Benign HEXA Mutations, C739T(R247W) and C745T(R249W), Cause β-Hexosaminidase A Pseudodeficiency by Reducing the α-Subunit Protein Levels*

(Received for publication, February 5, 1997, and in revised form, April 3, 1997)

Zhimin Cao‡, Emmanuel Petroulakis, Timothy Salo, and Barbara Triggs-Raine§
From the Department of Biochemistry and Molecular Biology, University of Manitoba, Winnipeg, Manitoba R3E 0W3, Canada

Two benign mutations, C739T(R247W) and C745T(R249W), in the α-subunit of β-hexosaminidase A (Hex A) have been found in all but one of the currently identified Hex A-pseudodeficient subjects. To confirm the relationship of the benign mutations and Hex A pseudodeficiency and to determine how the benign mutations reduce Hex A activity, we transiently expressed each of the benign mutations, and other mutations associated with infantile, juvenile, and adult onset forms of G M2 gangliosidosis, as Hex S (αα) and Hex A (αβ) in COS-7 cells. The benign mutations decreased the expressed Hex A and Hex S activity toward the synthetic substrate 4-methylumbelliferyl-6-sulfo-β-N-acetylglucosaminide (4-MUGS) by 60–80%, indicating that they are the primary cause of Hex A pseudodeficiency. Western blot analysis showed that the benign mutations decreased the enzymatic activity by reducing the α-subunit protein level. No change in heat sensitivity, catalytic activity, or the substrate specificity to the synthetic substrates, 4-methylumbelliferyl-β-N-acetylglucosaminide or 4-methylumbelliferyl-6-sulfo-β-N-acetylglucosaminide, was detected. The effects of the benign mutations on Hex A were further analyzed in fibroblasts, and during transient expression, using pulse-chase metabolic labeling. These studies showed that the benign mutations reduced the α-subunit protein by affecting its stability in vivo, not by affecting the processing of the α-subunit, i.e. phosphorylation, targeting, or secretion. Our studies also demonstrated that these benign mutations could be readily differentiated from disease-causing mutations using a transient expression system.

The lysosomal hydrolase, β-hexosaminidase (β-N-acetylhexosaminidase, EC 3.2.1.52), has two major isoenzyme forms, A (Hex A)1 and B (Hex B), and a minor form, S (Hex S) (reviewed in Ref. 1). These isozymes are dimers formed from the α-subunit encoded by HEXA and/or the β-subunit encoded by HEXB. The primary natural substrate of this enzyme, G M2 ganglioside, is only hydrolyzed by the αβ dimer, Hex A, with the help of the G M2 activator protein (GM2A gene), which binds and solubilizes the sphingolipid for hydrolysis (2, 3). The presence of mutation on both alleles of any one of the genes, HEXA, HEXB, or GM2A, can result in a loss of Hex A (αβ) activity in vivo and G M2 gangliosidosis (reviewed in Ref. 4).

HEXA mutations that cause a complete Hex A deficiency and Tay-Sachs disease, have a higher frequency among Ashkenazi Jews (5) and French Canadians of Eastern Quebec (6). Screening programs for the prevention of this disease have been established (7). They utilize a synthetic substrate, 4-methylumbelliferyl-β-N-acetylglucosaminide (4-MUG), in combination with a heat denaturation step, to differentiate the activities of the heat-stable Hex B (ββ) from Hex A (αβ) (8, 9). Through these programs, healthy individuals with a low in vitro Hex A activity (i.e. pseudodeficient) have been identified (10–15). Pseudodeficiency complicates prenatal diagnosis because a pseudodeficient fetus may be wrongly diagnosed as affected (14, 16).

Two benign mutations, C739T(R247W) and C745T(R249W), have been identified in subjects with Hex A pseudodeficiency (17, 18). Pseudodeficient subjects are compound heterozygotes, typically having the C739T mutation on one chromosome and a common Tay-Sachs disease mutation on the other. Studies of the percentage of Hex A in their samples using the synthetic substrate 4-MUG, revealed very low levels in serum (0–15%), and higher levels in leukocyte (13–24%) and fibroblast (8–26%) samples (10–15). Using G M2 ganglioside, fibroblast-loading studies and enzyme assays gave results in the low normal range (10, 11, 13, 14, 17). Metabolic labeling studies in fibroblasts from two subjects harboring the benign mutation C739T showed that the α-subunit protein was processed to its mature lysosomal size (13, 14). Although this analysis was not quantitative, the protein level was consistent with that which might be expected in a Tay-Sachs disease heterozygote. Some investigators proposed that the enzyme's capacity to hydrolyze the synthetic, but not the natural, substrate was reduced (10, 11, 17). Others suggested that there was a reduction in the activity of the enzyme toward both synthetic and natural substrates and that a differential tissue distribution accounted for the very low serum levels (13, 14).

Our aims were to determine 1) if the benign mutations are the primary cause of Hex A pseudodeficiency; 2) if the benign mutations can be differentiated from disease-causing muta-

---

* This work was funded in part by Medical Research Council of Canada (MRC) Grant MT-11708. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a Manitoba Health Research Council (MHRC) studentship. Current address: Dept. of Pathology, University of Louisville School of Medicine, Louisville, KY 40292.

