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Assessing the severity of the small inframe deletion mutation in the α-subunit of β-hexosaminidase A found in the Turkish population by reproducing it in the more stable β-subunit

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Summary: GM_2 gangliosidoses are a group of panethnic lysosomal storage diseases in which GM_2 ganglioside accumulates in the lysosome due to a defect in one of three genes, two of which encode the α- or β-subunits of β-*N*-acetylhexosaminidase (Hex) A. A small inframe deletion mutation in the catalytic domain of the α-subunit of Hex has been found in five Turkish patients with infantile Tay–Sachs disease. To date it has not been detected in other populations and is the only mutation to be found in exon 10. It results in detectable levels of inactive α-protein in its precursor form. Because the α- and β-subunits share 60% sequence identity, the Hex A and Hex B genes are believed to have arisen from a common ancestral gene. Thus the subunits must share very similar three-dimensional structures with conserved functional domains. Hex B, the β-subunit homodimer is more stable than the heterodimeric Hex A, and much more stable than Hex S, the α homodimer. Thus, mutations that completely destabilize the α-subunit can often be partially rescued if expressed in the aligned positions in the β-subunit. To better understand the severity of the Turkish *HEXA* mutation, we reproduced the 12 bp deletion mutation (1267–1278) in the β-subunit cDNA. Western blot analysis of permanently transfected CHO cells expressing the mutant detected only the pro-form of the β-subunit coupled with a total lack of detectable Hex B activity. These data indicate that the deletion of the four amino acids severely affects the folding of even the more stable β-subunit, causing its retention in the endoplasmic reticulum and ultimate degradation.

INTRODUCTION

The human lysosomal hydrolases, *N*-acetyl-β-hexosaminidase (Hex) A (αβ) and Hex B (ββ) (EC 3.2.1.52) degrade the carbohydrate moieties of glycosaminoglycans, glycoproteins, glycolipids and proteoglycans by removing the terminal β-glycosidically linked β-*N*-acetylglucosamine or β-*N*-acetylgalactosamine residues (Gravel et al 1995, 2001; Mahuran 1999). The α- and β-subunit genes are split into 14 exons spanning 35 and 40 kb (Neote et al 1988; Proia 1988; Proia and Soravia 1988). Each subunit is synthesized as a pre-pro-polypeptide and assembled into dimers in the endoplasmic reticulum (ER). Failure of the monomers to fold properly or to assemble results in their retention and degradation by the ER quality control system (Parodi 2000). After transport to the lysosome, the assembled pro-polypeptides undergo proteolytic processing to their mature forms. The homodimer of the β-subunit, Hex B, is more stable than heterodimeric Hex A, which in turn is more stable than the α-homodimer, Hex S. Although each subunit has its own active site, only the dimeric forms of the isozymes are catalytically active. Deficiencies in heterodimeric Hex A lead to severe inborn errors of metabolism known as GM_2-gangliosidoses. GM_2-ganglioside accumulates in the lysosome of the neuronal cell. Depending on the levels of residual Hex A activity, a wide spectrum in clinical severity of neurodegeneration appears. It has been estimated that as little as 10% of normal Hex A activity is necessary to prevent GM_2 storage. Although there are no therapeutic modes for the treatment of these diseases, two pharmacologically based therapeutic strategies have been proposed for mutations that allow some residual Hex A to be produced; substrate deprivation and pharmacological chaperones (Breunig et al 2003; Butters et al 2003; Perlmutter 2002; Tropak et al 2004). Estimating the severity of the mutation's effect on the translated protein's ability to fold and exit the ER is thus important in identifying candidates for these emerging therapeutic approaches.

