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A Pseudodeficiency Allele (D152N) of the Human β-Glucuronidase Gene

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Summary

We present evidence that a 480G→A transition in the coding region of the β-glucuronidase gene, which results in an aspartic-acid-to-asparagine substitution at amino acid position 152 (D152N), produces a pseudodeficiency allele (GUSBp) that leads to greatly reduced levels of β-glucuronidase activity without apparent deleterious consequences. The 480G→A mutation was found initially in the pseudodeficient mother of a child with mucopolysaccharidosis VII (MPSVII), but it was not on her disease-causing allele, which carried the L176F mutation. The 480G→A change was also present in an unrelated individual with another MPSVII allele who had unusually low β-glucuronidase activity, but whose clinical symptoms were probably unrelated to β-glucuronidase deficiency. This individual also had an R357X mutation, probably on his second allele. We screened 100 unrelated normal individuals for the 480G→A mutation with a PCR method and detected one carrier. Reduced β-glucuronidase activity following transfection of COS cells with the D152N cDNA supported the causal relationship between the D152N allele and pseudodeficiency. The mutation reduced the fraction of expressed enzyme that was secreted. Pulse-chase experiments indicated that the reduced activity in COS cells was due to accelerated intracellular turnover of the D152N enzyme. They also suggested that a potential glycosylation site created by the mutation is utilized in ~50% of the enzyme expressed.

Introduction

Human β-glucuronidase (E.C.3.2.1.31) is a lysosomal enzyme involved in the degradation of glucuronic acid containing glycosaminoglycans. The human β-glucuronidase cDNA and the human β-glucuronidase gene (GUSB) have been cloned and characterized (Oshima et al. 1987; Miller et al. 1990). At least seven unprocessed pseudogenes were detected in the human genome (Shipley et al. 1993*b*; Vervoort et al. 1993). Glycosylation sites and C-terminal processing of the protein have been studied using transient transfection of COS7 cells (Islam et al. 1993; Shipley et al. 1993*a*). Deficiency of β-glucuronidase causes the autosomal recessive disorder mucopolysaccharidosis VII (MPSVII) or Sly syndrome (Sly et al. 1973). Patients can be divided in groups on the basis of the clinical manifestation of the disease (Nelson et al. 1982; Sewell et al. 1982). All affected individuals have in common the presence of storage material in the lysosomes, elevated glycosaminoglycan excretion, and a β-glucuronidase activity of 0%–2% of average normal control values when measured with the synthetic substrate 4-methylumbelliferyl-β-D-glucuronide.

For many lysosomal enzymes, healthy individuals have been observed with an in vitro enzyme activity reduced to the range of activities seen in enzyme-deficient patients, but with no evidence of deficient degradation of natural substrates in vivo. This condition has been termed "pseudodeficiency" and has been reviewed recently (Zlotogora et al. 1983; Thomas 1994). In most pseudodeficiencies the low enzyme activity is probably also present in vivo but is thought to exceed a certain minimal threshold (threshold model). Thus, when tested with a loading assay, pseudodeficient cells generally behave as normal. In some cases, the low enzyme activity toward artificial substrates used in diagnosis is not found toward natural substrates. In these cases, the pseudodeficiency is explained as a change in substrate specificity rather than a decreased amount of enzyme. So far, all pseudodeficiencies studied at the molecular genetic level result from allelic mutations (Gieselmann et al. 1989; Dlott et al. 1990; Triggs-Raine et al. 1992; Cao et al. 1993).