§ Supported by a Medical Research Council scholarship. To whom correspondence and reprint requests should be addressed: Dept. of Biochemistry and Molecular Biology, University of Manitoba, 770 Bannatyne Ave., Winnipeg, Manitoba R3E 0W3, Canada. Tel.: 204-789-3218; Fax: 204-783-0864; E-mail: triggs@bldghsc.lan1.umanitoba.ca.

1 The abbreviations used are: Hex A, B, and S, β-hexosaminidase A, B, and S, respectively; 4-MUG, 4-methylumbelliferyl-β-N-acetylglucosaminide; 4-MUGS, 4-methylumbelliferyl-6-sulfo-β-N-acetylglucosaminide; α-MEM, α-minimum essential medium; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; G M2, G M2 ganglioside, GalNAcβ(1,4)-[N-acetylenuraminic acid (2,3)-]Galβ(1–4)-Glc-ceramide.
tions using a transient expression system, and 3) how the benig

EXPERIMENTAL PROCEDURES

Plasmids, Reagents, and Cell Lines—The vectors pBluescript (pBS) and pSVL were purchased from Stratagene and Pharmacia Biotech, Inc., respectively. The β-subunit expression vector pCD43 (19) and pTK18 plasmid (20) were gifts from Dr. Roy Gravel (McGill University, Montreal). The β-galactosidase expression vector, pRCoCMV-β-gal, was provided by Dr. D. Litchfield (University of Western Ontario, Canada). Double-stranded DNA sequencing kits were obtained from Pharmacia; Genecon II kits from BIO/CAN Scientific (Mississauga, Canada); restriction and DNA modifying enzymes from New England Biolabs or Life Technologies, Inc.; PansorbinTM from Calbiochem; methionine/cysteine-free minimum essential medium (Met/Cys-free medium) from ICN Pharmaceuticals; 4-MUG and 4-methylumbelliferyl-6-sulf-o-N-acetylglucosaminide (4-MUGS) from Toronto Research Chemicals Inc. (Toronto, Canada); dila-

Cell Culture—POS-7 and human fibroblast cell lines were cultured in α-MEM containing 10% fetal bovine serum and penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C in 5% CO2. For some experiments, 200 μg/ml leupeptin was added to the α-MEM when the cells were about 60% confluent.

DNA Transfection—Plasmid DNA for transfection was isolated using Nucleobond AX columns and quantitated by gel electrophoresis and absorbance at 260 nm. COS-7 cells, grown to 60–80% of confluence, were collected by trypsinization, washed, and resuspended in PBS containing 0.7% NaCl, 138 mM NaCl, 8.1 mM Na2HPO4, pH 7.6. Approximately 3 × 105 cells were mixed with the DNA in total volume of 400 μl of PBS for transfection (6.5 μg of opSVL, or its derivatives, alone for Hex S expression, and together with 2.3 μg of pCD43 for Hex A plus S expression). Two μg of pRCoCMV-β-gal vector, a concentration that did not influence the expressed Hex S and Hex A plus S activities, was co-transfected to express β-galactosidase as a measure of transfection efficiency. Expression vectors were introduced into the cells by electroporation using an Electro Cell Manipulator 600 (BTX Inc., San Diego, CA) with the settings of 150 charging volts, 48 ohms, and 1200 microfarads (33). The medium was changed at 24 h, and the cells were harvested at 72 h post-transfection.

Preparation of Cell and Medium Extracts—Cell extracts for enzyme assays were prepared differently from that for immunoprecipitation. For enzyme assays, the cell was washed with ice-cold PBS, scraped off into TEF buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl) and pelleted by centrifugation. Cells were resuspended in a 20 mM Tris-HCl, pH 7.0, and lysed by three rounds of freezing (−70 °C) and thawing (37 °C), and cell debris was removed by centrifugation at 14,000 × g for 20 min. For immunoprecipitation, cell and medium extracts were prepared according to a protocol by Proia et al. (34) except that lysis buffer and the solutions used in the preparation procedures contained 1 mM Pefabloc.

Protein Determination and Enzyme Assay—Protein concentrations were determined by the Bradford method (35) using γ-globulin as the standard. Hex S and Hex A plus S activities were measured using 4-MUG or 4-MUGS as a substrate (9). β-Galactosidase activity was determined using 4-methylumbelliferyl-β-D-galactoside as a substrate (36).

Separation of Hex A, B, and S Isozymes—Previously described protocols were modified to separate Hex A from Hex B and Hex S (1, 37). A DEAE-cellulose column (1.4-ml bed volume) was equilibrated with 10 mM phosphate buffer, pH 6.0, and 400–900 μl (1.2–3.0 mg of protein) of cell extract was loaded. After loading, the column was washed with normal MCH065, Tay-Sachs disease WG1881 (both from the Repository of Mutant Human Cell Strains, Montreal, PQ) and Sandhoff disease GM00294 (NIGMS Human Genetic Mutant Cell Repository, Camden, NJ), respectively. These samples were identified as subject B (GM04863) and F (TC72) in a previous study (17), where they were shown to be compound heterozygotes for the C739T[R247W] mutation and a second mutation associated with Tay-Sachs disease. The second allele in GM00294 was C745T [I294T] (24), and in F the donor was normal (25). The control fibroblast cell lines included normal WP09 (Winnipeg), normal MCH065, Tay-Sachs disease WG1881 (both from the Repository for Mutant Human Cell Strains, Montreal, PQ) and Sandhoff disease GM00294 (NIGMS Human Genetic Mutant Cell Repository, Camden, NJ).