Previously, a 12 bp deletion mutation in the catalytic domain of the α-subunit of Hex A was found in five Turkish patients with infantile Tay–Sachs disease (Özkara and Navon 1998; Sinici et al 2003). It has not been documented in other populations and is the only mutation found in exon 10 to date. Since new therapeutic techniques are emerging that can either decrease substrate synthesis or help stabilize mutant α-subunits in the ER, it is important to determine the degree to which missense mutations and small inframe deletions or insertions destabilize the folding patterns of the mutant α-subunit. One approach to assessing the degree to which a mutation has interfered with the folding of the α-subunit is to express the mutation in the aligned position in the β-subunit (Brown and Mahuran 1991). If any functional Hex B is produced, these new therapeutic approaches may also have a positive effect on residual Hex A activity *in vivo*. This strategy is based on previous data that show that the β-subunit of the Hex A heterodimer is much more stable than the α-subunit, and is considered to be an intramolecular chaperone necessary for the efficient export of the α-subunit-containing Hex A from ER (Proia et al 1984). Thus, in order to determine the degree to which the mutation prevents normal folding of the α-subunit, we

constructed the 12 bp deletion mutation (1267–1279) at the aligned position in the β-subunit cDNA by *in vitro* mutagenesis.

MATERIALS AND METHODS

Overlap extension PCR: The 12 bp deletion mutation was introduced in nucleotide positions 1267–1278 of human β-subunit cDNA of Hex by two-step PCR (Fujimaru et al 1998) as shown in Figure 1. In the first PCR reaction, the 5′ fragment (a:b) starting at the Kozak sequence of β-cDNA and terminating at the 5′ end of the 12 bp deletion was amplified using primers *a* (5′-CACCATG-GGCTGTGCGGGCGGGGCTGC-3′) and *b* (5′-CGCAAGCTTTGCTTTATCA-TCAAAAACCTCCTGCCAGACAATGGAGGTTGCAATAATATCCAAAAC-C-3′). The 3′ fragment (c:d) corresponding to the 3′ junction of the 12 bp deletion and extending to the stop codon was amplified using primers *c* (5′-GGTTTTGGA TATTATT-GCAACCTCCATTGTCTGGCAGGAGGTTTTGATGATAAAGC-AAAGCTTGCG-3′) and *d* (5′-CCTCCATTTTTTACATGTTCTCATG-3′). The resulting fragments (a:b 1283 bp in length and c:d 468 bp in length) were joined in a second PCR reaction by combining the products from the first reaction and amplifying them using primers *a* and *d*. Various concentrations of each primer and PCR conditions were evaluated to optimize the procedures. To lower PCR mutation frequency in amplifying long target β-cDNA, the KOD High Fidelity polymerase, which has a $3' \rightarrow 5'$ exonuclease proofreading activity, was used. All PCR reactions were performed in a total volume of 50 µl, containing 5 pmol/µl of each primer, 1 unit of KOD HiFi DNA polymerase (Novagen, Kita-ka Japan), 0.2 mmol/L dNTP mix, 5 µl 10× PCR buffer and 1 mmol/L $MgCl_2$. The reaction

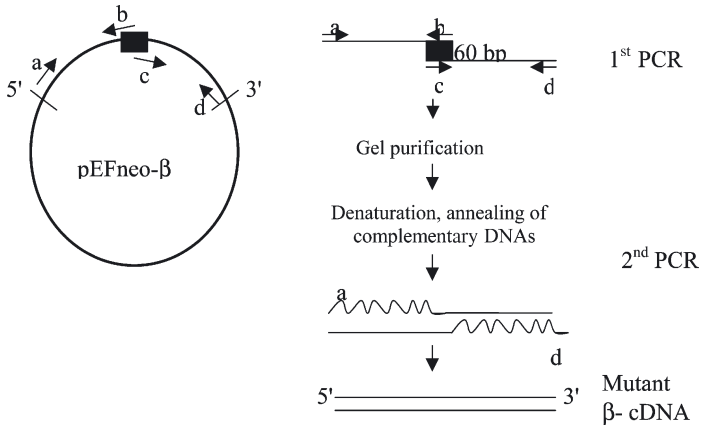

Figure 1 The artificial 12 bp deletion mutation was introduced in nucleotide positions 1267–1278 of the human β-subunit cDNA of Hex by two-step PCR. By deletion of 12 nucleotides in the primers *b* and *c*, the mutation was generated in the amplified DNA sequences. *a*, *b*, *c*, and *d* are primers; ■ indicates the mutation site

conditions used were one step of denaturation at 94°C for 4 min and 30 cycles comprising 1 min at 94°C (denaturation), 1 min at 60°C (annealing) and 1 min at 72°C (extension) and one step of extension at 72°C for 10 min using a Perkin Elmer Gene Amp PCR system 2400 thermal cycler (Perkin Elmer, MA, USA). PCR products were run on a 1% agarose gel and purified by QIAquick gel extraction kit (Qiagen, ON, Canada).

