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The Val[192]Leu Mutation in the α-Subunit of β-Hexosaminidase A Is Not Associated with the B1-Variant Form of Tay-Sachs Disease

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Summary

Substitution mutations adversely affecting the α-subunit of β-hexosaminidase A (αβ) (EC 3.2.1.52) result in Tay-Sachs disease. The majority affect the initial folding of the pro-α chain in the endoplasmic reticulum, resulting in its retention and degradation. A much less common occurrence is a mutation that specifically affects an "active-site" residue necessary for substrate binding and/or catalysis. In this case, hexosaminidase A is present in the lysosome, but it lacks all α-specific activity. This biochemical phenotype is referred to as the "B1-variant form" of Tay-Sachs disease. Kinetic analysis of suspected B1-variant mutations is complex because hexosaminidase A is heterodimeric and both subunits possess similar active sites. In this report, we examine a previously identified B1-variant mutation, α-Val[192]Leu. Chinese hamster ovary cells were permanently cotransfected with an α-cDNA-construct encoding the substitution and a mutant β-cDNA (β-Arg[211]Lys), encoding a β-subunit that is inactive but normal in all other respects. We were surprised to find that the Val[192]Leu substitution produced a pro-α chain that did not form α-β dimers and was not transported to the lysosome. Finally, we reexamined the hexosaminidase activity and protein levels in the fibroblasts from the original patient. These data were also not consistent with the biochemical phenotype of the B1 variant of Tay-Sachs disease previously reported to be present. Thus, we conclude that the Val[192]Leu substitution does not specifically affect the α-active site.

Introduction

There are two major β-hexosaminidase (Hex) isozymes in normal human tissue. Hex A is a heterodimer composed of an α- and a β-subunit, whereas Hex B is composed of two β-subunits. While each subunit contains an active site, dimerization is required for either to become functional (Kytzia and Sandhoff 1985). The presence of the β-subunit increases the stability of the dimer; thus Hex B is heat stable, and Hex A is heat labile. In vivo only the α-active site present in the Hex A heterodimer can catalyze the hydrolysis of the β-linked GalNAc residue from GalNAcβ(1-4)-[NANAα(2-3)-]-Galβ(1-4)-Glc-ceramide (G_{M2} ganglioside) to produce G_{M3} ganglioside. Thus, mutations in either the *HEXA* gene (chromosome 15), encoding the α-subunit, or the *HEXB* gene (chromosome 5), encoding the β-subunit, result in storage of G_{M2} ganglioside, primarily in the lysosomes of neuronal cells, and severe neurological disease, i.e., either Tay-Sachs or Sandhoff disease, respectively (Sandhoff et al. 1989). Hex S is an acidic, unstable α-dimer found only in small amounts in samples from patients with Sandhoff disease, and, despite its functional α-active sites, it cannot hydrolyze G_{M2} ganglioside in vivo (reviewed by Gravel et al. [1995]).

More than 50 mutations in the *HEXA* gene, leading to various forms of Tay-Sachs disease, have been documented (Gravel et al. 1995). Many of them produce unstable mRNA and/or an early stop codon that eliminate all Hex A activity. Demonstration of the relationship between various missense mutations and a clinical or biochemical phenotype is not straightforward. In the case of α-chain-substitution mutations, most of those that have been characterized affect the folding and/or assembly of the protein in the endoplasmic reticulum (ER), i.e., "folding mutations," leading to the accelerated degradation of the monomeric α-chains (reviewed by Mahuran [1991]). In patient cells, folding mutations produce normal levels of α-mRNA, but reduced or undetectable levels of α-cross-reacting material (CRM). If α-CRM is detected, it corresponds to the α-precursor form of the protein (~65 kD), which is assumed to be contained in the ER, rather than to the mature lysosomal α-form (~56 kD). Residual Hex A activity is reduced to 0%–5% of normal activity levels. The degree of reduction can be roughly correlated to the age at onset of clinical symptoms, which in turn correlates to the severity of the phenotype (Leinekugel et al. 1992).

