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Identification of Functional Domains within the α and β Subunits of β-Hexosaminidase A through the Expression of α−β Fusion Proteins†

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ABSTRACT: There are three human β-hexosaminidase isozymes which are composed of all possible dimeric combinations of an α and/or a β subunit; A (αβ), B (ββ), and S (αα). The amino acid sequences of the two subunits are 60% identical. The homology between the two chains varies with the middle > the carboxy-terminal ≫ the amino-terminal portions. Although dimerization is required for activity, each subunit contains its own active site and differs in its substrate specificity and thermal stability. The presence of the β subunit in hexosaminidase A also influences the substrate specificity of the α subunit; *e.g., in vivo* only the A heterodimer can hydrolyze G_{M2} ganglioside. In this report, we localize functional regions in the two subunits by cellular expression of α/β fusion proteins joined at adjacently aligned residues. First, a chimeric α/β chain was made by replacing the least well-conserved amino-terminal section of the β chain with the corresponding α section. The biochemical characteristics of this protein were nearly identical to hexosaminidase B. Therefore, the most dissimilar regions in the subunits are not responsible for their dissimilar biochemical properties. A second fusion protein was made that also included the more homologous middle section of the α chain. This protein expressed the substrate specificity unique to isozymes containing an α subunit (A and S). We conclude that the region responsible for the ability of the α subunit to bind negatively charged substrates is located within residues α132−283. Interestingly, the remaining carboxy-terminal section from the β chain, β316−556, was sufficient to allow this chimera to hydrolyze G_{M2} ganglioside with 10% the specific activity of heterodimeric hexosaminidase A. Thus, the carboxy-terminal section of each subunit is likely involved in subunit−subunit interactions.

β-*N*-Acetylhexosaminidase (Hex)[1] is a lysosomal hydrolase that cleaves terminal nonreducing β-1,4-linked *N*-acetylhexosamines from oligosaccharides, glycolipids, gangliosides, glycoproteins, and glycosaminoglycans. The human enzyme occurs as two major isozymes composed of α [encoded by the *HEXA* gene 15q23-q24 (Nakai et al., 1991)] and/or β [encoded by the *HEXB* gene 5q13 (Bikker et al., 1988)] subunits. Hex A is a heterodimer, αβ, whereas Hex B is a homodimer, ββ. A third, unstable isozyme, Hex S, is composed of two α subunits. Hex S was originally detected in very small amounts and characterized in human cells that do not synthesize a functional β subunit, *i.e.*, from Sandhoff patients (see below) (Ikonne et al., 1975). Although only dimeric forms of Hex are active, the existence of the three Hex isozymes (ββ, αβ, and αα) indicates that each subunit contains a potential active site. That the subunits share many structure−function relationships is not surprising since the *HEXA* and *HEXB* genes arose from a common ancestral gene (Korneluk et al., 1986; Proia, 1988). This relationship is evident from the nearly identical placement of the intron/exon junctions (13 of 14) and the close similarity in the deduced primary structures (60% identity) between the *HEXA* and *HEXB* genes and the α and β subunits, respectively.

Whereas the active sites of both the α and β subunits (in dimeric form) are able to hydrolyze many of the same neutral artificial, *e.g.*, MUG (Hou et al., 1996a), and several natural substrates [reviewed in Mahuran et al. (1985)], only the catalytic site in the α subunit can hydrolyze negatively charged substrates such as β-linked glucosamine 6-sulfate containing glycosaminoglycans (Hou et al., 1996a; Kresse et al., 1981; Kytzia & Sandhoff, 1985) and artificial substrates (Hou et al., 1996a; Kytzia & Sandhoff, 1985), *e.g.*,4-MUGS (Bayleran et al., 1984), and most importantly G_{M2} ganglioside. In the latter case, only the α subunit in its heterodimeric Hex A form is functional *in vivo* (Meier et al., 1991). Naturally occurring mutations in the *HEXA* gene cause Tay−Sachs disease. Two other inheritable diseases with clinically similar phenotypes are Sandhoff disease and the AB-variant form of G_{M2} gangliosidosis. These diseases arise from mutations in either the *HEXB* gene or the *GM2A*

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[1] Abbreviations: Hex, β-hexosaminidase; MUG, 4-methylumbelliferyl-β-*N*-acetylglucosamine; MUGS, methylumbelliferyl-β-*N*-acetylglucosamine 6-sulfate; MU, methylumbelliferone; G_{M2} ganglioside, GalNAcβ(1−4)-[NeuAc(2−3)-]-Galβ(1−4)-Glc-ceramide; ER, endoplasmic reticulum; wt, wild type; activator, G_{M2} activator protein; FCS, fetal calf serum; CAT, chloramphenicol acetyltransferase; PBS, phosphate-buffered saline; β*, mutant form of the β subunit of hexosaminidase containing an $Arg^{211}Lys$ substitution; Hex A* (αβ*), form of hexosaminidase A containing the inactive, mutant β* subunit.