The cases studied here include a phenotypically normal Spanish mother (Mrs. S.) of an MPSVII child (E.S.) who was the subject of an earlier report after she was found to have a low β-glucuronidase activity (6%–10% of controls) (Chabas et al. 1991). However, her fibro-

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blasts showed ^{35}S-sulfate incorporation and chase assays similar to controls. She had a normal excretion pattern of glycosaminoglycan. It was concluded that Mrs. S. was a compound heterozygote for a β-glucuronidase pseudodeficiency allele (GUSBp), which she did not transmit to her MPSVII offspring, and a genuine β-glucuronidase deficiency allele (MPSVII), which she did transmit to her son with MPSVII. The second case (B.G.) (case 1 described by Bernsen et al. 1987) was reported as an atypical MPSVII patient. Residual enzyme activity of B.G. and his affected sister was relatively high (3%–5% of controls). No mucopolysaccharidurie was observed, and the clinical findings, which might have been attributed to MPSVII (coarse facies, mental retardation, and epilepsy), were also present in a sibling with β-glucuronidase activity in the heterozygote range. These findings led us to suspect that this patient had a GUSBp/MPSVII genotype with clinical symptoms unrelated to MPSVII. Patient B.G. and his deficient sister both showed granulation in polymorphonuclear leukocytes not seen in the sibling with enzyme activity in the heterozygote range. The meaning of this granulation remains unclear (Bernsen et al. 1987).

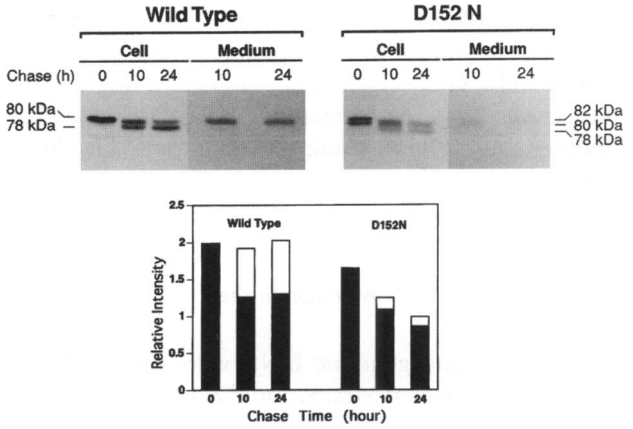

Figure 1 Metabolic labeling and pulse chase of wild-type and D152N β-Glucuronidase. COS cells were transfected as described in the note to table 1. At 50 h after transfection, COS cells were labeled with Trans^{35}S-label for 1 h and chased in unlabeled media for the hours indicated. Then, β-glucuronidase was immunoprecipitated by goat anti-human β-glucuronidase antibody and subjected to SDS-PAGE and fluorography (*upper panel*). The bands in each lane were then scanned using a LKB 2202 Ultra Scan Laser Densitometer. The areas under the peaks of the scan are presented as relative intensity in the lower panel. Blackened areas are from cells, while unblackened areas are from media.

Material and Methods

Total RNA Isolation, RT-PCR, DNA Isolation, and PCR Amplification

Total RNA was prepared using described procedures (Chomczynski and Sacchi 1987). RT-PCR was performed using the Gene Amp RNA PCR kit (Perkin-Elmer Cetus). Detailed protocols will be described elsewhere (R. Vervoort and W. Lissens, unpublished information). Genomic DNA of fibroblasts, leukocytes, and peripheral blood cells was prepared using standard procedures (Kunkel et al. 1977). Exon 3 was partially amplified from genomic DNA with PCR (Saiki et al. 1988). We used the sense primer 3f described by Shipley et al. (1993*b*) and a new antisense primer, HUG10, with sequence 5′-AGGTGTCAGTCAGGTATTGG-3′ (cDNA position 603–584). The sense primer RAF 44 is described in figure 1. Cycling conditions were as follows: denaturation at 94°C, annealing at 63°C, and extension at 72°C. Forty cycles were used to amplify the RAF44-HUG10 fragment and 35 cycles to amplify the 3f-HUG10 fragment. Cycling times were 30 s for fragment RAF44-HUG10 and 1 min for fragment 3f-HUG10. A final extension was used at 72°C for 7 min.