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Another biochemical phenotype is observed with rare α-substitution mutations that affect active site residues, i.e., residues involved in substrate binding and/or catalysis. As in other Tay-Sachs variants, the Hex A activity measured in these patient samples with 4-methylumbelliferyl-β-N-acetylglucosamine-6-sulfate (MUGS), which is hydrolyzed by only α-subunit–containing dimers (Hex A and S), is deficient. The key observations needed to link this finding to the B1 variant form of Tay-Sachs disease are that (*a*) patient samples have near normal levels of mature α-CRM, and/or (*b*) activity levels measured before and after heat denaturation of Hex A by using the common 4-methylumbelliferyl-β-N-acetylglucosamine (MUG) substrate, which is hydrolyzed by both α- and β-active sites, are near normal (reviewed by Sandhoff et al. [1989]; Mahuran [1991]; and Gravel et al. [1995]). Thus, Hex A with an active β-subunit is formed; because of the B1-mutation, however, the active site of the α-subunit is not functional.

Three missense mutations have been associated with the B1 variant phenotype, α-Arg^{178}His (exon 5) (Ohno and Suzuki 1988), α-Val^{192}Leu (exon 6) (Ainsworth and Coulter-Mackie 1992), and α-Asp^{258}His (exon 7) (Fernandes et al. 1992). Of these three, only the biochemical effect of the α-Arg^{178}His has been fully characterized (Brown et al. 1989; Brown and Mahuran 1991). In order to accomplish this characterization, we developed an alternative method of analysis for α-chain mutations that exploits the common evolutionary origins of the α- and β-subunits, i.e., conserved domains and residues in the α- and β-subunits serve similar functions. We expressed Hex B with the mutation in the homologous position in the β-chain, β-Arg^{211}His, transiently in COS cells (Brown et al. 1989). An inactive lysosomal Hex B was formed. However, some small abnormalities in the stability and rate of maturation of the mutant Hex B protein were also noted. These changes were normalized by introducing a more conservative Lys^{211}-substitution into Hex B. The β-Arg^{211}Lys-Hex B had a normal K_m with a V_{max} of only 0.25% of wild type (Brown and Mahuran 1991). Whereas this same approach could be used to characterize the above α-Asp^{258}His substitution (aligned β-residue is Asp^{290}), it cannot be used for the α-Val^{192}Leu substitution, since the aligned residue in the β-chain is Ala^{224}. Indeed, the absence of homology itself suggests that this is not a catalytic-site residue but could be part of the binding site for negatively charged substrates.

In this report, we introduce a new strategy for analyzing α chain mutations that can be used with substitutions that are not in residues conserved in the β-subunit, i.e., the α-Val^{192}Leu substitution (above). This method avoids the necessity of producing the unstable Hex S isozyme but still eliminates the problem of an active β-subunit in the Hex A heterodimer by the incorporation of the β-Arg^{211}Lys substitution (β*, see above). Thus, we cotransfect an α-cDNA construct encoding either wild-type or mutant α- with a mutant β-cDNA construct encoding the inactive β*-subunit (Brown and Mahuran 1991). We have recently shown that, when this procedure is used with a normal α-cDNA, a Hex A (αβ*) is formed that has nearly identical kinetic properties as the wild-type Hex A. These properties include the ability to hydrolyze G_{M2} ganglioside in the presence of human activator protein (Hou et al. 1996). Thus, this method allows us to differentiate between an α–active site mutation that affects substrate specificity and a mutation that acts to generally inactivate the α-subunit.

Material and Methods

DNA Construction and Mutagenesis

In order to create a pREP4-α construct encoding the Val^{192}Leu substitution, a 250-bp *Sac*I cDNA fragment from pSVL-α (Brown and Mahuran 1993) was subcloned into pBS+ (Stratagene). Mutagenesis was carried out by two-step PCR procedure. In the first PCR round, a 100-bp fragment was generated by using T7 primer and a synthetic primer, CTGGATCTCATGGCGTACAA, containing the G→C substitution necessary to produce the Val^{192}Leu substitution. The reactions were performed in 100 μl. Each reaction mix contained 10 ng plasmid DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each of dNTPs, 0.5 μg of each primer, and 5 U *Taq* polymerase (Boehringer Mannheim). The cycling steps used were as follows: 1 cycle of heat denaturation at 94°C for 7 min, 30 cycles each consisting of denaturation at 94°C for 2 min, annealing at 54°C for 2 min, and extension at 72°C for 2 min, in a Perkin Elmer-Cetus thermal cycler. The product of the first PCR amplification was purified and utilized as primer (≥1 μg) along with an antisense T3 primer for the second PCR reaction using the same conditions as mentioned above. The DNA fragment of ~250 bp from the second round was subcloned into a PCR fragment cloning vector by using TA cloning kit (InVitrogen). The mutation was confirmed by sequencing the entire 250-bp fragment by using T7 Pharmacia sequencing kit. The mutant insert was then subcloned into pREP4 to replace the *Sac*I segment. To obtain the mutant pEFNEO-β containing the Arg^{211}Lys substitution (pEFNEO was kindly supplied to us by Dr. D. Anson), a 2,000-bp partial *Bam*HI cDNA fragment from pHexB43 (Arg^{211}Lys) (Brown and Mahuran 1991) was isolated and ligated into *Bam*HI- and alkaline phosphatase–treated pEFNEO-β.