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gene [encoding the G_{M2} activator protein (see below), respectively; reviewed in Gravel et al., (1995)].

In order for Hex A to hydrolyze G_{M2} ganglioside, it requires the small, heat-stable G_{M2} activator protein (activator). The activator interacts with both the carbohydrate and the lipid portion of the ganglioside, solubilizing, or at least lifting, a ganglioside molecule from the membrane, and "presenting" it to Hex A for hydrolysis (Meier et al., 1991). *In vitro* assays demonstrate that detergents can be substituted for the activator; however, under these conditions, Hex S, as well as Hex A, but not Hex B, can efficiently hydrolyze G_{M2} ganglioside. Interestingly, Hex B can hydrolyze G_{A2}, the neutral, asialo derivative of G_{M2}, in the presence of detergent, but not in the presence of activator alone. Also, the activator, even in the absence of G_{M2}, can inhibit the hydrolysis of MUGS by both Hex A and Hex S [reviewed in Furst and Sandhoff (1992) and Sandhoff et al. (1989)]. These data indicate that the binding site for the complex is also located in the α subunit, but that elements of the β subunit [other than its active site (Hou et al., 1996a)] are necessary to somehow correctly orientate the complex and allow hydrolysis of the ganglioside. Further functions that have been identified for the β subunit are to greatly increase the stability of the resulting dimer and to facilitate the transport of the α subunit out of the ER (see below) [reviewed in Gravel et al., (1995) and Mahuran (1991)]. We have also recently shown that the presence of the β subunit in the Hex A heterodimer changes some of the kinetic properties of the α-active site, as previously assessed in the Hex S homodimer (Hou et al., 1996a). This conclusion was based on the analysis of a novel form of Hex A (Hex A*) which contained a normally folded but inactive β subunit (β*) with an $Arg^{211}Lys$ substitution mutation (Brown & Mahuran, 1991).

It is possible that if conserved regions account for some of the common properties of the α and β chains, *e.g.*, exon 5 which contains the aligned active site residues αAsp^{163}/βAsp^{196} and αArg^{178}/βArg^{211} (Brown & Mahuran, 1991; Brown et al., 1989; Tse et al., 1996), then nonhomologous domains may confer the aforementioned differences in substrate specificity and thermal stability. Interestingly, when the amino acid sequences of the α and β chains are aligned, the greatest homology is in the last three-fourths of the deduced sequence. The homology between the two chains at the N-terminal quarter, α^{1-131} and β^{1-164}, is minimal (Figure 1). This lack of homology is also reflected in the fact that it is only the placement of the exon 1/intron 1 junctions that is not conserved in the *HEXA* and *HEXB* gene structures (Neote et al., 1988).

Like other lysosomal and secretory proteins, the α and β chains of Hex are synthesized in the ER. ER to Golgi transport requires that proteins fold into their near-native conformation and that some, like Hex, form dimers [reviewed in Edgington (1992), Hurtley and Helenius (1989), Mahuran (1991), and Pelham (1989)]. Most deleterious missense mutations leading to Tay−Sachs disease prevent proper folding and/or dimerization and result in the detection of only small amounts of proα chain cross-reacting material (the ER form) [reviewed in Gravel et al. (1995)]. When pro-isozymes are able to exit the ER, they are transported to the lysosome where the single-chain pro-subunits undergo further proteolytic processing at exposed hydrophilic sites (Sagherian et al., 1993) to produce complex polypeptide structures held together by disulfide bonds in the mature subunits, *i.e.*, $[\alpha_p{}^{23-74}\alpha_m{}^{189-529}][\beta_p{}^{50-97}\beta_b{}^{112-311}\beta_a{}^{316-556}]$ (Figure 1) [reviewed in Gravel et al. (1995)].

