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Substitution of Alanine^{543} with a Threonine Residue at the Carboxy Terminal End of the β-Chain Is Associated with Thermolabile Hexosaminidase B in a Jewish Family of Oriental Ancestry

RITA DE GASPERI,* MIGUEL A. GAMA SOSA,* EUGENE E. GREBNER,† DEBORAH MANSFIELD,† STEFANIA BATTISTINI,* EDI L. SARTORATO,* SRINIVASA S. RAGHAVAN,* JESSICA G. DAVIS,‡ AND EDWIN H. KOLODNY*

Department of Neurology, New York University School of Medicine, New York, New York 10016; †Division of Medical Genetics, Thomas Jefferson University, Philadelphia, Pennsylvania 19107; and ‡Division of Human Genetics, Cornell University Medical College, New York, New York 10021

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Thermolabile forms of the lysosomal enzyme β-hexosaminidase B (Hex B), likely to result from different genetic defects, have been described. Ten individuals in five generations of a family of Oriental Jewish ancestry were identified biochemically as carriers of a thermolabile Hex B form. The β-chain thermolability was found to be associated with the presence of a G → A transition at nucleotide 1627 of the HEXB gene causing the substitution of Ala^{543} with a threonine. The same mutation was detected in an unrelated Oriental Jew whose Hex B was heat labile. Since thermolabile Hex B has been shown to occur more frequently among Jews of Oriental origin, the Ala^{543} → Thr mutation may be the common mutation associated with β-chain thermolability in this ethnic group.

Two major forms of the lysosomal enzyme β-hexosaminidase (β-Hex, E.C. 3.2.1.52), differing in their physicochemical properties and substrate specificity are normally found in human tissues. Hex A, the enzyme responsible for the catabolism of ganglioside G_{M2}, is a heat labile heterodimer composed of α and β subunits coded by separate genes (HEXA and HEXB, respectively), while Hex B is a heat stable homopolymer composed exclusively of β-chains. Serum contains a third major form, Hex I, which is also composed of β-chains. Deficiency of Hex A activity caused by mutations in the HEXA and HEXB genes results in a group of neurodegenerative disorders collectively known as G_{M2}-gangliosidoses (1). For the diagnosis of G_{M2}-gangliosidosis and for carrier identification the relative levels of Hex A and B in tissues and body fluids are measured by a simple heat inactivation assay which takes advantage of the different thermal stability of the two enzyme forms (1). This assay, however, becomes unreliable if Hex B is no longer thermostable. There have been several reports in the literature describing the existence of thermolabile forms of the β-chain (2–8). Depending upon the case, the thermolabile β-chain was found to form both Hex A and Hex B, albeit characterized by increased thermolability (5, 8), to associate only with the α-chain resulting in the total absence of Hex B (Hexosaminidase Paris variant) (2), or to lose its ability to associate with the α-chain, resulting in deficiency of Hex A activity (3, 4, 7, 9). These observations suggest that the β-chain thermolability is the result of more than one genetic defect.

Studies in the Jewish population have shown that a thermolabile Hex B form is found with the highest frequency in Jews of Oriental origin (10, 11). In this paper we report the biochemical characterization of a thermolabile Hex B form found in a family of Oriental Jewish descent and the identification of a mutation in exon 14 of the HEXB gene likely to be associated with the β-chain thermolability.

MATERIALS AND METHODS

Five generations of a family of Oriental Jewish descent (Iran) carrying the thermolabile Hex B trait were studied (see the pedigree in Fig. 1A). The proband, individual IV-4 in the pedigree, was originally identified as a potential carrier of a thermolabile β-chain through a community screening program for Tay-Sachs disease carriers. By the standard heat inactivation assay he was found to have only 9–12% residual heat stable β-Hex activity in serum and leukocytes. We have also studied another unrelated Jewish individual of Middle Eastern ori-