Cell Culture and DNA Transfection

Chinese hamster ovary (CHO) cells were grown in modified Eagle's medium with 10% FCS and antibiotics

at 37°C in 5% CO_2. Transfections were performed according to the lipofection protocol from GIBCO-BRL as described by Hou et al. (1996).

Immunoprecipitation Assay for Hex Activity

Hex A activity from cell lysates and media was determined by using an α-specific substrate, MUGS, while total Hex activity was assayed by a common substrate, MUG. The human Hex (A and B) in transfected cells were separated from CHO-endogenous Hex by immunoprecipitation with CHO-preabsorbed (the glycoprotein fraction from nontransfected CHO cell lysate bound on concanavalin A sepharose) sheep anti-Hex A antiserum coupled to protein G sepharose (Pharmacia) essentially as described by Brown and Mahuran (1991).

RNA Analysis

Total RNA was isolated by using TRIzol reagent (GIBCO BRL 5596UA/UB). Ten-microgram samples of RNA from each of transfected cell line and control CHO were separated by 1.0% agarose gel and then transferred to Hybond-N (Amersham). Hybridizations were performed at 65°C in a solution containing 7% SDS, 500 mM Na_2HPO_4, pH 7.2, 1% BSA, and 1 mM EDTA. The filters were washed two times with 5% SDS, 40 mM Na_2HPO_4, 1mM EDTA, and four times with 1% SDS, 40 mM Na_2HPO_4, 1mM EDTA, at 65°C. The probes were radiolabeled using BRL random primer labeling kit. The probes that were used to detect α- or β-RNA were a 2.0-kb cDNA fragment encoding the α-chain from pSVL-α and a 2.0-kb *Bam*HI cDNA insert encoding the β-chain from pHexB43, respectively. As well, a 500-bp Pst DNA insert encoding β-actin was used as a probe to confirm that equal amounts of total RNA were separated and analyzed (data not shown).

Western Blot Analysis

Equal amounts of total proteins from each sample of cell lysates were resolved by SDS-PAGE by the Laemmli gel system (12.5% gel) using a Bio-Rad mini-gel system. Proteins were transferred to nitrocellulose overnight at 4°C. The filter was blocked and then incubated with 1:800 dilution of rabbit anti-human Hex A or anti-human α-subunit IgG. The filter was developed using Amersham ECL system according to the manufacturer's instructions and as described by Xie et al. (1992).

Indirect Immunofluorescence

Transfected cells were grown at 37°C in 5% CO_2 on chamber slide (Lab-Tek). After 24 h of incubation, the cells were fixed and gently permeabilized with 100% cold methanol at −20°C for 30 min. The fixed cells were then washed three times with PBS, 5 min/wash. Immediately after the third wash, blocking solution (PBS containing 1% BSA [w/v; Sigma] and 2% normal goat serum) was added to the wells and allowed to stand for 30 min, prior to incubation with the primary antibody. Cells were incubated with either of two primary antibodies diluted 1:100 with blocking solution for 1 h in a moist chamber. The IgGs were (*a*) a rabbit anti-Hex B IgG or (*b*) a rabbit anti-Hex A IgG, which was preabsorbed with an excess amount of Hex B to produce an anti-α-IgG. Cells were then washed and the secondary antibody (a fluorescein-labeled goat-anti-rabbit IgG), diluted 1:100 with blocking solution, was added. The cells were then washed three times with PBS and were mounted with elvanol containing p-phenylenediamine (fluorescence preservatives). In control cultures, the preimmune rabbit IgG was substituted for the primary antibody. The slides were analyzed using a fluorescent microscope (Olympus AN-3) at 400× magnification.