One possible method to bypass the quality control system in the ER for structure/function studies of the two Hex subunits is to make chimeric proteins joined at adjacent aligned residues. Since evolutionarily-related proteins retain nearly identical three-dimensional structures, the exchange of aligned sequences should produce minimal effects on the folded structure, allowing efficient transport from the ER to the Golgi. In this report, we demonstrate that this hypothesis is at least partially true.

MATERIALS AND METHODS

PCR-Mediated Fusion of the α and β cDNAs. The PCR (polymerase chain reaction)-mediated gene fusion technique (Yon & Fried, 1989) was utilized in the construction of two chimeric proteins consisting of N-terminal portions of the α chain replacing the corresponding aligned segments of the β chain of human Hex. The PCR technique allows the amplification of DNA sequences by using two specific primers. By incorporating extra nucleotide sequences into these synthetic primers, one is able to generate amplified DNA sequences with modified ends. Based on this concept, a third DNA primer (oligo 869, fusion 1; or oligo 113, fusion 2) termed the linking-oligo is constructed in such a way that the (5′) upstream half of its sequence is complementary to the cDNA encoding the α subunit of Hex, whereas the other (3′) half is complementary to the β subunit encoding cDNA. Together with the 5′ primer (oligo 868, fusion 1; or oligo 143, fusion 2) complementary to an αcDNA sequence and a 3′ primer (oligo 870, fusion 1; or oligo 112, fusion 2) complementary to a βcDNA sequence, the linking-oligo is able to mediate the fusion of the αcDNA to the βcDNA at precise locations. Direct translation of this amplified construct gives rise to chimeric proteins with their NH_2-terminal portion from the α subunit and the rest of the protein molecule from the β-subunit.

The whole process was carried out in a single step with all three primers and both cDNA templates present, to generate "fusion 1", as was suggested in the original procedure (Yon & Fried, 1989). For "fusion 2", a more consistently successful method was developed employing two major steps. In step 1, 30 cycles of amplification are performed using the linking oligo/primer along with the 5′ end primer and the αcDNA template. An intermediate product is then generated which contains the α−β fusion point. In step 2 of the process, the intermediate fragment acts as a primer in conjunction with the 3′ end primer and the βcDNA template to yield the final product of the reaction.

Whereas transiently transfected COS-1 cells were used to produce the fusion 1 protein, permanently transfected CHO cells were used to express fusion 2. The necessity for this variation was the exhaustion of our human-specific anti-Hex B antiserum which did not cross-react with endogenous COS Hex (Brown et al., 1989). Other antisera were less specific. If they were absorbed with COS cell extract, they lost cross-reactivity to both human and COS cell Hex. In order to allow absorption of an antiserum without a coordinate loss in reactivity to the human-derived Hex, cells from a species further removed from man than monkey were needed for the transfections experiments. CHO cells were found to