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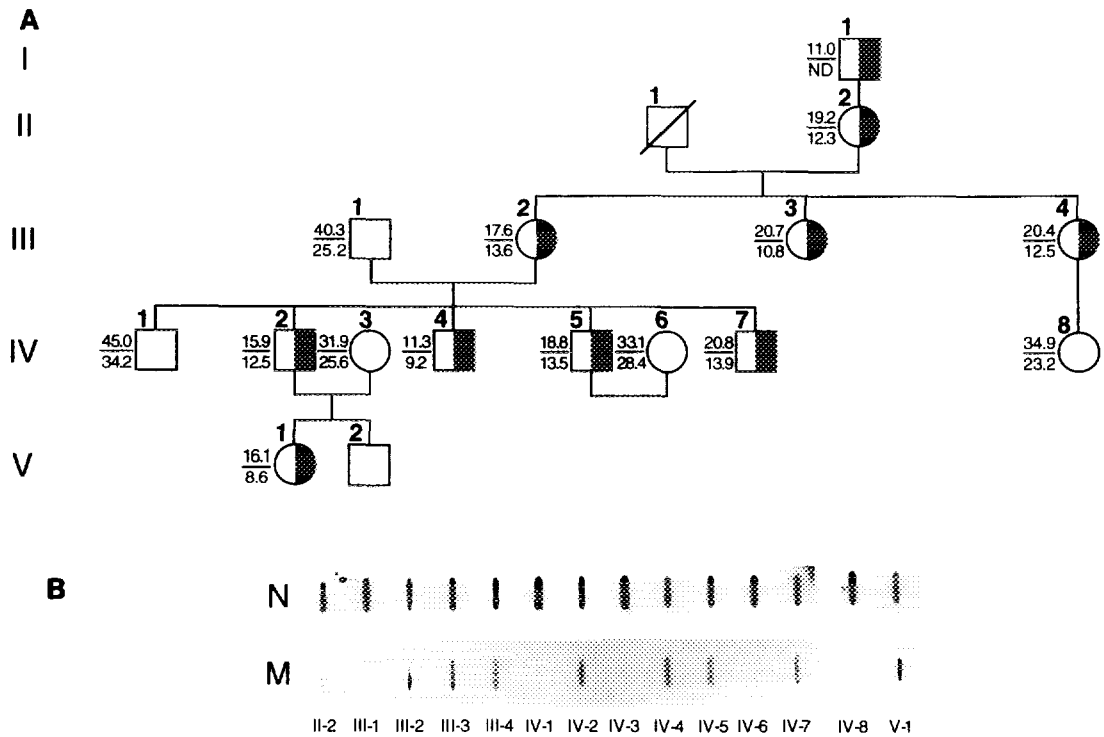

FIG. 1. (A) Pedigree of the Middle Eastern Jewish family presented in this study. The values on the left of each individual represent the percentage of thermostable β-Hex activity in serum (value above the line) and in leukocytes (value below the line) determined by heat inactivation assay at 50°C. ND, the leukocytes of I-1 were not tested. Individuals II-1 and V-2 were not tested. The dark area represents the thermolabile β-chain allele. (B) Analysis of the $Ala^{543} \rightarrow Thr$ mutation in exon 14 of the HEXB gene by allele specific oligonucleotide hybridization. Exon 14 was amplified from the genomic DNA of the family members in A (with the exception of I-1, II-1, and V-2) and hybridized to ^{32}P-labeled oligonucleotide probes specific for the normal sequence (N) (G^{1627}, Ala^{543}) and for the mutated sequence (M) (A^{1627}, Thr^{543}), respectively.

gin (Syria) with 11% residual heat stable β-Hex activity in his leukocytes, suggesting to us that he too may be a carrier of the thermolabile allele.

Total β-Hex activity was measured in serum and leukocytes with the fluorogenic substrate 4-methylumbelliferyl-β-*N*-acetylglucosaminide (MUG) (12). Heat inactivation assays were performed by incubating serum and leukocyte extracts diluted in 50 mM Na citrate phosphate buffer, pH 4.4, containing 0.5% bovine serum albumin at 50°C for the indicated period of time.

The β-Hex forms were isolated by ion-exchange chromatography through a DE-52 column (Whatman, Clifton, NJ) equilibrated with 10 mM Na phosphate buffer, pH 6.0. After collecting the Hex B activity in the unadsorbed fraction, a linear NaCl gradient (0–0.2 M) was applied to the column to elute Hex I, followed by Hex A. The fractions containing the highest activity of each enzyme component were dialyzed against 10 mM Na citrate phosphate buffer, pH 4.4, for subsequent studies.