Separation of Hex Isozymes by Diethylaminoethyl (DEAE) Ion-Exchange Chromatography

Proteins (5 mg) from control CHO or transfected cell lysates were applied to a 3-ml column of DEAE sepharose CL-6B (Pharmacia). The unbound Hex B fraction was collected by washing the column with 10 mM Na_2HPO_4 (pH 6.0). To remove most of the endogenous CHO Hex A, the column was washed with 50 ml of 0.075 M NaCl in 10 mM sodium phosphate (pH 6.0). In order to separate Hex A from Hex S, a 0.075—0.25 M NaCl gradient was used (Hou et al. 1996). Three-milliliter fractions were collected and assayed for Hex activity. The concentration of salt in each fraction was determined by conductivity measurements (Mahuran and Lowden 1980).

Results

CHO cells were permanently cotransfected with cDNAs encoding either the wild-type pro-α chain and β-Arg^{211}Lys substituted pro-β chain (αβ*), or the α-Val^{192}Leu substituted pro-α chain with the pro-β Arg^{211}Lys chain (α*β*). The α-constructs also contained a hygromycin-selectable marker, while the β-construct contained a neomycin selectable marker. Cells were grown in the presence of both chemicals and individual surviving clones selected for analysis. Confirmation that each CHO clone was expressing both cDNA inserts was obtained by Northern blot analysis (fig. 1*A*).

Individual CHO cell clones that were transcribing from either the α- and β*- or the α*- and β*-cDNAs (fig. 1*A*) were assayed using the common MUG substrate (hydrolyzed by both α- and β-active sites of Hex A) or the α-specific MUGS substrate (table 1). Since the β*-subunit is virtually inactive, any of the activities in table 1 obtained after immunoselection (Bound), to remove endogenous CHO-Hex, are a direct result of hydrolysis by the α-subunit of Hex A. Our immunoprecipitation

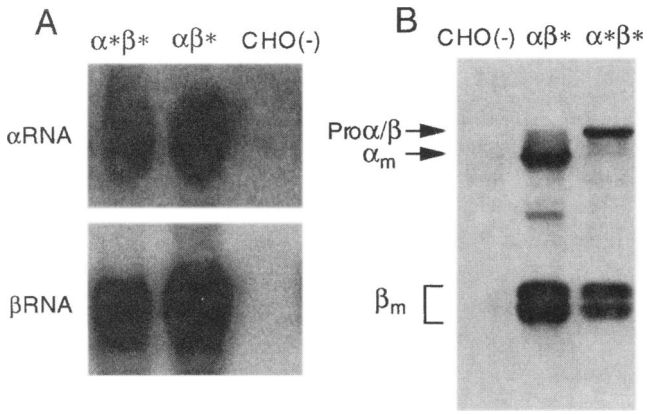

Figure 1 Northern blots (*A*) and Western blots (*B*) of mock transfected CHO cells, CHO(−), and CHO cells cotransfected with either α and β* cDNA (encoding wild-type α- and β-Arg^{211}Lys) or α*- and β*-cDNA (encoding α-Val^{192}Leu and β-Arg^{211}Lys). As shown in panel *A*, total RNA from each line was electrophoretically separated and visualized by probing with either [^{32}P]-normal α- or β-cDNA. As shown in panel *B*, total cell lysate protein from each cell line was separated by SDS-PAGE and visualized using a preabsorbed (untransfected CHO cell lysate) anti-human Hex A IgG, the positions corresponding to the pro-α/-β chains, mature-α, and mature-β chains are indicated.

of the human isozymes followed by our solid-state assay (Brown et al. 1989) produced >100- or >900-fold increases in MUG or MUGS activity, respectively, in CHO cells transfected with the α- and β*-cDNAs as compared to untransfected cells. However, when the α-Val^{192}Leu was substituted for the wild-type α, activities of only approximately twofold above background were detected with either substrate (table 1, α*β*, Bound). These data are consistent with either an inactive α*β*-Hex A, an unstable α*β*-Hex A that is rapidly degraded, or an incorrectly folded α* monomer that cannot form dimers and exit the ER.