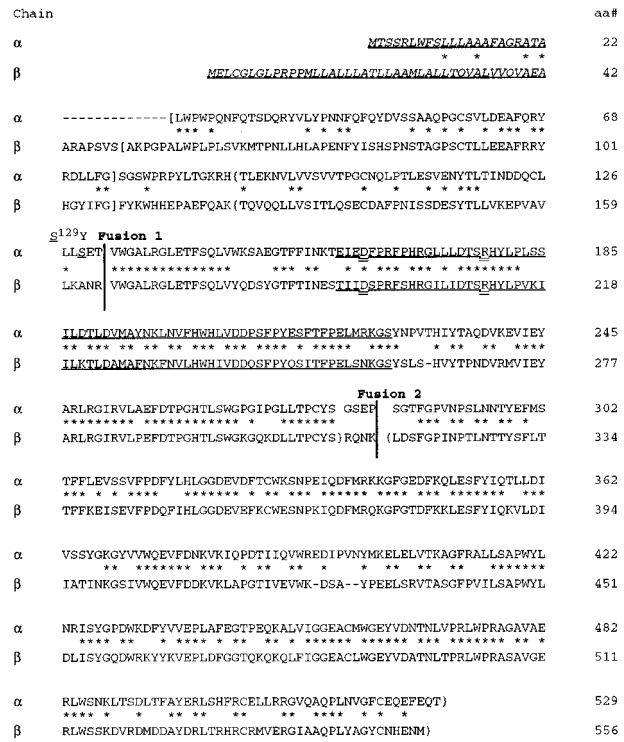

FIGURE 1: Alignment of the deduced primary structures of the preproα and preproβ chains of human β-hexosaminidase A. The signal peptide is in italics and underlined. The mature α_p and β_p chains are enclosed in brackets. The mature α_m and β_b chains are enclosed in braces. The mature β_a chain is enclosed in parentheses. Residues between the end of one set of brackets and the beginning of another are believed to be removed during maturation. Residues believed to be involved in the active sites (Brown & Mahuran, 1991; Tse et al., 1996) are double-underlined. The most highly conserved domain within 15 aligned Hex-related sequences is underlined (Tse et al., 1996). The α/β junctions of fusion 1 (Fusion 1) and fusion 2 (Fusion 2) are indicated by vertical lines. The fusion 2 junction has been previously shown to be located in a hydrophilic loop structure (Sagherian et al., 1993).

serve this purpose. However, because they lacked the COS cell's ability to replicate the expression vector (through the SV40 *ori*), transient expression did not result in sufficient protein synthesis to allow detailed biochemical analyses to be carried out. Expression in selected "highly producing" permanently transfected CHO cell clones was found to be necessary (data not shown).

Fusion 1. Translation of this construct would produce a chimeric polypeptide with its N-terminus consisting of αMet^1 to αThr^{131} and the rest of the β chain from βVal^{164} to βMet^{556} (Figure 1). This strategy was used to prevent the disruption of any unique N-terminal proα chain secondary structure. The cDNAs encoding the Hex α and β subunits had previously been subcloned into the eukaryotic expression vector pSVL (referred to as pSVL-α and pSVL-β, respectively) (Brown & Mahuran, 1993; Neote et al., 1990a). The three oligonucleotides utilized in the PCR reactions were the following: (a) Oligo 868, which binds to the antisense strand of pSVL-α in the region corresponding to the junction of the vector and the αcDNA, had the following sequence: 5′-TAAAAGCTCCTCGAGGTCGCCGCT-3′, within the center of which is a unique *Xho*I site (underlined) originally used in subcloning the αcDNA. It is this oligo that initiates the PCR reaction from the 5′ end of the αcDNA. (b) The linking oligo, Oligo 869, had the following sequence (antisense): 5′-CCTCGTAATGCTCCCCAAACAGTCTCAGA-GAGGAGTAAAC-3′. Twenty nucleotides at its 5′ end are complementary to the sequence that extends 3′ to the junction of the sense strand of the βcDNA, and 20 nucleotides at the 3′ end are complementary to the sequence that is 5′ to the junction of the sense strand of the αcDNA. (c) Oligo 870 is complementary to the sense strand of the βcDNA around its second internal *Eco*RI site (underlined), and has the following sequence: 5′-GCAGGACTCGAATTCCTCG-TAATC-3′. Various concentrations of each primer and PCR conditions were evaluated to optimize the procedure. The conditions that resulted in the generation of the fusion cDNA are as follows. PCR reactions were carried out in a 50 μL volume containing buffer (final concentrations: 100 mM Tris, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.1% gelatin w/v), 200 μM each of deoxynucleotide triphosphates, 1 μM each of Oligo 868 and Oligo 870, 0.01 μM Oligo 869, 10 ng of pSVL-α DNA, and 100 ng of a restriction fragment generated from the digestion of pSVL-β by *Eco*RV and *Bam*HI (encompassing the region needed for PCR fusion). Taq polymerase, 1.25 units (from Perkin Elmer Cetus), was added after the reaction mix was heated to 94 °C. Thirty-five cycles of PCR were performed on a Perkin Elmer Cetus DNA Thermal Cycler with 30 s denaturation at 94 °C, 2 min annealing at 40 °C, and 2 min primer extension at 70 °C.

(A) Production of the pSVL-αβ1 Expression Vector. General cloning procedures were as described by Sambrook (Sambrook et al., 1989). Briefly, the PCR product generated from the above procedure was isolated by agarose gel electrophoresis, and purified with the Geneclean Kit (Bio 101). After being digested with restriction enzymes *Xho*I and *Eco*RI and subcloned into a Bluescript vector (Stratagene), the insert was sequenced by the dideoxy chain termination method (Sanger et al., 1977). To allow expression in mammalian cells, the insert was subsequently subcloned into pSVL-β, replacing the corresponding 5′ segment of the βcDNA.

