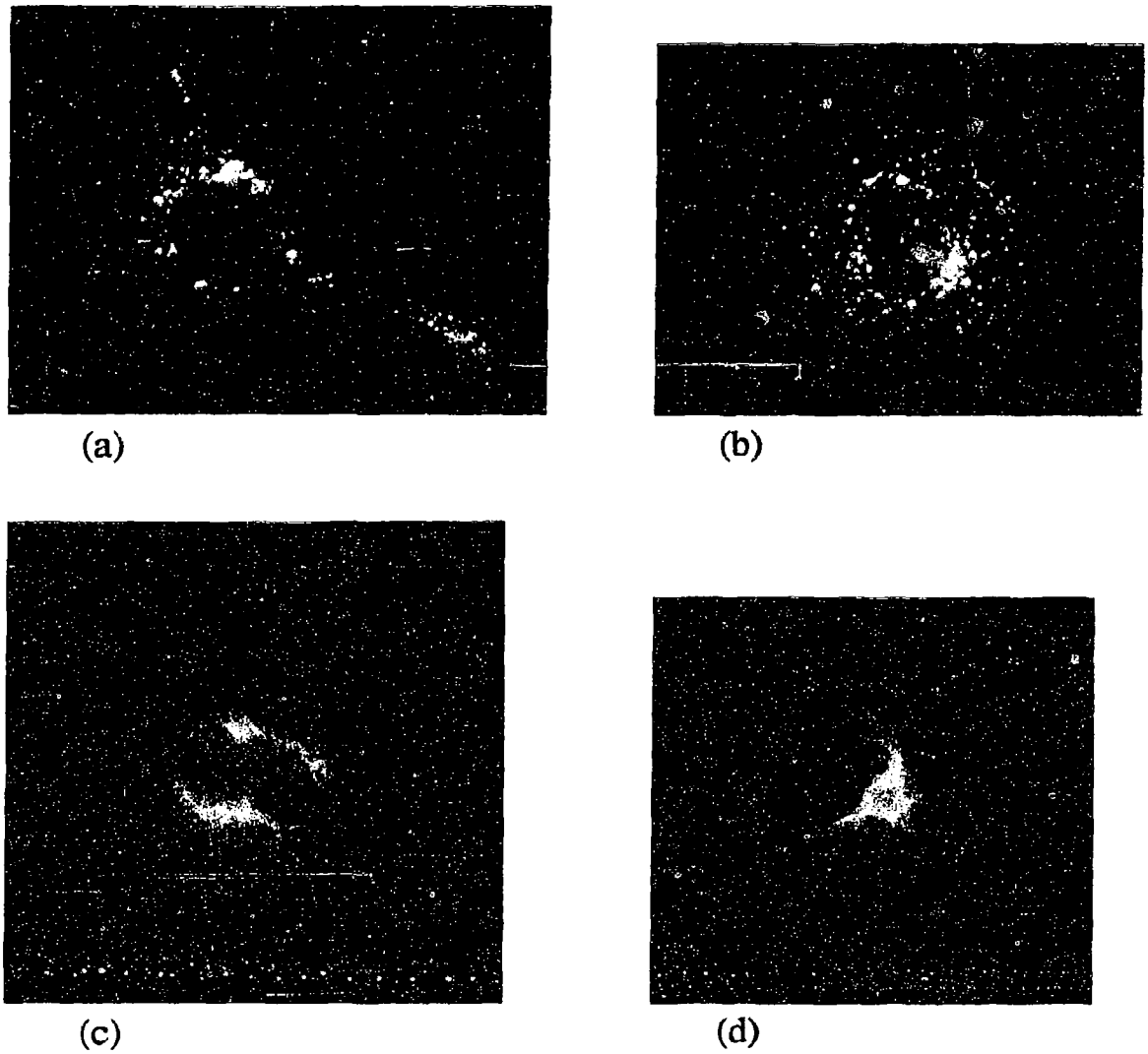

Fig. 6. Immunofluorescent detection by light microscopy of normal and mutant lysosomal glycosidases in transfected COS-1 cells. Immune complexes were visualized with goat anti-rabbit-fluorescein. (a) pSVL/RLCB1 (di-*N*-acetylchitobiase)-transfected cell; (b) pSVL/HPAsn.6 (normal glycosylasparaginase [3])-transfected cell; (c) pSVL/AGU$^{\Delta Fin}$ (Finnish mutant glycosylasparaginase [6])-transfected cell; (d) pSVL/AGU$^{\Delta exon8}$ (patient TC79-842 mutant glycosylasparaginase)-transfected cell.

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