Cloning and transformation: PCR product generated by overlap extension PCR was cloned into pcDNA3.1D/V5-His-TOPO according to manufacturer's instructions (Invitrogen, CA, USA). Clones were isolated by QIAprep Kit (Qiagen, Canada). To confirm the presence of the 12 bp deletion mutation and exclude extraneous mutations, all candidate mutant β-cDNAs were sequenced in their entirety (ACGT Corp, Toronto, ON, Canada). Owing to low levels of expression, the mutant β-cDNA was cloned into the pEFneo vector (made by Dr D.S. Anson, previously laboratory of D.J.M.) previously shown to express high levels of β-Hex (Hou et al 1996b; Sharma et al 2003). Because of the lack of restriction map, restriction endonuclease digestions with *Eco*RV, *Bsu*36I, *Afl*II, *Afl*III, *Nco*I, *Pst*I, *Bam*HI *Hin*dIII and *Not*I and double digestions with *Bam*HI–*Hin*dIII, *Not*I–*Bam*HI and *Not*I–*Hin*dIII were conducted to determine the appropriate cloning sites in the 'home-made' pEFneoβ plasmid. The mutant cDNA was cloned into the *Bam*HI–*Not*I site derived from pcDNA3.1D/V5-His-TOPO-Hex by performing a triple ligation. Restriction endonuclease digestions of pEFneoβ plasmid were performed as follows. (1) Digestion with *Bam*HI and then *Hin*dIII to obtain a clear appropriately sized DNA band. (2) Double digestion with *Hin*dIII and *Not*I. The mutated site was obtained from plasmid pcDNA3.1D/V5-His-TOPO-Hex by double digestion with *Bam*HI–*Not*I. Triple ligation was performed overnight at 4°C by T4 ligase (Roche, Penzberg, Germany). Mutant pEFneoβ constructs were transformed into competent *E. coli* cells and clones were verified by DNA sequencing of the entire β-cDNA.

Cell culture and DNA transfection: CHO cells were grown in α-MEM (minimal essential medium) with 10% FCS (fetal calf serum), penicillin and streptomycin at 37°C in 5% CO_2. Cells (40% confluent) were transfected with 10 μg of pEFneoβ plasmid, containing a wild-type or mutant β-cDNA insert, and 10 μg of pEFneo using Superfect (Qiagen) according to the manufacturer's protocol. After 48 h, the cells were trypsinized and diluted tenfold in α-MEM plus FCS containing 600 μg/μl neomycin (Fujimaru et al 1998; Sharma et al 2003). Following 2 weeks growth in drug-containing medium, stable transfected neomycin-resistant cells were used to assay Hex activity and expression levels.

Hex activity assay: Transfected CHO cells were lysed in 10 mmol/L sodium phosphate buffer, pH 6.0, and 5% glycerol containing protease inhibitor cocktail by six freeze–thaw cycles. Protein from cell lysates was quantified according to the Bradford method (Bio-Rad, CA, USA) (Bradford 1976). β-Glucuronidase and Hex activities from cell lysates were determined using the fluorogenic substrates 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (MUG) and 4-methylumbelliferyl β-glucuronide (Brown and Mahuran 1991; Hou et al 1996a).