(B) Cell Culture and DNA Transfection. Monkey kidney cells (COS-1) (American Type Culture Collection) were maintained and propagated in α-minimal essential media (Flow Laboratories) supplemented with 10% FCS v/v, 100 μg/mL streptomycin, and 100 μg/mL penicillin, at 37 °C in 5% CO_2. Ten micrograms of purified pSVL containing the βcDNA or the fusion cDNA in the eukaryotic expression vector pSVL, plus 5 μg of pBLCAT2 containing the cDNA encoding the bacterial CAT gene, was transfected into these cells as previously reported (Brown et al., 1989).

(C) β-Hexosaminidase Assays. Cell lysates, obtained from freezing and thawing the transfected COS-1 cells in PBS, were assayed for human Hex activity using a solid-state immunoprecipitation method (Brown et al., 1989). The immobilized human Hex was assayed with both the common MUG and α-isozyme-specific MUGS substrates (Brown & Mahuran, 1993). Lysate protein was determined by the Lowry method.

(D) Western Blot. COS-1 cell lysates (treated with $^1/_3$ volume of CCl_4 to reduce their lipid content) were analyzed by Western blotting as previously reported (Brown et al., 1989). The rabbit anti-human Hex B IgG used primarily cross-reacts with the β_a chain portion (Figure 1) of the proβ chain or the mature β subunit (McInnes et al., 1992).

(E) Stability Study. The appropriate amount of COS-1 lysates was incubated with temperature-preequilibrated cit-

rate−phosphate buffer, pH 4.1, containing 0.15% human serum albumin w/v as a stabilizing agent at 60 °C. Aliquots were taken at different time points (0, 10, 20, 30, and 50 min) and assayed using the solid-state immunoprecipitation methods described above. The log of percentage of residual bound activity (after subtracting the corresponding value given by nonspecific binding of the COS cell enzymes) was plotted against the time of exposure to 60 °C. From this graph, the time at which 50% of the original activity remained was calculated from the best-fit line, $T_{1/2}$ 60 °C.

Fusion 2. A new construct was produced that encoded a chimeric protein composed of αMet^{1}−αPro^{283} fused to βLeu^{316}−βMet^{556} (Figure 1). The cDNAs encoding the Hex α and β subunits have previously been subcloned into the eukaryotic expression vector pSVL (see Fusion 1). The three oligonucleotides utilized in the PCR reactions were as follows: (a) Oligo 143, which binds to the antisense strand of pSVL-α in the region corresponding to the junction of the vector and the αcDNA, had the following sequence: 5′-TAAAAGCTCCTCGAGGTCGCCGCT-3′, within the center of which is a unique *Xho*I site (underlined) originally used in subcloning the αcDNA. It is this oligo that initiates the PCR reaction from the 5′ end of the αcDNA. (b) The linking oligo, Oligo 112, had the following sequence (antisense): 5′-ATAGGTCCAAAAGAGTCCAAGGGCTCA-GACCCAGAGTAGC-3′. Twenty nucleotides at its 5′ end are complementary to the sequence that extends 3′ to the junction of the sense strand of the βcDNA, and 20 nucleotides at the 3′ end are complementary to the sequence that is 5′ to the junction of the sense strand of the αcDNA. (c) Oligo 113 is complementary to the sense strand of the βcDNA around its unique *Pst*I site (underlined), and has the following sequence: 5′-TCGACCATCCTGCAGCGGTGC-CTT-3′. Various concentrations of each primer and PCR conditions were evaluated to optimize the procedure. The conditions that resulted in the generation of the fusion cDNA are as follows: In both steps, the reaction was carried out in a 100 μL volume in reaction buffer composed of 100 mM Tris, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.1% gelatin w/v with 200 μM each of deoxynucleotide triphosphates. Taq polymerase, 5 units (from Perkin Elmer Cetus), was added after the reaction mix was heated to 94 °C. Thirty cycles of PCR were performed on a Perkin Elmer Cetus DNA Thermal Cycler with 30 s denaturation at 94 °C, 2 min annealing at 54 °C, and 2 min primer extension at 70 °C. In step 1, 0.3 μM of each Oligo 143 and Oligo 113 and 10 ng of pSVL-α DNA were used. In step 2, 0.3 μM oligo 112, 10 ng of pSVL-β DNA, and 100 ng of the "intermediate" fragment (generated from step 1) were used.