SSCP Analysis and Sequence Analysis

To amplify fragments for SSCP analysis of the cDNA, ^{35}S-dATP and ^{35}S-dCTP (1,000 Ci/mmol, Amersham) were incorporated during PCR. SSCP analysis (Orita et al. 1989) was performed using a combination of methods described elsewhere. Samples were heat denatured and loaded on an 8% polyacrylamide gel, with or without 5% glycerol and with 0.5 × or 1 × TBE-buffer. Gels were run overnight at constant power (5 or 10 W), at room temperature or at 4°C. Double-stranded PCR products amplified from cDNA and genomic DNA were directly sequenced with Sequenase 2.0 (United States Biochemicals).

Polymorphism Analysis

The 1766T/C (T or C at cDNA position 1766) and 1972C/T (C or T at cDNA position 1972) polymorphisms were detected in the mRNA and in the genomic DNA by using PCR-based methods. In the presence of the 1766C polymorphic allele, a *Bsa*AI site is created in exon 11, which was detected in PCR fragments spanning this nucleotide amplified from total RNA, and in the 11f-11r fragment amplified from genomic DNA (Shipley et al. 1993*b*). This restriction site is absent in the presence of the 1766T polymorphism. The 1972C/T polymorphism in exon 12 was detected in the RAF17-HUG16 fragment, which was amplified from total RNA and from genomic DNA. The RAF17 primer (sense, 5′-TCACAATGTTTGGAAAACAGGC-3′, cDNA position 1950–1971) introduces an *Hae*III restriction site in the PCR product amplified from the 1972C allele, but not from the 1972T allele. The HUG16 primer has sequence 5′-CCGTGAACAGTCCAGGAGGC-3′ (antisense), with cDNA position 2072–2053.

Expression Studies

Expression studies in COS7 cells, were performed as described elsewhere (Oshima et al. 1987). Site-directed mutagenesis was performed as described elsewhere (Shipley et al. 1993*a*), using the Sculptor™ kit (Amersham).

Results

Detection of the D152N Mutation in the Pseudodeficiency Allele

Total RNA and genomic DNA were prepared from cultured fibroblasts of Mrs. S. and E.S. of the first family. The coding region of the cDNA was amplified with PCR and screened for mutations with PAGE to detect length changes and/or heteroduplexes. Since no abnormal fragments were detected with PAGE, SSCP analysis of PCR-amplified fragments was performed. Only one fragment showed an abnormal pattern on SSCP gels. Different running conditions were used but disclosed no additional changes. Direct sequencing showed the MPSVII child (E.S.) to be homozygous for a C-to-T transition at cDNA position 552 (552C→T) in exon 3, predicting a leucine-to-phenylalanine substitution at amino acid position 176 (L176F). Mrs. S. was heterozygous for this L176F allele. The presence of both a G (normal) and an A (mutant) at cDNA position 480 in exon 3 indicated that she was also heterozygous at this position. This 480G→A substitution predicts an aspartic-acid-to-asparagine change at position 152 (D152N). Genomic analysis of exon 3 of Mrs. S. and of E.S. confirmed these results (fig. 2). Sequencing of overlapping fragments, spanning the complete coding region of the cDNA, of both individuals did not disclose additional changes from the normal sequence.

The L176F mutation was shown elsewhere to be the only change in two American Mennonite siblings with the MPSVII phenotype (Wu et al. 1994). The independent segregation of the D152N and L176F mutations in the S. family shows they are on different alleles (fig. 2). Thus, Mrs. S. is a compound heterozygote for the MPSVII allele and another allele with a D152N change, the postulated pseudodeficiency allele. Comparison of the intensity of the bands on a sequencing gel at the position of the mutated nucleotides indicates that both alleles are present in comparable amounts in the β-glucuronidase mRNA of Mrs. S. (data not shown).

Two polymorphisms have been described in the β-glucuronidase coding region that are helpful in haplotype analysis. These are 1766T/C in exon 11 (no amino acid change) and 1972C/T in exon 12 (P/L649). By sequencing, we determined those polymorphisms for Mrs. S. and E.S., and we deduced the haplotype of the GUSBp allele of Mrs. S. (nt 480A, 552C, 1766C, 1972C) and that of the MPSVII allele (480G, 552T, 1766C, 1972T). Comparison of band intensities at the position of the 1972C/T polymorphism confirmed that the GUSBp and the MPSVII alleles are present in almost equal amounts in the β-glucuronidase mRNA of Mrs. S.