Vector Construction and Site-directed Mutagenesis—The α-subunit cDNA fragment from pTK18 (NarI/PstI) was subcloned into pBSC (~5' AccI/StuI) to create pHEXa2 and into pSVL to create opSVL as described (25). The C739T mutation was introduced into the opSVL cDNA by replacing a NdeI/SnaBI fragment with the same fragment from a polymerase chain reaction product generated from fibroblast cDNA contain-

Analysis of Hex A Pseudodeficiency Mutations
with a mix of 3.8 ml of this medium, 150 μl of dialyzed fetal bovine serum, and 1 μl of 32P (500 mCi/ml).

For immunoprecipitation, two methods were used. The first method was according to Proia et al. (34) except that three different polyclonal antibodies, against human α-subunit, Hex B, and Hex A, were used for immunoprecipitation. In the second method, total α-subunit was first immunoprecipitated using anti-Hex A antibody and following the method of Proia et al. (34). The immunoprecipitated complexes were then dissociated by suspending the antibody-antigen-Pansorbin cell pellet in 50 μl of a denaturing solution (20 mM Tris-HCl, pH 7.4, 1% SDS, 20 mM dithiothreitol) and incubating at 100 °C for 10 min followed by a centrifugation. This process was repeated once, and supernatants were combined and mixed with 0.9 ml of lysis buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P40, 0.02% NaN₃, 1 mM Pefabloc) containing 1% bovine serum albumin and 4 mM N-ethylmaleimide. N-Ethylmaleimide was used as a chelator of dithiothreitol in the denaturing solution. The total α-subunit in the mix was then immunoprecipitated using 4 μl of anti-α-subunit antibody and following the method of Proia et al. (34). The process was repeated once to completely recover the α-subunit.

**High Porous Gradient SDS-PAGE**—High porous 5–12% gradient polyacrylamide separating gels (0.075 × 12 × 16 cm) and 4% polyacrylamide stacking gels were prepared and run as described previously (41, 42).

**Fluorography and Autoradiography**—Signals from 35S-labeled proteins were detected by fluorography according to the instruction provided by the manufacturer of DuPont Entensify solution.

Autoradiography was used to detect the signals of the 32P-labeled proteins. The gel was incubated twice for 30 min in 50 ml of fixing solution (acetic acid:methanol:H₂O = 10:20:70), dried, and exposed to Kodak X-omat film.

**RESULTS**

To determine the effects of the benign mutations, C739T(R247W) and C745T(R249W), on Hex A activity and the α-subunit protein, both a transient expression system and fibroblast cell lines from normal and Hex A pseudodeficient subjects were employed. Hex S was expressed and analyzed in this study because it is an α-subunit dimer, and if the benign mutation was present on both of the subunits in Hex S, their effects might be more obvious than on Hex A (αβ). Hex A was also expressed to mimic the physiological form of the enzyme.

**Analysis of Expressed Hex S Activity and α-Subunit Protein**—To determine the effects of the benign mutations on the activity of Hex S and the α-subunit protein level, normal and mutant Hex S was expressed in COS-7 cells. Four mutant vectors, containing C739T(R247W), C745T(R249W), G805A(G269S), or C508T(R170W) mutations associated with Hex A pseudodeficiency (two vectors), adult onset and infantile forms of GM2 gangliosidosis, respectively, were expressed. The results showed that normal Hex S activity (1026 ± 200 nmol/h/mg of protein) was expressed to a level more than 10 times that of the β-hexosaminidase activity in COS-7 cells (82 ± 10 nmol/h/mg of protein). For comparison, the wild-type Hex S activity, after subtracting the COS-7 cell background and normalization, was expressed as 100%. The activities of the various mutant Hex S isozymes were converted to a percentage of the wild-type (Fig. 1). Data revealed that the activity of Hex S harboring the benign mutations, R247W and R249W, was about 20–35% of the wild-type Hex S activity and was substantially higher than that of Hex S containing the G269S substitution that is associated with adult onset GM2 gangliosidosis.

The level of the mature α-subunit protein containing the benign mutations was also decreased to about 40% of the normal level (Fig. 2), consistent with its reduced enzyme activity. No detectable mature form, but a normal level of the precursor form, of the α-subunit corresponding to the G269S- and R170W-containing Hex S was observed.