Genomic DNA was isolated from peripheral blood leukocytes. Poly(A)+ mRNA was isolated from cultured fibroblasts of individual IV-4 as described (13). The poly(A)+ mRNA was reverse transcribed and the β-chain cDNA amplified in six overlapping fragments using the primers described by Dlott *et al.* (14). The reaction mixture contained 60 mM Tris–HCl, pH 8.5, 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 μg of each primer, and 2.5 U of *Taq* polymerase (Perkin–Elmer, Norwalk, CT). The amplification was carried out for 35 cycles as follows: 94°C for 15 s, 55°C for 30 s, and 72°C for 45 s. The PCR-amplified products were cloned in the vector pCRII (Invitrogen, San Diego, CA) and the resulting clones sequenced by the dideoxynucleotide termination method using Sequenase (USB, Cleveland, OH). Exon 14 was amplified from the genomic DNA of IV-4 using the following primers: 5′ TCATGTTATCTACAGACGTGGA and 5′ GTAGTACAGATTGCTGTGGCC (14), and the resulting product cloned and sequenced as above. For allele specific oligonucleotide hybridization (ASOH), an aliquot of the amplified exon 14 DNA was applied to Immo-

bilon S (Millipore, Bedford, MA) and hybridized overnight at 42°C to the following ^{32}P-labeled oligonucleotides: 5′ GAATAGCT**G**CACAACCT (normal probe) and 5′ GAATAGCT**A**CACAACCT (mutant probe). The filters were then washed in 6× SSC, 0.1% SDS, at 50°C for 10 min and autoradiographed.

Site-directed mutagenesis of the β-chain cDNA was performed using the mutagenic oligonucleotide 5′ GAGGTTGTGTAGCTATTCC as described (15). The wild-type and the mutant β-chain cDNA were subsequently subcloned into the eukariotic expression vector pSVL (15) and the resulting constructs purified by CsCl-ethidium bromide isopycnic centrifugation. Each construct (5 μg) was transfected into COS-7 cells using the calcium phosphate coprecipitation method (16). As an internal control for efficiency, 2 μg of the indicator plasmid pBLCAT2 containing the chloramphenicol acetyl transferase gene (CAT) (17, 18) was included in each transfection experiment. After 48 h, the cells were harvested and the β-Hex and CAT activity (19) was determined in cell extracts. In some experiments, 20 μM leupeptin was added to the culture medium after transfection (20).

RESULTS

Biochemical Characterization of β-Hex

In heat inactivation assays the serum, leukocytes, and cultured fibroblasts of individual IV-4 (see pedigree in Fig. 1A) were found to have reduced levels of Hex B. Separation of the β-Hex forms by ion-exchange chromatography showed that the levels of Hex B in the fibroblasts of individual IV-4 were similar to those found in the fibroblasts of a control individual, suggesting that the apparently reduced level of heat stable β-Hex in IV-4 was due to the presence of a thermolabile Hex B. Thermal stability studies using the Hex B fraction isolated by ion-exchange chromatography showed that at 50°C the enzyme from IV-4 lost 50% of its activity in the first 20 min. This was followed by a slower inactivation phase so that after 180 min only 25% of the original activity was heat stable. Under the same conditions, the control Hex B was stable (Fig. 2A). Heat inactivation assays at different temperatures from 46 to 52°C confirmed the intrinsic thermolability of the Hex B from IV-4 (Fig. 2B). Similar results were obtained with Hex I (results not shown). The Hex A isolated from IV-4 also appeared to be slightly more thermolabile than the control Hex A at 50°C (Fig. 2C). This became more evident when the stability of Hex A was studied as a function of the temperature. As shown in Fig. 2D, at 46°C the control Hex A lost about 40% of its activity in 2 h, while under the same conditions the Hex A isolated from IV-4 lost 60% of the original activity.