(A) Production of the pEFNEO-$\alpha\beta$2 Expression Vector. General cloning procedures were as described by Sambrook (Sambrook et al., 1989). The isolation and purification of the PCR product are the same as for Fusion 1. The insert is treated with restriction enzymes *Xho*I and *Pst*I (complete and partial digestion, respectively) and subsequently subcloned into pSVL-β, replacing the corresponding 5′ segment of the βcDNA. The construct pSVL-fusion, due to the lack of appropriate restriction sites, was then subjected to various subcloning steps in order to produce a eukaryotic expression construct containing a suitable selection marker for the permanent transfection of CHO cells. The vector used was pEFNEO (kindly supplied to us by Dr. Anson) (Anson et al., 1992), which has a neomycin (G418) resistance marker. These subcloning procedures are described as follows: (i) *Xho*I and *Cla*I digestion of the construct pSVL-fusion and ligation of the insert into a Bluescript vector (Stratagene); (ii) *Apa*I and *Xba*I digestion of the construct pBS-$\alpha\beta$2 and ligation of the insert into the super-linker vector, pSL301-(Invitrogen); and, finally, (iii) transferring of the fusion cDNA to the vector pEFNEO by *Eco*RI partial digestion: the insert was then sequenced by the dideoxy chain termination method (Sanger et al., 1977).

(B) Production of Expression Vectors, pEFNEO-β, and pREP4-α.* The construction of pEFNEO-β containing the wild-type βcDNA, pREP4-α containing the wild-type αcDNA, and pEFNEO-β* encoding a β subunit with an Arg^{211}Lys substitution has been previously described (Hou et al., 1996a). The latter β* substitution produces a nearly inactive dimeric Hex B* which can also interact with human α chain to produce a Hex A* isozyme fully capable of hydrolyzing G_{M2} ganglioside in the presence of human activator protein, but with an inactive β* subunit (Brown & Mahuran, 1991; Hou et al., 1996a). Expression of this construct in CHO cells was used as a control to demonstrate the lack of interspecies dimer formation, *i.e.,* human Hex subunits interacting with endogenous CHO Hex subunits.

(C) Cell Culture and DNA Transfection. The same methods that were used to culture COS cells (above) were used to culture Chinese hamster ovary cells (CHO). Transfections were carried out with 10 μg of purified plasmid DNA (the β or the fusion cDNA in the eukaryotic permanent expression vector pEFNEO), or with 20 μg of pREP4-α with or without 10 μg of wt pEFNEO-β. These were mixed with 70 μg of Lipofectin reagent (GIBCO BRL) in 200 μL of serum-free MEM, as previously reported (Hou et al., 1996a).

(D) β-Hexosaminidase Assays. CHO cell lysates were obtained and analyzed through similar procedures as described in Fusion 1 (Brown et al., 1989), except that the antibody used in this case was a sheep anti-human Hex A-IgG. The antibody was preabsorbed with the glycoprotein fraction from normal CHO lysate, bound to a concanavalin A−sepharose column (Pharmacia), in order to remove the majority of the endogenous CHO cell Hex binding capacity. This antibody retained its ability to recognize the human isozymes (this was not the case if COS lysate was used for the absorption). The buffers were also slightly modified: PBS (pH 7.0) or citrate−phosphate buffer (pH 4.1) each containing 2.5% w/v human serum albumin and 5% v/v glycerol, was utilized for washing the protein G−Sepharose beads (Pharmacia) and in the actual enzyme reaction, respectively.

(E) Western Blot. CHO cell lysates were analyzed by the method as described above for Fusion 1. However, the blot was developed by the ECL procedure (Amersham) (Hou et al., 1996a), and a rabbit anti-Hex A IgG preparation was used as the primary antibody. This antibody cross-reacts more strongly with the reduced α chain and the β_p segment of the proβ chain (which runs off the gel if the major component in the lysate is the reduced, mature β subunit), rather than with the β_a chain (Figure 1) which is the primary antigen for our rabbit anti-Hex B IgG.

(F) Chemical Cross-Linking with Dithiobis(succinimidyl Propionate). Cross-linking with 0.6 mM dithiobis(succinimidyl propionate) using a 6 mM stock solution in dimethyl sulfoxide was performed as previously repoted (Tse et al., 1996). Transfected CHO cell lysates were prepared as

described above except that the PBS lysis buffer was substituted with the following: 50 mM sodium phosphate, pH 7.0, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% Triton X-100 v/v, 5 mM β-glycerophosphate, and 0.05% w/v bovine serum albumin, to prevent nonspecific protein aggregation and equalize protein concentrations. Denatured but nonreduced lysates were subsequently analyzed by Western blotting.