To help differentiate between the above possibilities, we analyzed the cotransfected CHO cells by western blotting with anti-Hex A rabbit IgG (fig. 1*B*). Blots failed to detect any CRM in nontransfected CHO cells (fig. 1*B*, CHO[−]), detected large amounts of mature (lysosomal) β- and α-CRM in α- and β*-cotransfected cells (fig. 1*B*, αβ*), and large amounts of mature β but smaller amounts of only pro-α* CRM in CHO cells cotransfected with α*- and β*-cDNA constructs (fig. 1*B*, α*β*). The lack of detectable mature α*-chain is inconsistent with the B1-biochemical phenotype and instead suggests that the α*β*-Hex A is either unstable in the lysosome or that the pro-α* monomer cannot exit the ER.

Because our data contradict those reported by others, who concluded that α-Val^{192}Leu substitution specifically affected the α-active site, we confirmed and extended our results with two additional experiments. The first utilized indirect immunofluorescence to determine whether there were detectable amounts of pro-α* chains in the lysosomes of cotransfected cells. We utilized either anti-β-rabbit IgG or anti-Hex A rabbit IgG absorbed with excess purified human placental Hex B (Mahuran and Lowden 1980), i.e., effectively an anti-α-IgG, as the primary antibody. We found that, whereas the β*-protein detected by the anti-β-IgG produced a distinct punctate pattern indicating a lysosomal localization, whether it was cotransfected with the normal or mutant a construct (fig. 2*D*, *F*), only cells cotransfected with the wild-type α-cDNA produced such patterns when probed with the anti-α-IgG (fig. 2*C*). When the α*β* cotransfected cells were analyzed with the anti-α-IgG, only a diffuse central fluorescence pattern was observed, which suggests that the residual pro-α* protein is retained and degraded in the ER (fig. 2*E*).

Finally, we demonstrated that no inactive pro-α*β* dimers were being formed in the ER of cotransfected CHO cells. Lysates from cells cotransfected with either α- and β*- or α*- and β*-cDNAs were separated by DEAE sepharose 6B-CL. This procedure separates homodimeric Hex B (pI = 6.9) and Hex S (pI = 3.6) from heterodimeric Hex A (pI = 4.8) (Mahuran and Lowden 1980). Pooled fractions eluting between 0.1 and 0.14 M NaCl (containing the peak MUG or MUGS activity) from the α- and β*-cDNA cotransfected cell lysate and the corresponding fractions (containing no activity to-

Table 1

Assay of Hex A from Transfected and Nontransfected (CHO[−]) CHO Cell Lysates with the Common, MUG, or the α-Specific, MUGS, Substrates

	Activity (nmol MU/mg/h [cell lysate])					
	Lysate[a]		Free[b]		Bound[c]	
Hex A	MUG	MUGS	MUG	MUGS	MUG	MUGS
CHO(−)	2.1	.48	2.0	.44	.05	.005
αβ*	8.5	5.4	1.4	.39	6.5	4.5
α*β*	2.1	.41	1.9	.33	.13	.01

NOTE.—Two forms of Hex A were expressed in CHO cells. Cells were permanently cotransfected with two cDNAs encoding either (1) wild-type α- and βArg^{211}Lys, αβ*, or (2) αVal^{192}Leu and βArg^{211}Lys, α*β*. Immunoprecipitation was carried out with a sheep anti-Hex A IgG that had been preabsorbed with excess amount of CHO cell lysate (see Material and Methods) and bound to sepharose protein-G beads.

[a] Hex activity (endogenous CHO and expressed human) in the total CHO cell lysate, i.e., before immunoprecipitation.

[b] Hex activity (endogenous CHO) remaining in the supernatant after immunoprecipitation with anti-Hex A antibody (this precipitates any active human Hex S as well as Hex A).

[c] Hex activity (expressed human) bound to the protein G beads and assayed directly.