From the Dutch patient (B.G.) only frozen leukocytes were available, from which genomic DNA was prepared. By direct sequencing of exon 3, we found that B.G. was a carrier of the D152N change, which we had shown to be present on the postulated pseudodeficiency allele of Mrs. S. Since we expected B.G. to carry another inactivating allele based on his low β-glucuronidase activity in comparison to normal controls, PCR-amplified exons (Shipley et al. 1993*b*) were screened for mutations found in MPSVII alleles. This analysis indicated that B.G. was heterozygous for a C-to-T transition at cDNA position 1095 (1095C→T) in exon 7, replacing an arginine codon with a termination codon (R357X) (data not shown). This substitution was previously described in a black American patient (Shipley et al. 1993*b*). We also found it in homozygous form in one of our Belgian MPSVII patients (V.D.P.). Since neither RNA from B.G. nor DNA from other members of his family were available, we were unable to prove that B.G.'s D152N and R357X changes are on different alleles. PCR-based haplotype analysis at the polymorphic positions 1766C/T and 1972C/T showed that both alleles of B.G. had the 1766C/1972C haplotype.

In order to estimate the frequency of the postulated GUSBp allele, we used a PCR-based method to screen 100 unrelated normal Belgian individuals for the 480G→A nucleotide change (fig. 3). We found one carrier of this substitution, allowing a rough estimate of the allele frequency at 0.5%. PCR-based haplotype analysis showed this carrier was heterozygous for a 1766T/1972C and a 1766C/1972C allele.

Properties of the D152N Enzyme Expressed in COS Cells

The mutant cDNA encoding the D152N mutant enzyme was produced by in vitro mutagenesis, was subcloned into the expression vector pJC119, and was expressed in COS cells. Table 1 shows that the level of total activity expressed in COS cells from the mutant cDNA was only 27% that seen in COS cells transfected with the wild-type cDNA. Furthermore, the fraction of total expressed enzyme that was secreted was smaller (19%) than that secreted following transfection with the wild-type cDNA (30%). To determine whether NH_4Cl would divert more newly synthesized enzyme from lysosomes to the secretory pathway (as seen with the wild-type enzyme), both wild-type and D152N transfected cells were exposed to 15 mM NH_4Cl for 50–74 h after transfection (in the presence of mannose-6-phosphate to prevent recapture of secreted enzyme). The secretion of the wild-type enzyme was doubled, while the total wild-type enzyme produced increased only 4.5%. The secre-