(G) Indirect Immunofluorescence. Transfected cells were grown at 37 °C in 5% CO_2 on a chamber slide (Lab-Tek) in 1mL wells containing α-MEM complete medium. Indirect immunofluorescence was carried out as previously reported (Hou et al., 1996b), using a rabbit anti-Hex A primary antibody. In control cultures, the preimmune rabbit IgG substituted for the primary antibody.

(H) Stability Study. The methods used here were the same as described in Fusion 1 except that the incubation temperature was 45 °C. The same type of buffer was utilized as a stabilizing agent at 45 °C except that it contained 0.3% w/v human serum albumin.

(I) G_{M2} Hydrolysis. Natural substrate assays using tritium-labeled G_{M2} ganglioside and human activator protein purified from transformed bacteria (Klima et al., 1993) were done using equal MUGS units, rather than total cell protein, of human Hex S, human Hex A, endogenous CHO Hex A, or fusion 2 Hex expressed from the transfected pEFNEO-αβ2 vector (at least three different amounts of MUGS units from each isozyme were tested, and the slope of the best-fit line was calculated) as previously reported (Hou et al., 1996a).

RESULTS

Fusion 1. A fusion cDNA was generated by PCR. Direct sequencing of the fragment demonstrated that it was comprised of portions from both the α and β cDNA sequences extended from Oligo 868 and Oligo 870, respectively. The PCR fragment was subcloned into Bluescript, and individual clones were sequenced to confirm that there were no PCR artifacts generated. Three independent cDNA clones, found to have the correct sequence, fusion 1-(3−5), and one mutant cDNA clone, containing a single nucleotide substitution (TCT to TAT) encoding a $Ser^{129}Tyr$ change in the α portion of the fusion cDNA, were subcloned into the vector pSVL-β and transiently expressed in COS-1 cells.

To confirm that each construct caused a stable fusion protein to be synthesized and transported into the lysosome, Western blot analysis was carried out using equal amounts of cell lysate protein (corrected for transfection efficiency by CAT assays) (Figure 2). Under reducing conditions, wt Hex B produces an immunoreactive band corresponding to about 30 kDa, *i.e.*, the mature β_a chain (Brown et al., 1989). Processing from its 65 kDa precursor form (proβ-polypeptide chain) to its mature polypeptides (Figure 1) has long been taken as evidence for incorporation of the enzyme into the lysosome (Hasilik & Neufeld, 1980a,b). The pattern of immunoreactive bands produced from cell lysates transfected with fusion 1−3 or fusion 1−4 showed the same pattern as the wt Hex B (Figure 2). However, there appeared to be less immunoreactive protein in the cells transfected with either of the fusion 1 cDNAs, as compared to the wt βcDNA. Interestingly, no proβ or β_a bands were detected in lysates from cells transfected with the mutant ($Ser^{129}Tyr$) fusion 1 cDNA. However, a very dark band corresponding to the

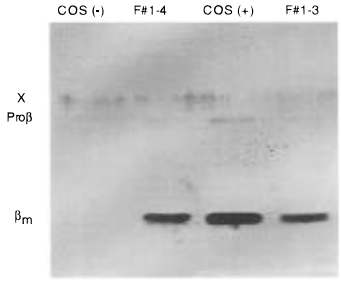

FIGURE 2: Western blot of COS-1 cell lysates using an anti-Hex B IgG (strongly reacts with the β_a chain) from nontransfected cells, COS (−); cells transfected with a wt βcDNA construct, COS (+); and cells transfected with the fusion 1 cDNA construct from two independent cDNA clones, F#1−3 and F#1−4. The positions of the proβ chain, Proβ (65 kDa), and the single immunoreactive mature β chain, β_a (β_m, 30 kDa), are indicated on the left. As well, a nonspecific band, X, can be seen in the COS (−) lysate and other samples.