**Analysis of Expressed Hex A plus S Activity and α-Subunit Protein**—To examine the effects of the benign mutations, C739T(R247W) and C745T(R249W), on Hex A plus S activity and the α-subunit protein, normal and mutant Hex A plus S were transiently expressed by introducing pCD43, together with αpSVL or its mutant variants (including the G479AαpSVL construct, which contains a mutation associated with juvenile onset G479A gangliosidosis) into COS-7 cells. Once again, the specific activity of the expressed normal Hex A plus S was about 10-fold above the COS-7 cell background (Fig. 1). The specific activity of Hex A plus S harboring the benign mutation, R247W and R249W, was about 38 and 22% of that of the normal Hex A plus S, respectively (Fig. 1). Hex A plus S
Analysis of Hex A Pseudodeficiency Mutations

FIG. 2. Levels of expressed α-subunit protein containing the benign and disease-causing mutations. The α-subunit cDNA and its various mutant derivatives were transfected alone to express Hex S (A) or together with the β-subunit to express Hex A plus S (B and C). The amount of protein loaded for each sample within a panel (A and B) or together with the β-subunit to express Hex A plus S (B and C) was normalized to the co-expressed β-galactosidase activity, with the exception of the mock (25–30 μg) and fibroblast (30–50 μg) samples. The α- and β-subunits of β-hexosaminidase were detected with a polyclonal anti-Hex A antibody. A, lane 1, αPSVL (normal); lane 2, C739TαPSVL (benign); lane 3, C745TαPSVL (benign); lane 4, G805αapolSVL (adult onset); lane 5, C508TαSVL (infantile); lane 6, MCH065fibroblast (normal); lane 7, WG1881 fibroblast (Tay-Sachs disease); lane 8, GM00294 fibroblast (Sandhoff disease); lane 9, mock-transfected COS-7 (background); lane 1, αPSVL (normal); lane 2, C739TαSVL (benign); lane 3, C745TαSVL (benign); lane 4, G805αapolSVL (adult onset); lane 5, G749AαapolSVL (infantile); lane 6, C508TαSVL (normal); lane 7, mock-transfected COS-7 (background); lane 8, WP09 fibroblast (normal); lane 9, WG1881 fibroblast (Tay-Sachs disease); C, lanes 1–7, the same as in B; lane 8, WG1881 fibroblast (Tay-Sachs disease); lane 9, GM00294 fibroblast (Sandhoff disease). ×, cross-reacting protein of unknown identity.

with the adult onset disease G269S mutation in the α-subunit had about 11% of the normal level of activity, significantly less than either benign mutation. The activity of Hex A plus S containing the G749A/G250D mutation was less than 4% of the normal, consistent with a juvenile onset clinical phenotype (43). The Tay-Sachs disease mutation R170W expressed a specific activity similar to the COS-7 cell background (Fig. 1).

The levels of the mature α-subunit protein expressed as Hex A plus S were also correspondingly decreased by the benign mutations (Fig. 2, B and C), but the level of the precursor α-subunit containing the benign mutations was similar to that of the normal. The mature α-subunit was not detected in the extracts prepared from expression of disease-causing mutations, although a significant amount of the precursor was detected. The differences between the benign mutations and disease-causing mutations appeared more obvious at the level of the mature α-subunit protein than at the activity level.

The co-transfection of pCD43 and αPSVL in COS-7 cells under the conditions used in these studies (see “Experimental Procedures”) resulted in the formation of both Hex A and Hex S; Hex A was predominant, although there was a significant level of Hex S produced (Fig. 3). Anion exchange chromatography showed that Hex A was formed when the β-subunit was co-expressed with the α-subunit carrying the benign mutations (data not shown).

The level of normalized Hex S and Hex A plus S activities was found to be influenced by the transfection efficiency. When transfection efficiency was low, higher levels of both β-hexosaminidase activity and α-subunit protein were expressed from mutant vectors in comparison with the normal vector. In Fig. 2B, expressed levels of α-subunit protein were higher than those in Fig. 2C, where the transfection efficiency was higher.

Analysis of Hex A in Cultured Fibroblasts—The Hex A activity and the α-subunit protein in fibroblasts from Hex A pseudodeficient subjects were compared with that from the normal. Hex A-pseudodeficient fibroblasts had 36–41% of normal Hex A activity (Table I) and a comparable level of mature α-subunit protein (data not shown). This is consistent with previous studies where pseudodeficient fibroblasts had Hex A activity that was 23–28% of the total β-hexosaminidase activity compared with 49–65% of Hex A for the normal (13). Leupeptin, a lysosomal protease inhibitor at 200 μM (44) did not