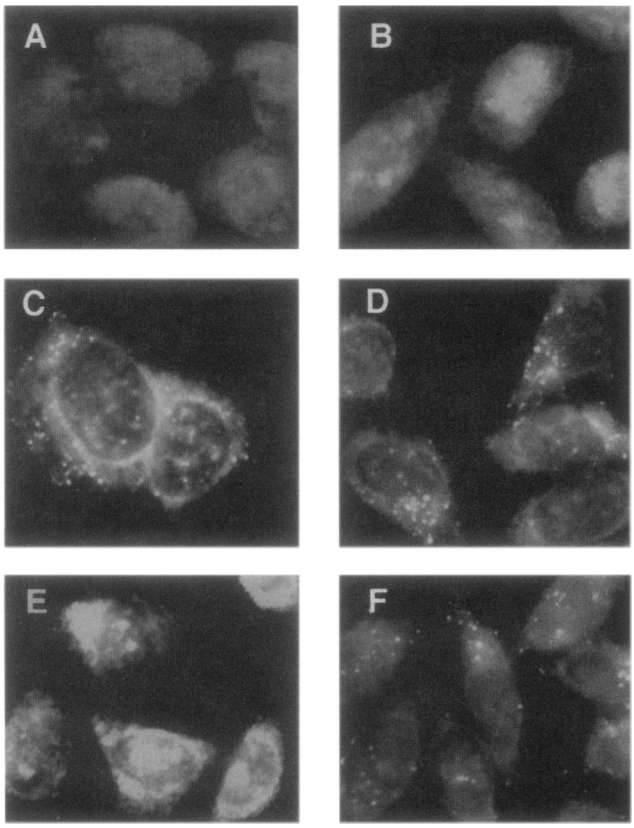

Figure 2 Indirect immunofluorescence of nontransfected CHO cells (*A* and *B*) or cells cotransfected with either α- and β*- (*C* and *D*) or α*- and β*- (*E* and *F*) cDNAs. In panels *A*, *C*, and *E*, cells are visualized utilizing an anti-α IgG as the primary antibody, whereas cells in panels *B*, *D*, and *F* were probed using an anti-Hex B (anti-β) IgG as the primary antibody.

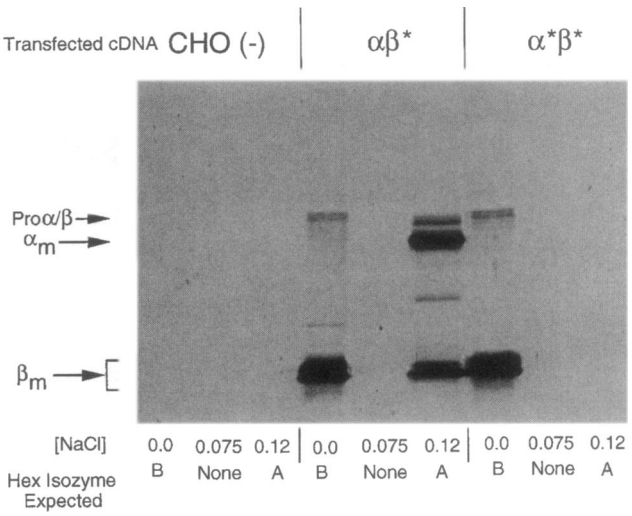

Figure 3 Western blot analysis of the concentrated pools of fractions from the DEAE column separation of untransfected CHO cells, CHO (−), or cell cotransfected with either α- and β*- or α*- and β*-cDNAs. The identities of the subunits producing the immunoreactive bands with the anti-Hex A antiserum are indicated on the left and are of the expected M_r (mature α = 56 kD; mature β = 26–30 kD). The expected Hex isozyme to be eluted at the given NaCl concentrations are shown at the bottom of the figure.

ward either substrate) from the α*- and β*-cDNA cotransfected cell lysate, as well as an inactive sample from the unbound fraction, 0 M NaCl (inactive β*β*-Hex B), were concentrated and analyzed by western blotting (fig. 3). While α- and β-CRM was easily visible in the Hex A (αβ*), 0.12 M NaCl fraction, none was detectable in the Hex A (α*β*) 0.12 M NaCl fraction. The unbound fractions from both cotransfected CHO cell clones produced large amounts of mature β-CRM. Thus, all the data from the various cotransfection studies indicate that the Val^{192}Leu substitution prevents the mutant α-chain from forming heterodimers and exiting the ER.