Western blot analysis: Samples of transfected and nontransfected CHO cell lysates, carrying equal β-glucuronidase activities (60 nmol/h per μg protein), were resolved by polyacrylamide gel electrophoresis in sodium dodecyl sulphate by the Laemmli gel system (12.5% gel) using Bio-Rad mini-gel system (Laemmli 1970). Proteins were electrophoretically transferred to nitrocellulose overnight at 4°C. The filter was blocked in 5% powdered skim milk in TBST (10 mmol/L Tris base, 150 mmol/L NaCl, 0.05% Tween 20, pH 7.5) for 4 h followed by overnight incubation with 1/800 dilution (1% skim milk in TBST) of rabbit anti-human Hex B, which does not cross-react with CHO cell Hex (laboratory of D.J.M.) (Tse et al 1996). Nitrocellulose was washed four times with (1% skim milk in TBST) and was incubated with a 1/10 000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immuno Research, PA, USA) in 1% skim milk in TBST for 1 h. Bands were visualized using chemiluminescent substrate as described in the Amersham ECL system (Amersham, Bucks, UK) (Hou et al 1996b).

RESULTS

The 12 bp deletion mutation was introduced in the β-cDNA, which was then permanently transfected into CHO cells. Extracts from mock-transfected cells (negative control), cells transfected with the wild-type β-cDNA (positive control) and those transfected with the mutant β-cDNA were analysed by Western blotting using rabbit anti-human Hex B, which does not cross-react with CHO cell Hex. Extracts from wild-type transfection contained both the pro-β-chain and the processed mature β-chains of the β-subunit of Hex, whereas mock-transfected cells contained

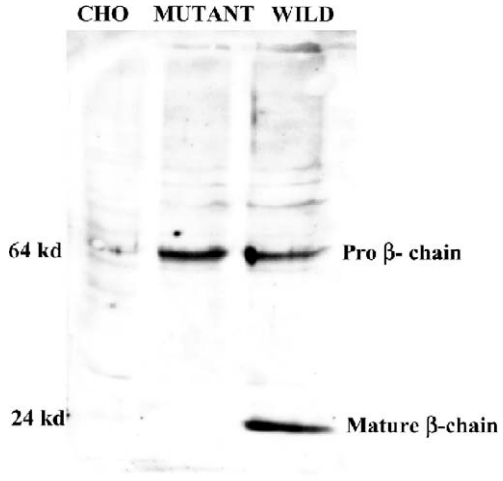

Figure 2 CHO cells were transfected with pEFneo carrying a wild-type cDNA of the human Hex β-subunit (WILD) or a 12 bp deletion mutation (ΔIle398–Asn–Lys–Gly401) (MUTANT). Mock-transfected CHO cells were also used as a control (CHO). The positions of the pro-β-chain and the mature β-chain are indicated

Table 1 Hexosaminidase activities in transfected CHO cells expressing mutant and wild type Hex B

	Hex activity (nmol/h per μg protein)	*Hex activity* (%)
Nontransfected CHO	56.14	56.65
Wild type	99.10	100
Mutant type	45.65	46.06

none of these. On the other hand, extracts from mutant transfections showed only pro- β-chain (Figure 2). This indicates that the mutation prevents even the inherently more stable pro-β monomers from being able to dimerize and exit the ER for transportation to the lysosome and processing into its mature form.

Hex activities were assayed in cellular extracts with MUG to evaluate the total Hex activities (Table 1). Hex activity was found 2-fold higher in wild-type-transfected cells than in mock-transfected cells. As expected from the western blot data, lysates of mutant-transfected cells contained only background levels of activity, similar to the Hex activities found in mock-transfected cells.

DISCUSSION

To date, five infantile homozygote Turkish Tay–Sachs patients have been reported carrying a 12 bp deletion mutation in the α-subunit gene of Hex (Özkara et al 1998; Sinici et al 2003). The deletion mutation in exon 10 seems to be the most frequent Tay–Sachs mutation in Turkey and also the only mutation so far localized to exon 10. It results in the absence of four amino acid residues in protein (Tyr366–Gly369). The expression analysis demonstrated detectable levels of an inactive mutant α-protein in its pro-form (Özkara and Sandhoff 2003).