FIG. 3. Separation of the β-hexosaminidase isoenzyme forms Hex A, Hex B, and Hex S by anion exchange chromatography. Cell extract (1.7 mg of protein) prepared from α/β-subunit cDNA co-transfected COS-7 cell was loaded onto a DEAE-cellulose column (1.4-ml bed volume), that had been equilibrated with 10 ml of 10 mM phosphate buffer, pH 6.0, and the column was eluted with a linear and then step gradient of NaCl extending to 0.5 M NaCl. The linear NaCl gradient is shown as ———A. Start points for the steps of the gradient are denoted by a down arrow, the number at the top of the arrow indicates the molar concentration of NaCl. Column fractions representing each peak of activity (numbered 1–4) were pooled and concentrated. 20 μg of fractions 1 and 2 and ~40 μg of fractions 3 and 4 were separated by SDS-PAGE followed by Western blot analysis to determine the identity of the peaks using the anti-Hex A antibody. Inset, lane 1, no NaCl (Hex B); lane 2, 0–0.215 M NaCl (Hex A); lane 3, 0.3 M NaCl (Hex S); lane 4, 0.5 M NaCl (Hex A-related; function unknown); lane 5, extract of COS-7 cells transfected with pCD43/αPSVL (normal); lane 6, WG1881 fibroblast (Tay-Sachs disease); lane 7, GM00294 fibroblast (Sandhoff disease).
increase the percentage of the specific activity of Hex A (Table I) or the level of mature α-subunit protein (data not shown) in either the normal or mutant fibroblasts.

Isolation and Property Studies of Mutant Hex A—Hex A was separated from Hex B and Hex S as shown in Fig. 3. The 0−0.215 mM NaCl gradient, followed by 0.215 mM NaCl, eluted almost all Hex A from the column, and 0.3 mM NaCl eluted all of the Hex S (Fig. 3). The activity peaks corresponding to Hex A and Hex S possessed activities toward both the synthetic substrates, 4-MUG and 4-MUGS. The Hex B peak had activity only toward 4-MUG.

The identity of the eluted activities was confirmed by Western blot (Fig. 3, inset). Only the β-subunit was detected in the Hex B fractions; α- and β-subunits were in the Hex A fractions; and only the α-subunit was in the 0.3 mM NaCl-eluted Hex S fraction. An additional peak, of unknown identity, was eluted with 0.5 mM NaCl. This fraction contained both the α- and β-subunits and has previously been observed (37).

Kinetic Studies—The benign mutations, R247W and R249W, do not have a significant effect on the $K_m$ of Hex A for the synthetic substrates, although the apparent $V_{max}$ was substantially decreased (Table II). To determine if the lower apparent $V_{max}$ was the result of a change in catalytic activity of Hex A or a reduction of the α-subunit protein, the α-subunit protein levels of equal Hex A activities toward 4-MUGS were analyzed by Western blot. The levels of protein in normal and Hex A-pseudodeficient fibroblasts and the COS-7 cells transfected with pCD43/αpSVL or pCD43/C739TαpSVL were similar (Fig. 4). This indicated that the benign mutations affect Hex A by reducing the α-subunit protein level, not by affecting Hex A’s affinity toward the synthetic substrates and/or Hex A’s catalytic activity.

Heat Stability of Benign Mutation-containing Hex A plus S—The activity of Hex A and S harboring the benign mutation, R247W or R249W, in the α-subunit, decreased at a rate similar to that of the normal enzyme at 45 or 50.1 °C (data not shown). The results from fibroblast and expressed Hex A plus S were shown to be similar (data not shown). Similar results were also previously reported using fibroblast extract and a treatment at 37 °C (14). Hex A plus S containing the adult onset mutation G269S was shown to be more sensitive than normal (data not shown; Ref. 25). These suggested that the benign mutations do not increase the heat sensitivity of the α-subunit in vitro.

Optimal pH of Benign Mutation-containing Hex A—The optimal pH for the hydrolysis of the synthetic substrate by Hex A in the cell extracts from MCH065 and TC72 fibroblasts and the COS-7 cells, transfected with pCD43/αpSVL or pCD43/C739TαpSVL or pCD43/C745TαpSVL, was 4.0–4.4 (data not shown).

Effect of the Benign Mutations on α-Subunit Processing and Stability in Vivo—To determine the effects of the benign mutations on Hex A and S in transfected COS-7 cells and on Hex A in the fibroblasts from a normal and a Hex A-pseudodeficient subject with the C739T/R247W on one chromosome and a null mutation on the other, pulse-chase metabolic labeling was used.

The specificity of the antibody against human Hex A, which was used for immunoprecipitation, was analyzed; it was shown to recognize both the free and β-subunit-associated forms of the α-subunit (data not shown). The anti-Hex B and anti-α-subunit antisera were confirmed to have the specificity described by Proia (34). The anti-Hex B antibody recognizes all β-subunit monomers and β-subunit containing dimers, while the anti-α-subunit antibody recognizes only the α-monomer.

Effect of the Benign Mutation, R247W, on Phosphorylation of the α-Subunit—Fibroblasts from normal, Hex A-pseudodeficient, Tay-Sachs disease and Sandhoff disease subjects were pulse-labeled with $^{32}P$. The radiolabeled forms of β-hexosaminidase were immunoprecipitated using antibodies against human Hex A, Hex B, and the α-subunit, separated by gradient SDS-PAGE, and detected by autoradiography. Three separate experiments showed a considerable amount of precursor α-subunit in the free and β-subunit-associated forms in both normal and the C739T benign mutation-containing fibroblasts (data not shown), suggesting that Hex A with the benign mutation was normally phosphorylated. The Tay-Sachs disease sample did not show a $^{32}P$-labeled band corresponding to the α-subunit, and the Sandhoff disease sample did not exhibit a band corresponding to the radiolabeled β-subunit.