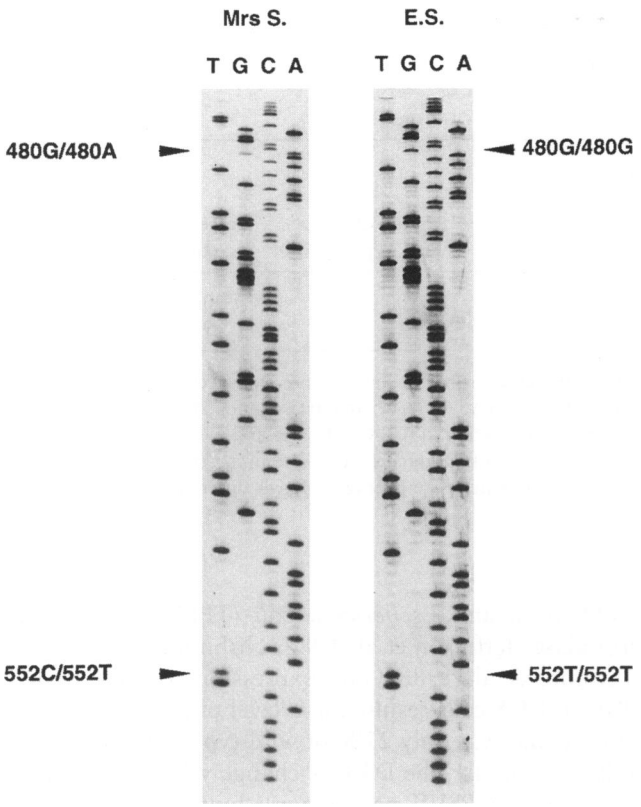

Figure 2 Independent segregation of the D152N (nucleotide change 480G→A) and L176F (nucleotide change 552C→T) mutations. Exon 3 of the β-glucuronidase gene was partially amplified with primers 3f-HUG10. PCR products were directly sequenced with primer HUG10, for the pseudodeficient mother (Mrs. S.) and her affected child (E.S.).

tion of the D152N enzyme increased 4.6 times the low level secreted in the absence of NH_4Cl, and the total amount produced increased 18% (table 2). These data suggest that the D152N enzyme is protected somewhat by NH_4Cl, which raises the pH of internal compartments and diverts a larger fraction of the enzyme from receptor-mediated delivery to lysosomes to the secretory pathway.

Figure 1 presents the data from a pulse-chase experiment meant to compare the rate of turnover of the D152N enzyme to that of the wild-type enzyme. Following metabolic labeling for 1 h, COS cells transfected with the wild-type and D152N cDNA were incubated for 0, 10, and 24 h before immunoprecipitation of the β-glucuronidase from the cell lysate and media. The immunoprecipitate from each time point of chase was analyzed by SDS-PAGE and fluorography, and the fluorograms were quantitated by laser densitometry. When one sums the secreted and intracellular radioactivity, it is evident that the wild-type enzyme shows no degradation over 24 h. There are several interesting differences between the metabolically labeled D152N enzyme and the wild-type enzyme. First, the amount of mutant enzyme synthesized during the labeling period is comparable to the amount of wild-type enzyme. Second, the mutant enzyme gives two bands at zero time, one the same size as that produced initially by the wild-type cDNA and one slightly larger. The larger band presumably represents the fraction of the enzyme in which the fifth glycosylation site, an extra site introduced by the mutation, is utilized. Third, a smaller fraction of radioactivity is secreted, as was also observed for the catalytic activity (table 1). Fourth, the radioactive D152N enzyme disappears with time, the total radioactivity falling to ~60% of that present at the zero time point by 24 h. Thus, the D152N enzyme shows accelerated degradation in comparison with the wild-type enzyme.

Since active β-glucuronidase is a homotetrameric protein, D152N subunits might have a dominant negative effect on wild-type subunits in mixed tetramers. To address this question, the wild-type and the D152N cDNAs were coexpressed in COS cells, using equimolar amounts of each expression vector (table 3). The enzyme activity measured in cells coexpressing both cDNAs (52% of wild type alone), was slightly lower than the average of the enzyme activities expressed separately