We then studied five generations of the family of IV-4 as seen in Fig. 1A and were able to identify nine additional individuals who appeared to have inherited the thermolabile Hex B trait (see Fig. 1A). These included the oldest member of the family (I-1), a 104-year-old man. The β-Hex elution profiles obtained by ion-exchange chromatography of the sera of all the family members tested were essentially identical, again suggesting that those individuals who had reduced levels of Hex B in heat inactivation assays actually had normal amounts of the enzyme. As shown in Table 1, the Hex B fraction isolated from the sera of III-1, IV-1, IV-3, IV-6, and IV-8 was stable after 180 min at 50°C, while the enzyme isolated from the remaining family members was thermolabile and lost, depending upon the individual, about 55 to 75% of the original activity. Similar results were obtained with the Hex I fraction (Table 1). The biochemical results also suggested that the 10 individuals with heat labile Hex B must be heterozygous for the allele causing β-chain thermolability, otherwise no residual thermostable Hex B activity would have been found after 2 h at 50°C.

Molecular Analysis of the β-Chain Coding Sequences

To understand the molecular basis of the Hex B thermolability, we sequenced the β-chain cDNA amplified from the mRNA isolated from the fibroblasts of IV-4. A G → A transition at position 1627 of the coding sequence was the only base alteration found in the entire β-chain cDNA. Sequence analysis of PCR-amplified genomic sequences containing exon 14 confirmed the presence of the $G^{1627} \rightarrow A$ mutation in IV-4 (Fig. 3). This mutation was found in about half of the clones analyzed, which was consistent with the biochemical data suggesting a carrier status for the thermolabile allele. As a result of the mutation, Ala^{543}, which is located at the C-terminal end of the enzyme, is substituted by a threonine. Analysis of the $Ala^{543} \rightarrow$ Thr mutation in the HEXB gene of the other family members showed that this mutation was carried only by those individuals whose Hex B was thermolabile (see Figs. 1A and 1B). The same $Ala^{543} \rightarrow$ Thr mutation was also found in another Middle Eastern Jewish individual unrelated to the family described above, whose bio-