Effect of the Benign Mutation, R247W, on the Secretion of Hex A—Data derived from three repeated experiments showed that the secretion of the precursor α-subunit from both normal and Hex A-pseudodeficient fibroblasts was enhanced by growth in the presence of NH$_4$Cl (Fig. 5). The effect of NH$_4$Cl on the

---

**Table I**

**β-Hexosaminidase A activity in cultured fibroblast cells**

The activities were determined by incubating a 30-μl reaction mix containing 4–8 μg of cell extract protein and 20 μl of 4 mM 4-MUGS in citrate-phosphate buffer, 0.3% bovine serum albumin, pH 4.4, for 120 min at 37 °C. The activities are defined as nmol of 4-MUGS hydrolyzed/h/mg protein. The data represent the average of four experiments using three plates of each fibroblast cell line. The enzyme assay on each plate was carried out in triplicate.

|                          | MCH065 | TC72 | GM04863 | WG1881 | GM00294 |
|--------------------------|--------|------|---------|--------|---------|
| α-Leupeptin              | 332 ± 64 | 120 ± 16 | 137 ± 19 | 13 ± 3 | 43 ± 3  |
|                          | 100 ± 36 | 41 ± 4 | 4 ± 13 | 41 ± 4 | 4 ± 13  |
| + Leupeptin              | 100 ± 36 | 41 ± 4 | 4 ± 13 | 41 ± 4 | 4 ± 13  |
| Percentage              | 100 ± 34 | 37 ± 4 | 7 ± 19 | 37 ± 4 | 7 ± 19  |

a ± leupeptin indicates the presence (+) or absence (−) of leupeptin in the cell culture medium.

b Hex A activities of the Hex A pseudodeficient cell lines, TC72 and GM04863, a Tay-Sachs disease cell line WG1881, and a Sandhoff disease cell line, GM00294, are presented as a percentage of the activity of a normal fibroblast cell line MCH065 (100%).

---

**Table II**

**Kinetic studies of Hex A expressed in COs-7 cells and fibroblasts**

The Hex A activities were determined by incubating a reaction mix containing a similar level of DEAE-column-isolated Hex A activity with 7–9 various concentrations of 4-MUG (0.133−8.133 mM) or 4-MUGS (0.133−10.66 mM) in citrate/phosphate buffer, 0.3% bovine serum albumin, pH 4.4, in 30 μl at 37 °C for 30 min (120 min for 4-MUGS). The apparent $K_m$ and $V_{max}$ values were determined using the direct linear plot method (45).

|       | 4-MUG                | 4-MUGS               |
|-------|----------------------|----------------------|
| $K_m$ | μM                   | $V_{max}$            | μmol/h/mg           |
|       |                      |                      |                     |
| WP09a | 0.83 ± 0.17 (n = 2)  | 62.0 ± 3.6 (n = 3)   |                      |
| TCT7/R247Wa | 0.65 ± 0.10 (n = 2) | 15.9 ± 1.14 (n = 2) |                      |
| COS-7/R247Wa | 0.60 ± 0.10 (n = 2) | 10.46 ± 0.93 (n = 2)|                      |

a Hex A was isolated from normal (WP09) and Hex A pseudodeficient (TC72) fibroblasts.

b Hex A was isolated from pCD43/αpSVL-R249W-transfected COS-7 cells. $n$ denotes the number of experiments. Each experiment included two or three separate sets of assays.
The mature subunit of Hex A is less stable than normal. The mature subunit, at 20 h of chase, was significantly less abundant than normal, and its level, at 20 h of chase, was significantly less than normal.

The delay in the decrease of the maturesubunit suggests the rate of conversion from the precursor subunit to its mature form is delayed.

**Fig. 4.** Comparison of the α-subunit protein and Hex A plus S activity. Portions of fibroblast cell extracts, containing 8250 fluorescent units/90 min of activity (lanes 1–3), and of transfected COS-7 cell extracts, containing 4000 fluorescent units/90 min of activity (lanes 4–6), measured using 4-MUGS as the substrate, were separated by SDS-PAGE. Western blot analysis of the protein was done with the polyclonal anti-Hex A antibody. Lanes 1–3, MCH065 (normal); lane 2, CT72 (benign R247W); lane 3, GM04863 (benign R247W); lane 4, pCD43/pSVL (normal); lane 5, pCD43/C739T/pSVL (benign); lane 6, pCD43/C745T/pSVL (benign). Panels a and b indicate cell extracts from duplicate cell culture plates. Lane 7, WG1881 (Tay-Sachs disease); lane 8, GM00294 (Sandhoff disease).

**Fig. 5.** Effect of benign mutation on the secretion of Hex A. Fibroblasts from two cell lines, as indicated, were grown to confluency in tissue culture dishes (20 × 100 mm). Each cell line was pulse-labeled with Trasensel-label (0.3 mCi) for 3 h and chased for 20 h in the presence (+) and absence (−) of 10 mM NH₄Cl. Similar amounts of protein in medium extracts were analyzed. Three antibodies, as indicated, were used for immunoprecipitation, and one-fourth of the sample was analyzed on a high porous 5–12% gradient SDS-PAGE gel. The radioactive signals were detected by fluorography. Fetal bovine serum was not used in the culture medium to avoid the hydrolysis of the secreted α-subunit by the proteases that may be present in the fetal bovine serum.