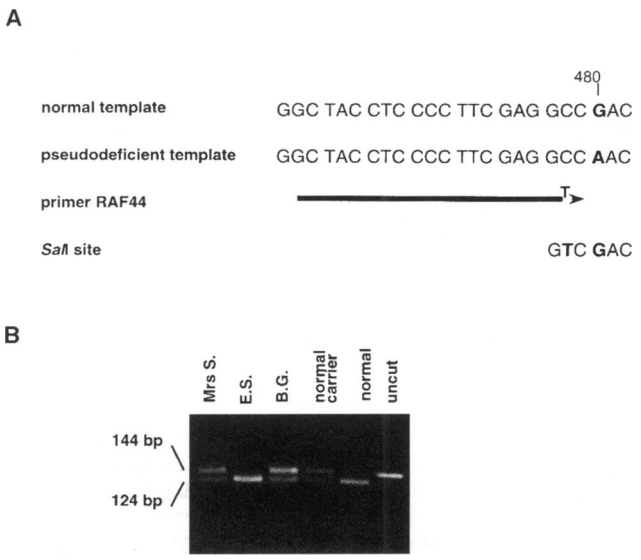

Figure 3 PCR-based detection of the GUSBp allele. *A*, Exon 3 was partially amplified from genomic DNA with PCR, using primers designed to amplify the gene but not the pseudogene sequences: the antisense primer HUG10 and a sense primer (RAF44) designed to introduce a *SalI* site in the normal allele but not in the pseudodeficient allele, with sequence 5′-GCTACCTCCCCTTCGAGGTC-3′ (cDNA position 460–479). *B*, PCR products were digested with *SalI* and run on a 3% agarose gel; results are shown for the pseudodeficient mother (Mrs. S.), her affected child (E.S.), the postulated unrelated pseudodeficient individual (B.G.), an unaffected unrelated individual found to be a carrier of the 480G→A mutation during population screening, a homozygous normal individual, and the uncut fragment.

Table 1

Expression of Wild-Type and D152N Mutant Enzymes in COS Cells

Construct	β-Glucuronidase Activity: Units/mg Cell Protein minus Endogenous			Percent of Wild Type	Percent Secretion
	Cell	Media	Total		
Wild type	1,697	709	2,406	100	30
D152N	527	122	649	27	19

NOTE.—COS cells grown in p60 plates were transiently transfected, using 10 μg of expression vector pJC119 containing wild-type or mutant (D152N) β-glucuronidase cDNA. At 76 h after transfection, the media were collected and the cells lysed in 0.6 ml of 0.25% deoxycholic acid (DOC). Cell lysates and media were assayed for β-glucuronidase activity by using 4-methylumbelliferyl-β-D-glucuronide. One unit is the amount of activity that releases 1 nmol of 4-methylumbelliferone/h. Values are averages of two different experiments.

(64%). This reduction was observed in cells (55% vs. 66%) and in media (41% vs. 57%).

Discussion

The genetic evidence presented here supports the hypothesis that the D152N enzyme is encoded by a pseudodeficiency allele of the β-glucuronidase gene. It was found in a healthy mother of a MPSVII child with low residual enzyme activity, and it was shown to be present on the allele that was not transmitted to her affected child (fig. 2). No other mutation could be detected in her cDNA, in sequencing the whole coding region of the gene. Moreover, the D152N change occurs in a conserved region in human, rat, and mouse (LPFEADI), and *Escherichia coli* (TPFEADV) β-glucuronidase (Jefferson et al. 1986; Nishimura et al. 1986; D'Amore et al. 1988), and expression of the mutated allele in COS cells resulted in a level of β-glucuronidase activity that was only 27% of wild-type enzyme activity (table 1). Finally, the D152N change was also found in an atypical MPSVII patient with low β-glucuronidase activity but with clinical features similar to those of a sibling with enzyme activity in the heterozygote range. In this patient a genuine MPSVII mutation was also found (R357X), but, unfortunately, because of lack of appropriate materials, we have not been able to demonstrate that the D152N and R357X changes are present on different alleles and that the familial distribution of mutated and normal alleles explains the differences of β-glucuronidase activity between the family members.

Table 2

Effect of NH_4Cl on the Expression of Wild-Type and D152N Enzymes in COS Cells

	NH_4Cl	β-Glucuronidase Activity (Units/mg Cell Protein minus Endogenous)		
		Cell	Media	Total
Wild type:				
24 h	–	1,933	558	2,491
24 h	+	1,472	1,130	2,602
D152N:				
24 h	–	816	67	883
24 h	+	773	308	1,041

NOTE.—At 50 h after transfection, media were replaced with 2 ml of media with or without 15 mM NH_4Cl and 5 mM mannose-6-phosphate. After 24 h, the media were collected and the cells lysed in 0.25% DOC. Cell lysates and media were assayed for β-glucuronidase activity as in table 1.