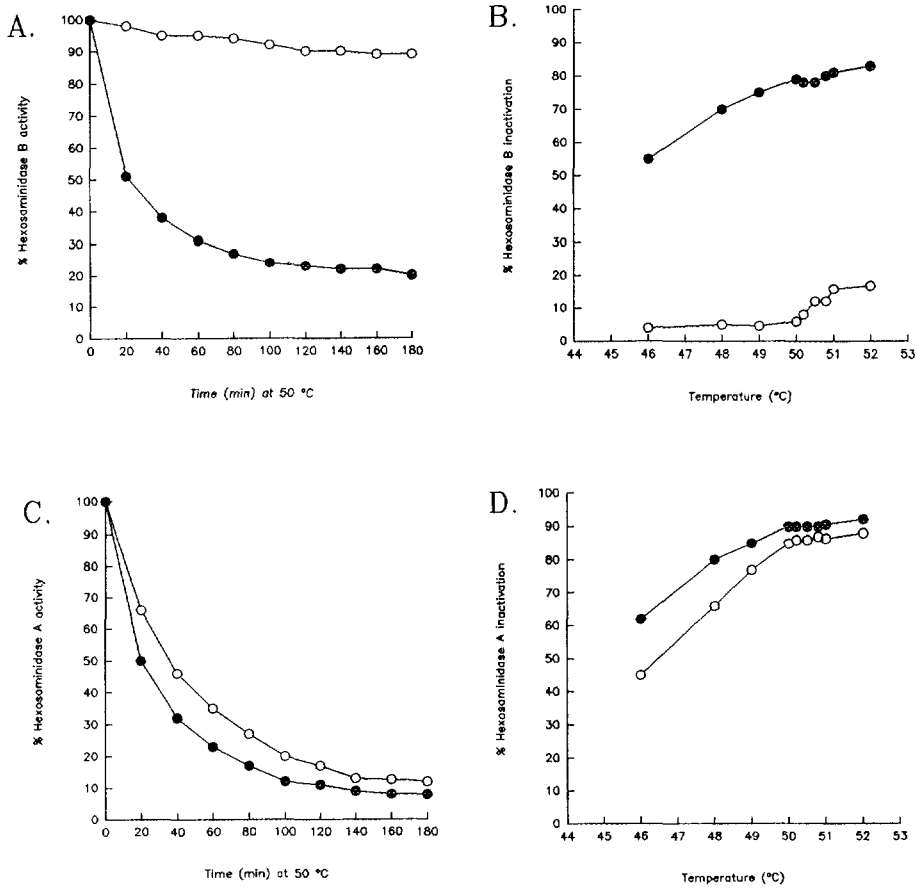

FIG. 2. Thermal inactivation (A) and thermal stability at different temperatures (B) of Hex B isolated from the fibroblasts of IV-4 (●) and of a control individual (○). The Hex B fraction isolated by ion-exchange chromatography was incubated for the indicated periods of time at 50°C (A) or for 2 h at the indicated temperatures (B) and the residual activity measured with MUG. (C and D) Same as A and B, except that the experiments were performed with the Hex A fraction isolated from the fibroblasts of IV-4 (●) and of a control individual (○).

chemical data had suggested that he may also be a carrier of heat labile β-chain (Fig. 4).

A mutant β-chain cDNA containing the $G^{1627} \rightarrow A$ mutation was prepared by site-directed mutagenesis and cloned into the eukariotic expression vector pSVL. Transfection of two independent mutant constructs in COS-7 cells did not result in the expression of any Hex B activity over background. Under the same conditions, the Hex B activity in COS cells transfected with the wild-type β-chain was on average 13-fold over the activity of mock-transfected cells. Cotransfection of each mutant construct separately with the plasmid containing the wild-type cDNA resulted in the expression of thermostable Hex B activity only. Changes in the amount of DNA transfected and in the method of transfection as well as addition of 20 μM leupeptin to the medium after transfection (20) did not result in expression of any mutant Hex B activity. Cotransfection of the mutant β-chain cDNA with the α-chain cDNA also failed to result in increased Hex A activity. The amount of CAT activity expressed in the cells transfected with the mutant constructs and with the wild-type plasmid was comparable, indicating that the lack of expression of the mutant enzyme was not due to a poor efficiency of transfection. Also sequence analysis of the entire mutant cDNA did not reveal the presence of any other alteration which could explain the lack of expression in COS cells.

DISCUSSION

We have identified 10 individuals belonging to five generations of a Jewish family of Middle Eastern origin in which the allele conferring heat lability to the Hex β-chain had segregated. The biochemical results indicated that they must be heterozygotes for the heat labile β-chain allele because of the pres-

TABLE 1

Thermostability of Hex B and Hex I Isolated by Ion-Exchange Chromatography from the Sera of Family Members and Controls

Case[a]	%Hex B[b]	%Hex I[b]
II-1	51.3	85.8
III-1	92.4	90.7
III-2	41.1	45.1
III-3	42.8	83.7
III-4	42.0	57.1
IV-1	84.0	88.0
IV-2	44.7	45.3
IV-3	94.2	99.6
IV-4	24.3	52.7
IV-5	31.7	20.8
IV-6	96.1	91.4
IV-7	36.7	47.2
IV-8	94.5	100.0
V-1	37.0	64.6
Control[c]	92.4 (±3.8)	96.4 (±5.3)

[a] See pedigree in Fig. 1A.

[b] Residual activity of Hex B and Hex I after 2 h at 50°C expressed as percentage of the original activity.

[c] Average of the residual activities of Hex B and Hex I isolated from the sera of six control individuals. The standard deviation is indicated in parentheses.