The results of this may reflect an effect on folding or dimerization.

**Effect of the Benign Mutation, R247W, on the Stability of the α-Subunit in Hex A-pseudodeficient Fibroblasts—**To test the stability of the benign mutation-containing α-subunit in vitro, the experiments were designed differently from those in the previous reports (13, 14). The differences are that 1) equal amounts of cell extract protein were used for immunoprecipitation, and 2) two approaches were used in immunoprecipitation. The first approach used three types of antibodies to immunoprecipitate both precursors and the mature forms of the α- and β-subunit; the second method used anti-α-subunit antibodies in combination with anti-Hex A antibodies to immunoprecipitate only α-subunit. Results derived using the first method (data not shown) and the second method (Fig. 7) appeared similar. The benign mutation-containing fibroblasts, at 3 h of pulse, produced about half the level of the α-subunit precursor produced from normal fibroblasts. The benign mutation-containing mature α-subunit reached its maximal level at about 14 h of chase, similar to the normal. However, by 20 h of chase, the level of benign mutation-containing α-subunit was much lower than that of the normal consistent with a defect in stability.

**Effect of Protease Inhibitors, E-64 and Leupeptin, on the Stability of the α-Subunit—**Fibroblasts from normal and Hex A-pseudodeficient subjects were analyzed by pulse-chase metabolic labeling in the presence of 280 μM E-64 or 105 μM leupeptin. The lysosomal protease inhibitors had no apparent effect on the stability of the α-subunit containing the benign mutation R247W (data not shown). Leupeptin (105 μM) has successfully been used to increase the activity and protein levels of arylsulfatase A in the fibroblasts from adults with metachromatic leukodystrophy (46).

**DISCUSSION**

The benign mutations, C739T (R247W) and C745T (R249W), were originally identified in subjects with Hex A pseudodeficiency and in enzyme-defined Tay-Sachs disease carriers (6, 16, 17, 42). These mutations were clearly associated with enzyme...
deficiency, but we could not rule out the possibility that additional mutations, i.e. regulatory mutations, on the same allele were the cause of the enzyme deficiency.

Using a COS-7 expression system, the relationship between the benign mutations and Hex A pseudodeficiency was confirmed. The levels of expressed Hex S and Hex A plus S activities and their corresponding α-subunit protein were significantly decreased by the benign mutations C739T and C745T (Figs. 1 and 2). Hex A-pseudodeficient fibroblasts also demonstrated similar results (Table I). This is the first direct evidence that the benign mutations are the primary cause of the Hex A pseudodeficiency.

Benign mutations, C739T and C745T, were readily differentiated from the mutations associated with adult onset and other forms of GM2 gangliosidosis using transient expression (Figs. 1 and 2). Normally there is an overlap between the percent Hex A activity that is associated with various phenotypes, including pseudodeficiency, when Hex A activity is determined in serum, leukocytes, or fibroblasts (10, 13–15). Our results extended a previous study by Brown and Mahuran (25), who showed that α/β-subunit co-expression could distinguish the adult onset mutation from those associated with more severe forms of GM2 gangliosidosis. Our studies showed that the disease-causing mutations resulted in a lower percentage of Hex A plus S, compared with normal, than that previously reported for the α/β-subunit co-expression (25). This may be because 1) whole cell extracts were used, instead of immunoprecipitated enzyme, to determine Hex A plus S activity, and 2) there were differences in the ratio of spSVL:pCD43, the DNA concentration, and the method used for DNA transfection.

Data derived from the current studies and previous studies suggest that the benign mutations do not cause disease. The levels of Hex A activity in fibroblasts and leukocytes of Hex A-pseudodeficient subjects with the R247W and R249W amino acid changes (10–15, 17–18) are usually slightly higher than that found in a patient with juvenile or adult onset GM2 gangliosidosis (15). Results from in vitro and in situ GM2 ganglioside hydrolysis assays (13, 14), together with the results from the current transient expression studies, suggest that the level of the enzyme associated with benign mutation is above the critical threshold required for normal GM2 ganglioside hydrolysis. Indeed, several pseudodeficient subjects are presently more than 40 years old (17–18), and patients with the adult onset mutation G805A/G2698S typically show symptoms before the age of 40 years (15, 47). However, one cannot rule out the possibility of a late onset phenotype in these subjects.

The pH optimum of Hex A was not altered by the benign mutations, indicating that the Arg247 and Arg249 residues are not required for proton transfer in the catalytic reaction and are not involved in the catalytic reaction. This is consistent with the prediction that these residues are located on the surface of the α-subunit molecule (48), not in the active site where the substrate binding and catalytic reaction occur.