Molecular studies in fibroblasts and expression of the D152N change in COS cells allow one to draw several conclusions concerning the mutated enzyme. The reduced β-glucuronidase activity is primarily due to accelerated degradation of the protein and not to lowered expression of the mRNA or reduced synthesis of the protein. Indeed, analysis of mRNA of fibroblasts of Mrs. S. showed the D152N and the L176F mRNAs are present at equal levels, and the latter has been shown previously to be expressed at wild-type levels (Wu et al. 1994). Furthermore, the amount of mutant enzyme present after a labeling period of 1 h in a pulse-chase experiment in transiently transfected COS cells is only slightly reduced when compared with wild type. In contrast, 40% of the metabolically labeled D152N enzyme is degraded during a 24 h chase period, whereas the wild-type enzyme remains undegraded (fig. 1). Moreover, when one compares the ratio of enzyme activity and radioactivity of the wild-type and the D152N enzymes secreted, our results suggest that the specific activity of

Table 3

Coexpression of Wild-Type and Mutant Enzymes in COS Cells

Construct	β-Glucuronidase Activity				
	Units/mg Cell Protein Minus Endogenous				
	Cell	Media	Total	Percent of Wild Type	Percent Secretion
Wild type	1,810	616	2,426	100	25
D152N	581	86	667	28	13
Wild type + D152N	995	255	1,250	52	20

NOTE.—COS cells were transfected with wild-type and/or D152N cDNA (10 μg plasmid was used for each plate; for coexpression 5 μg of each construct was used). At 77 h after transfection cells were collected and β-glucuronidase activity was measured as described in table 1.

the enzyme toward artificial substrate is not significantly reduced by the mutation (558/1.4 and 67/0.2, respectively; combining data presented in table 2 and in fig. 1). The D152N mutation reduces the fraction of enzyme secreted, which may be related to the additional glycosylation site, which is introduced by the mutation and which is partly utilized in COS cells (fig. 1). If phosphorylated, this additional oligosaccharide chain could improve the efficiency of sorting of the newly synthesized D152N enzyme to lysosomes, and thereby reduce the fraction secreted. The enhanced secretion of the D152N enzyme by NH_4Cl is consistent with this suggestion (table 2; Gonzalez-Noriega et al. 1980). Coexpression experiments of wild-type and D152N cDNAs in COS cells have demonstrated some reduction of the activity as compared with the expected average of both cDNAs expressed separately, in cells as well as in media. An attractive hypothesis to explain this observation is a dominant negative effect of D152N monomers in mixed tetramers; thus, D152N monomers could decrease secretion and increase cellular retention of mixed tetramers, possibly as a result of their additional glycosylation site.

The frequency of various pseudodeficiency alleles has been estimated, and was relatively high in some cases (for a review, see Thomas 1994). For example, 10%–20% of the population may be carriers of the arylsulfatase A pseudodeficiency mutation. In the non-Jewish population, 32%–42% of enzyme-defined carriers of Tay-Sachs disease carry the C739T-pseudodeficiency allele of β-hexosaminidase A, and 6% carry the C745T-pseudodeficiency allele (Triggs-Raine et al. 1992; Cao et al. 1993). In a first attempt to estimate the frequency of the GUSBp allele of the β-glucuronidase gene, we screened 100 unrelated Belgian individuals and found one carrier of the D152N change. Although this is only a rough estimate, the GUSBp allele might be more frequent (0.5%) than MPSVII alleles. As for other diseases in which pseudodeficiency alleles exist, the identification of a pseudodeficiency allele for β-glucuronidase could have important consequences on a practical level (Thomas 1994). It is still premature to make definitive conclusions about the phenotypical consequences of the GUSBp allele, since the presence of the D152N mutation in combination with certain other MPSVII alleles might be more deleterious than its combination with L176F in Mrs. S. Since β-glucuronidase deficiency mucopolysaccharidosis (MPSVII) is a rather rare disease, the D152N allele may be more common than alleles known to produce MPSVII. Its major importance may be related to possible errors in prenatal diagnosis and to misdiagnosis of atypical patients.

Acknowledgments

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