ence of residual thermostable activity in serum and leukocytes after incubation at 50°C. The Hex B heat inactivation curve (such as the one shown in Fig. 2A) suggested that the enzyme was likely to be a mixture of three forms, one containing both thermolabile β-chains ($\beta^t\beta^t$) reflected in the initial rapid loss of activity, one containing one thermolabile and one thermostable β-chain ($\beta^t\beta$) reflected in the slower inactivation phase, and the ββ form responsible for the residual heat stable activity. The differences in the amount of thermostable Hex B in the various individuals (Table 1) indicated that the ratio among the three Hex B forms was variable, at least for the enzyme isolated from serum. Assuming the presence of equal amounts of both types of β-chain and their free association, the binomial theorem predicts the proportion of the thermostable fraction to be 25% (5), which was indeed the case for individual IV-4. In addition, the labile β-chain appeared to associate also with the α-chain as shown by the increased heat lability of the Hex A. From the above data we concluded that the thermolabile β-chain could associate with itself or with the wild-type β-chain to produce Hex B and with the α-chain to form Hex A. It is therefore different from those thermolabile β-chain forms which failed to associate with the α-chain (3, 4, 7) or which appeared to form only Hex A (2).

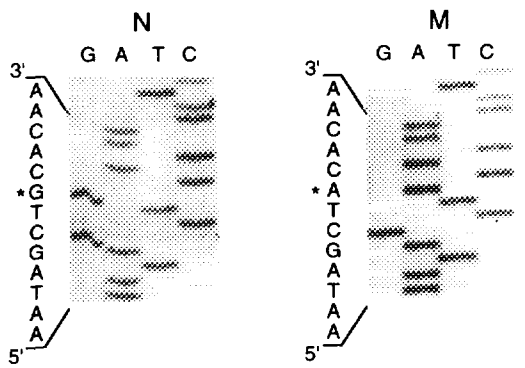

FIG. 3. Nucleotide sequence of exon 14 of the HEXB gene from individual IV-4. The sequences of two representative clones representing, respectively, the normal allele (N) (G^{1591}) and mutant allele (M) (A^{1627}) are shown.

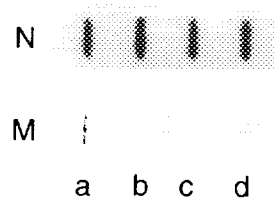

FIG. 4. Identification of the $Ala^{543} \rightarrow$ Thr mutation in the individual with heat labile Hex B unrelated to the family shown in Fig. 1. Exon 14 was amplified from the genomic DNA isolated from the above individual (a) and from three controls (b–d), and analyzed by allele specific oligonucleotide hybridization. N, normal probe (G^{1627}); M, mutant probe (A^{1627}).

Analysis of the HEXB gene showed that the individuals with heat labile Hex B were carriers of a missense mutation causing the substitution of Ala^{543} with a threonine residue at the C-terminal end of the β-chain. Interestingly, the same mutation was detected in another unrelated Jewish individual, also of Oriental descent, who also appeared to carry a thermolabile Hex B form.

Transient expression studies were performed to demonstrate that the $Ala^{543} \rightarrow$ Thr substitution results in β-chain thermolability. However, no mutant Hex B activity could be expressed in COS-7 cells. Since, under the same condition, the wild-type Hex B could be expressed, it can be concluded that the β-chain carrying the $Ala^{543} \rightarrow$ Thr mutation may be particularly unstable in COS cells. Stabilization of the mutant β-chain could not be achieved by the addition of leupeptin, a thiol protease inhibitor which is known to increase the stability of proteins in lysosomes (20) or by coexpression of either the wild-type β-chain or the α-chain. It is interesting to point out that the mutation here described involves an amino acid residue located at the C-terminal re-

gion of the β-chain, which has been suggested to be important for the proper folding of the protein (14). Mutations causing the in-frame insertion of six or eight amino acids in this area were found to result in the production of an elongated β-subunit which did not fold properly (14), while a mutation at Arg^{505} ($Arg^{505} \rightarrow Gln$) was shown to result in Hex B thermolability (7). It is also noteworthy that Ala^{543} is part of a four-amino-acid stretch which is conserved in the α-chain (21), suggesting that this residue is likely to play a role in the proper folding of both subunits.

Since a thermolabile Hex B form has been reported to occur more frequently in Jewish individuals of Oriental origin (10, 11), it will be of interest to determine whether the $Ala^{543} \rightarrow Thr$ mutation here described may be the common mutation associated with thermolabile Hex B in this ethnic group.

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Note added in proof. Ala^{543} hereby reported corresponds to Ala^{531} in the sequence reported by Proia (21).

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