Because the benign mutation did not alter Hex A’s affinity for the synthetic substrates and there was clearly a reduction in the α-subunit protein level, we attempted to determine the basis of the decreased protein level. Triggs-Raine et al. (17) had previously shown the α-subunit cDNA could be amplified after reverse transcription of mRNA isolated from Hex A-pseudodeficient fibroblasts. This indicated that the HEXA gene with the benign mutation, C739T(R247W), was normally transcribed, because the other allele in the subject had the 1278ins4 mutation that does not produce a stable α-subunit mRNA (49).

Previous studies (13, 14) also showed an apparently normal amount of the precursor α-subunit protein in Hex A-pseudodeficient fibroblasts. This was also observed in our Western blot analysis (Fig. 2), and pulse-labeling analysis (Figs. 6 and 7). Together these results suggest that the benign mutations do not affect the α-subunit biosynthesis.

The benign mutation, C739T(R247W), did not affect the phosphorylation of the α-subunit or the secretion of Hex A (Fig. 5), indicating that 1) the benign mutations did not cause Hex A to be trapped in the endoplasmic reticulum or the Golgi apparatus, and 2) the mutant Hex A could be normally targeted to the lysosome or to the previ-lysosomal compartments. These results did not explain the absence of serum Hex A in pseudodeficient subjects.

Further studies using both Hex A in fibroblasts and Hex S expressed in COS-7 cells demonstrated that the benign mutations significantly decreased the stability of the mature α-subunit (Figs. 6 and 7). A slight delay in the precursor conversion to its mature α-subunit as expressed Hex S was also indicated, but this was not seen in the fibroblasts. This difference might have resulted from the effect of Hex S overexpression in COS-7 cells. These results indicated that the effects of the benign mutations on the α-subunit at the previ-lysosomal level may also exist.

The protease inhibitors, leupeptin and E-64, did not have an effect on the decreased level of the mature α-subunit by the benign mutations. This may be because the benign mutations render the α-subunit susceptible to a lysosomal protease(s) that cannot be inhibited by these protease inhibitors, or the mutant α-subunit may be susceptible to nonenzymatic factors.

The residues Arg247 and Arg249 fall in a highly conserved region of exon 7 of β-hexosaminidase from mammals, bacteria, and yeast, suggesting that these residues are important in maintaining normal Hex A function. They have been predicted to locate on the surface of the α-subunit molecule and might be involved in maintaining the stability of the α-subunit and/or in interacting with the β-subunit or GM2 activator protein.

Acknowledgments—We are grateful to Dr. Hans Jacobs for critical review of the manuscript.

REFERENCES

1. Mahuran, D., Novak, A., and Lowden, J. A. (1985) Isozymes Curr. Top. Biol. Med. Res. 12, 229–286.
2. Conzelmann, E., and Sandhoff, K. (1979) Hoppe-Seyer’s Z. Physiol. Chem. 360, 1837–1849.
3. Furst, W., and Sandhoff, K. (1992) Biochim. Biophys. Acta 1196, 1–16.
4. Gravel, R. A., Clarke, J. T. R., Kaba- c, M. M., Mahuran, D., Sandhoff, K., and Suzuki, K. (1995) The Gm2 Gangliosidosis: The Metabolic Basis of Inherited Disease, 7th Ed. (Sriv- er, C. R., Beaudet, A. L., Sly, W. S., Valle, D., eds) McGraw-Hill, Inc., New York.
5. Petersen, G. M., Rotter, J. I., Cantor, R. M., Field, L. L., Greenwald, S., Lim, J. S. T., Roy, C., Schon-efeld, V., Lowden, J. A., and Kaba- c, M. M. (1983) Am. J. Hum. Genet. 35, 1258–1269.

FIG. 7. Effect of the benign mutation R247W on the stability of the α-subunit of Hex A in fibroblasts: immunoprecipitation with antiserum against the α-subunit. Fibroblasts from four cell lines, as indicated, were grown to confluence in tissue culture dishes (20 x 100 mm). Each cell line was pulse-labeled with Tran35S-label (0.3 mCi) for 3 h and chased for various intervals as indicated. The cell extracts were prepared for immunoprecipitation in 0.65 ml of lysis buffer, and 2 μl of cell extract was used for the measurement of the protein concentration. Cell extracts (480–510 μl) containing equal amounts of protein (1.20 mg) were mixed with lysis buffer containing 1% bovine serum albumin to a final volume of 600 μl. Primary and secondary immunoprecipitations were then done as described under “Experimental Procedures.” One-fourth of the sample was analyzed on a high porous 5–12% gradient SDS-PAGE gel. The labeled proteins were detected by fluorography.
Benign HEXA Mutations, C739T(R247W) and C745T(R249W), Cause β-Hexosaminidase A Pseudodeficiency by Reducing the α-Subunit Protein Levels

Zhimin Cao, Emmanuel Petroulakis, Timothy Salo and Barbara Triggs-Raine

J. Biol. Chem. 1997, 272:14975-14982.
doi: 10.1074/jbc.272.23.14975

Access the most updated version of this article at http://www.jbc.org/content/272/23/14975

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 46 references, 11 of which can be accessed free at http://www.jbc.org/content/272/23/14975.full.html#ref-list-1