Although α- and β-subunit genes map to different chromosomes, they are believed to have evolved from a common ancestral gene. Primary amino acid sequences of α- and β-chains revealed a 60% sequence identity, mostly in the catalytic domains (Korneluk et al 1986; Myerowitz et al 1985). Areas of identity between aligned primary sequences coincide with common functional domains. The β-subunit of the Hex A heterodimer has been shown to be much more stable than the α-subunit, and is considered to be an intramolecular chaperone necessary for the efficient export of the α-subunit-containing Hex A from ER (Proia et al 1984). Currently, there are efforts for therapy of GM_2-gangliosidoses: one of which is the use of pharmacological chaperones. This approach is based on the critical threshold hypothesis of Sandhoff, which indicates that, *in vivo*, as little as 10% of normal Hex A levels are needed to turn over all its substrates (Conzelmann and Sandhoff 1983). Small molecules, which function either as agonists or competitive inhibitors, have been used to rescue several misfolded proteins from ER-associated degradation (Ellgaard and Helenius 2003; Fan 2003). It is not clear whether these chaperones act by stabilizing the monomers, the dimers or both. The juvenile and adult types of Tay–Sachs disease are more suitable for treatment with pharmacological chaperones as they have some residual Hex A activity (Tropak et al 2004). Although the infantile type Tay–Sachs mutations

are severe, only a small increase in Hex A activity is needed for improved life. As the β-subunit acts as an intramolecular chaperone for the α-subunit, by determining the severity of the mutation for β-subunit it should be possible to treat the patients using pharmacological chaperones.

One approach to assessing the degree to which a mutation has interfered with the folding of the α-subunit is to express the mutation in the aligned position in the β-subunit. If any functional Hex B is produced, these new therapeutic approaches may also have a positive effect on residual Hex A activity *in vivo*. The above approach has been carried out for the 'B1 variant' of Tay–Sachs, which affects a known active-site residue in the α-subunit (Arg178) (Mark et al 2003). When the aligned residue in the β subunit (Arg211) was mutated and Hex B was expressed, the isozyme was also inactive (Brown and Mahuran 1991). Thus, the chemical chaperone approach to therapy would not be feasible in this case. On the other hand, the expression of a mutant Hex B (βGly301Ser), mimicking the major mutation associated with adult Tay–Sachs (αGly269Ser), for which candidate chemical chaperones have been identified (Tropak et al 2004), produced mutant Hex B activity levels comparable to those of transfected cells expressing the wild-type β-subunit (Brown and Mahuran 1993). Interestingly, the heat stability ($T_{1/2}$) of the mutant Hex B was reduced from ~20 to ~5 min at 60°C. When the mutation was placed in the α-subunit, no Hex S (αα) could be detected in transfected cells. Co-transfections with normal β-cDNA did produce an active mutant Hex A, but its heat stability dropped from 200 min to 10 at 45°C. These data demonstrate the greater inherent stability of the β-subunit, and that the major effect of the mutation, in either α or β, is to reduce the difference in free energy between the folded and unfolded forms of the subunits (Dill and Shortle 1991). These conclusions are fully supported by the recent three-dimensional crystal structure of Hex B and the model of Hex A derived from it (Mark et al 2003). Thus, the severity of the most common Tay–Sachs mutation in the Turkish population, which causes a four-amino-acid deletion in the protein, was analysed by reproducing it in β-cDNA and expressing the mutant β-subunit in CHO cells. Western blot analysis detected only the pro-β-form in lysates from transfected cells. These cells also produced no detectable Hex B activity. The absence of mature β protein is a good measurement of stability as intracellularly the pro-β-chain is found only in the ER. This has been shown for other misfolded α- and β-subunits by immunofluorescence (Hou et al 1996b; Sagherian et al 1994). The possibility of the protein being properly folded (stable), but unable to form dimers is not likely, as we know from the 3D structure of Hex B that the mutation does not directly affect the areas involved in dimerization. Glycosylation is also unlikely to be affected because it is a co-translational event and the mutation does not directly effect a known site of glycosylation. These data indicate that the β-deletion mutant, like the α-subunit mutant, is probably misfolded and retained in the ER and does not undergo lysosomal processing. Thus, this is a severe mutation that is not likely to be treated by the emerging chemical chaperone and/or substrate depletion therapies.