Conclusive evidence for the above conclusion was obtained by a reevaluation of the biochemical phenotype expressed in fibroblasts from the original Tay-Sachs patient (table 2, fig. 4). Lysates from these cells were assayed by using either (*a*) the α-specific substrate or (*b*) the common MUG substrate before (total Hex) and after (Hex B) heat denaturation. In addition, these lysates were analyzed by western blotting using an anti-Hex A IgG. The results were similar to those obtained from the fibroblasts of a classical Jewish Tay-Sachs patient (table 2, fig. 4). Thus, no heterodimeric Hex A with mature, functional β- and mature, nonfunctional α-subunits is present in the patient's fibroblasts, and the Val^{192}Leu substitution is not associated with the B1 variant phenotype.

Discussion

In a previously published report, two substitution mutations were identified in a single *HEXA* allele, Val^{192}Leu and Val^{200}Met, from a patient apparently ex-

Table 2

Hexosaminidase Activity in Human Fibroblasts before (Total Hex A and B) and after (Hex B) Heat Inactivation with the Common MUG Substrate and Measured Directly (Hex A) with the α-Specific MUGS Substrates

Sample	Total (MUG)	%A (MUG)[a]	MUGS[b]
Normal	10,469	47	500
Patient's (B1?) father	9,446	38.7	346
Patient (B1?)	6,192	4.8	10
Classical Tay-Sachs patient	5,752	4.3	14

NOTE.—Units of Hex activity are in nmol MU/mg/h.

[a] Determined by heat denaturation method, 100× (total-heat stable)/total.

[b] Alpha-specific substrate, nmol MU/mg/h. See Gordon et al. (1988).

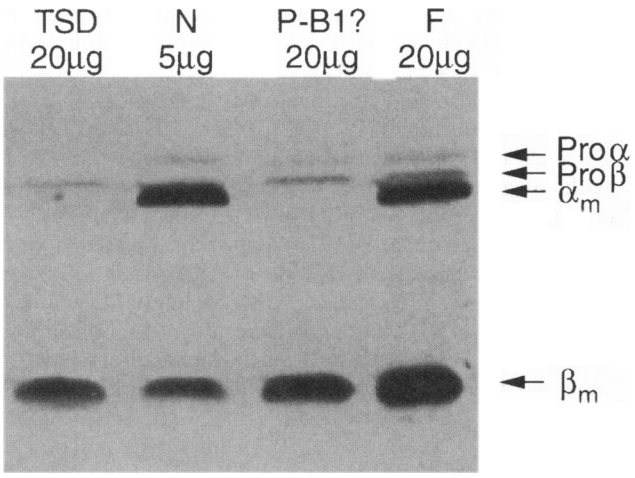

Figure 4 Western blot analysis of lysates from human fibroblasts. TSD is lysate (20 μg) from cells of a Jewish Tay-Sachs patient, homozygous for the common 4-bp insertion mutation in exon 11, which produces unstable mRNA and thus no α-protein (Gravel et al. 1995); N is lysate (5 μg) from normal cells; P-B1? is cell lysate (20 μg) from the patient previously identified as having the B1 variant form of Tay-Sachs disease; F is cell lysate (20 μg) from the patient's, P-B1?, father.