Our results were also supported by articles describing the crystal structure of the β-subunit that appeared in the literature concurrently with this work (Maier et al

2003; Mark et al 2003). The crystal structure of the β-subunit indicates that the β-subunit is a kidney-shaped, two-domain protein. The two-domain structure of the β-subunit is conserved and represents a fundamental fold present in all family 20 glycosidases found in species ranging from prokaryotes to humans. Domain I (residues 50–201) consists of a six-stranded antiparallel β-sheet that buries two parallel α-helices against domain II. Domain II (202–556) is a $(\beta/\alpha)_8$ barrel structure that houses the active site within loops extending from the C termini of the strands constituting the β-barrel. The four residues (Ile398–Asn–Lys–Gly401) deleted in the mutant β-subunit correspond to a loop connecting the β5 strand with the α4 helix (Figure 3). The deleted residues are not in a position to directly participate

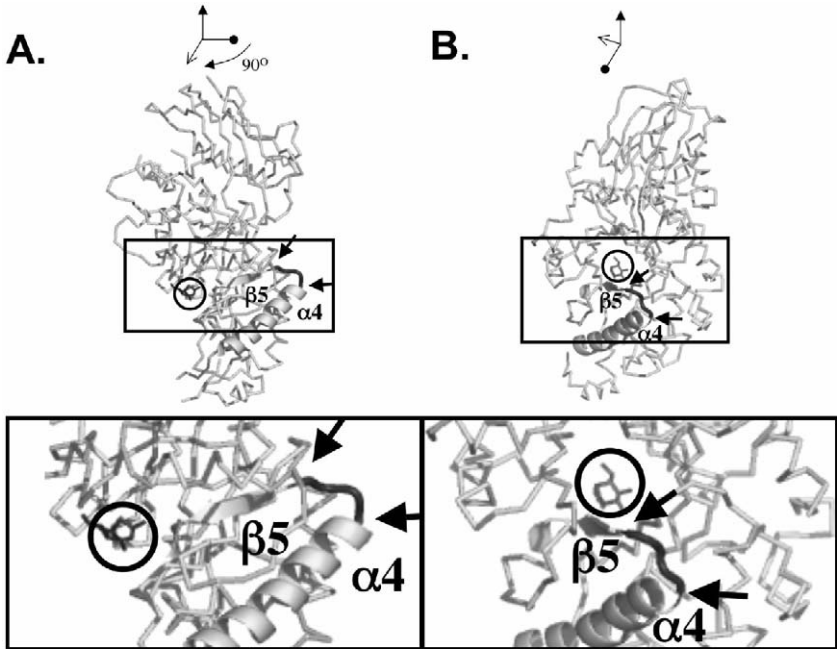

Figure 3 3D ribbon diagram of Hex B showing location of deleted amino acids (398–401). Panels (A) and (B) show two orientations (of a ribbon diagram of a single β-subunit from Hex B). Relative to panel (A) the subunit in panel (B) is rotated 90° counterclockwise around the vertical axis (indicated above the models). Deletion of amino acids 398–401 in the β-subunit removes a loop (black tube indicated by solid black arrows) connecting β-strand β5 and α-helix α4 (represented as cartoons) in one subunit of Hex B. The boxed region in the upper portions of panel (A) and (B) is shown magnified in the lower portions of the panels. Panel (A) shows a side view of the β-subunit. Note that the deleted loop is far removed from the entrance to the substrate-binding pocket denoted by the position of NAG-thiazoline (circled). In panel (B) (rotated 90° counterclockwise relative to the model in panel (A)), looking into the substrate-binding pocket (denoted by the position of the circled NAG-thiazoline) from behind, it is apparent that the loop also connects one of the β-strands lining the substrate recognition site. Ribbon diagrams were prepared using MacPyMOL

in the formation of the active site or the dimer interface and as such may indirectly affect activity by destabilizing the overall structure of the enzyme. Thus, the experimental data are consistent with the deduced structure of Hex B, indicating a critical role for the four-amino-acid loop between the β-strand and the α-helix in overall folding of both the α- and β-subunits.

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