pressing the biochemical phenotype associated with the B1-variant form of Tay-Sachs disease (Ainsworth and Coulter-Mackie 1992). The second mutant allele was later identified as a splicing mutation caused by an G→A transversion at the +1 position of intron 9 (Coulter-Mackie 1994). This splice-junction mutation has been previously linked to the most severe infantile onset form of Tay-Sachs disease, consistent with lack of detectable normal α-mRNA in homozygous patient cells, even by PCR analysis (Akli et al. 1991; McDowell et al. 1992). Of the two substitution mutations, only α-cDNA encoding the Val^{192}Leu or both the Val^{192}Leu and Val^{200}Met substitutions produced little MUGS activity (0%–7%) when coexpressed with a normal β-cDNA in COS-1 cells. However, Hex A activity as measured by MUG hydrolysis after heat denaturation was reported to be ~60% of wild type α-β-cDNA cotransfected cells in both cases, which is suggestive of the B1-phenotype. Thus, the Val^{200}Met substitution was found to be a neutral mutation, and the Val^{192}Leu substitution was identified as the deleterious mutation (Coulter-Mackie 1994). Our earlier report characterizing the COS cell cotransfection method by using normal or mutant α-cDNA along with normal βcDNA (Brown and Mahuran 1993) revealed two major weaknesses in the methodology used to examine the α-Val^{192}Leu substitution (Ainsworth and Coulter-Mackie 1992). We showed that (*a*) Hex B levels cannot be used to normalize Hex A levels in transiently cotransfected COS cells, i.e., correct for variations in transfection efficiency, as was done. This is because total MUG activity (Hex B levels) can double when the cotransfected α-cDNA encodes a mutation that severely inhibits α-chain folding as compared to the wild-type α-cDNA (a phenomenon that can also occur in samples from infantile Tay-Sachs patients [Sandhoff 1969; Sandhoff et al. 1971]). An independent cDNA, e.g., pBLCAT2, a plasmid containing the chloramphenicol acetyltransferase gene, must also be transfected to normalize for transfection efficiency between culture dishes. (*b*) It is always necessary to analyze the transfected cell lysates by western blotting to determine the proportion of pro-α (ER) versus mature-α (lysosomal) chains and to compare the amounts of α-CRM with the units of MUG and MUGS activities, i.e., to obtain an estimate of the Hex A–specific activities. This was not done in the previous report. These data are necessary because residual Hex A activities produced by cotransfected COS cells are much higher than in patient samples if the mutation affects protein folding because of overexpression (Brown and Mahuran 1993; Banerjee et al. 1994; Wu et al. 1994).

The previously reported data (Ainsworth and Coulter-Mackie 1992) when compared to the original analysis of the patient's samples (Gordon et al. 1988) were very suggestive of the B1-phenotype. However, it was difficult to rationalize a role for a neutral amino acid (Val^{192}) in the active site of the α-subunit. The most likely possibility was that it was close to the predicted unique α-binding site for negatively charged substrates, e.g., MUGS. This hypothesis was also consistent with the fact that α-Val^{192} is not conserved in the β-chain. Thus, this mutation presented us with the ideal situation in which to test our new Hex A, CHO cell–expression system. We predicted that the mutation would produce a Hex A (α-Val^{192}Leu:β^{211}-Lys, α*β*) that was active toward the MUG substrate but not toward the MUGS substrate. We unexpectedly found that the α-Val^{192}Leu substitution produced a mutant pro-α chain in cotransfected CHO cells that could not form heterodimers and exit the ER. Thus, no detectable mutant Hex A isozyme was formed with activity toward either substrate. These characteristics are typical of mutations affecting protein folding (Mahuran 1991). Because of the previous report to the contrary, we performed a number of experiments to confirm our conclusion. We (*a*) independently confirmed the sequence of our mutant cDNA insert through the Network of Centres of Excellence, Canadian Genetics Diseases Network's DNA sequencing facility, (*b*) confirmed that the steady-state levels of RNA transcribed by the isolated CHO cell clone from the α- and β*-cDNAs were comparable to the selected clone containing the α*- and β*-cDNAs (fig. 1*A*), and (*c*) demonstrated that both pro-β* and pro-α* were also translated by the latter cloned cells; but unlike the pro-β* chain, the pro-α* was not converted into its mature lysosomal form (fig. 1*B*). (*d*) Whereas the mature α- and β*-pro-

teins were localized to the lysosome in cotransfected CHO cells by immunofluorescence microscopy, the pro-α* and the mature-β* chains were localized to the ER and lysosomes, respectively, in cells coexpressing these proteins (fig. 2). (*e*) We used ion-exchange chromatography to separate any momeric subunits and homodimeric Hex isozymes from heterodimeric Hex A (αβ). Western blot analysis was then used to demonstrated that no inactive α*β* dimers were present in the area where Hex A is expected to elute (Mahuran and Lowden 1980; Mahuran et al. 1985) (fig. 3). (*f*) Finally, we were able to reexamine cultured fibroblasts from the original patient. The analyses of the Hex isozymes by activity measurements, and the individual levels of mature subunit protein by Western blotting, in lysates from these cells were not consistent with the B1-biochemical phenotype (table 2, fig. 4). These data emphasize the care that must be taken in the diagnosis of the B1-variant of Tay-Sachs and in the interpretation of results from transfected COS cells.

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