Table 1: Expression of Fusion 1 and wt Hex B in Transiently Transfected COS Cells

Hex cDNA	β- MUG	β-MUGS
COS(−)	(0.04)[a]	(0.003)[a]
fusion 1−3[c]	0.20[b]	0.001[d]
fusion 1−4	0.26[b]	0.002[d]
fusion 1−5	0.20[b]	0.001[d]
wt βcDNA	0.65[b]	0.001[d]

[a] The nmol of MU hr^{-1} mg^{-1} of human Hex activity immunoprecipitated from mock-transfected COS cell lysate, COS(−), values were subtracted from those below. [b] The nmol of MU hr^{-1} mg^{-1} of Hex activity produced from the wild-type β and fusion 1 cDNA constructs after (a) immunoprecipitation of the human Hex, (b) the subtraction of the residual COS(−) activity, and (c) normalization for the transfection efficiency by factoring in the specific CAT activity. [c] Three fusion cDNAs in the pSVL vector are referred to as clones 3, 4, and 5, all of which contained the correct sequence of the fusion cDNA. [d] The nmol of MU hr^{-1} mg^{-1} of Hex activity produced from the wild-type β and fusion 1 cDNA constructs after (a) immunoprecipitation of the human Hex and (b) subtraction of the residual COS(−) activity. CAT values were used to confirm transfection of the cells (data not shown). However, Hex values were considered too low to be significant and were thus not normalized with CAT.

proβ chain was seen when the membrane fraction from these lysed cells was reextracted with detergent (Sagherian et al., 1994) (data not shown). These data emphasize the severe impairment on ER to Golgi transport that can occur because of a single amino acid substitution. Some impairment to the transport of the fusion protein can be inferred from the decrease in the levels of both immuno-cross-reactivity (Figure 2) and Hex activity (see below, Table 1) as compared to wt Hex B.

The level of Hex activity associated with the fusion 1 protein was assessed using our solid-state assay procedure for both MUG and MUGS substrates. This procedure reduces the COS cell background by selectively precipitating the human cDNA-derived Hex activity in transfected cells (Brown et al., 1989; Neote et al., 1990a). These Hex activities were normalized for transfection efficiency through their CAT activities and compared (Table 1). It is clear that expression of the fusion cDNA produces an active Hex with a substrate specificity very similar to the wt Hex B, *i.e.*, the same K_m for MUG and very little activity toward MUGS (Tables 1 and 2). The reduced levels of immunoreactive protein seen in the Western blots were comparable to the reduced levels of activity produced by the three fusion constructs (30−40% of wt Hex B). Also in keeping with

Table 2: Summary of Kinetic and Thermal Stability Data from Various cDNA Constructs Expressed in Transfected Cells

Hex	MUG K_m[a]	MUGS K_m[a]	G_{M2}/MUGS[b]	$T_{1/2}$ (45 °C) (min)[a]	$T_{1/2}$ 60 °C (min)[a]
B ($\beta\beta$)	0.71 ± 0.05	UD[c]	ND[d]	UD[e]	13.8
A ($\alpha\beta$)	0.75 ± 0.05	0.25 ± 0.05	1.3	230	2.2
S ($\alpha\alpha$)	1.55 ± 0.04	0.30 ± 0.06	0.0000	73	ND
fusion 1[f]	0.75 ± 0.05	UD[c]	ND	UD[e]	10.6
fusion 2	0.79 ± 0.05	0.30 ± 0.05	0.076	160	ND
A* ($\alpha\beta$*)[g]	0.92 ± 0.05	0.21 ± 0.05	1.2	ND	ND
CHO[h]	ND	ND	0.0000	ND	ND

[a] Assays were made using a solid-state assay immunoprecipitation system, ±standard error where given. [b] [G_{M2} ganglioside] hydrolyzed × 10^3/[MUGS] hydrolyzed. Values for G_{M2} hydrolysis were obtained in a free solution assay (endogenous CHO cell enzyme was present but found to be inactive toward the human activator protein/ganglioside complex[h]). Units of MUGS activity were determined utilizing the solid-state assay system except in the case of the negative control, *i.e.*, untransfected CHO cells, which was assayed in free solution. [c] Activity levels were too low to determine an accurate value. [d] The value was not determined for this isozyme. [e] Undetectable decrease in activity over the incubation period. [f] The fusion 1 protein was transiently expressed in COS cells. All other values are for constructs permanently transfected into CHO cells. [g] Data from Hou et al. (1996a) for a Hex A* isozyme with a normal α subunit and an inactive β subunit. [h] Endogenous CHO cell Hex, negative control.

Table 3: Immunoprecipitation Assay of Hex Activity in Transfected and Nontransfected CHO Cells

transfected cDNA	Hex	MUG[a] bound	MUGS[a] bound	MUG/MUGS[a]
none	CHO	(0.15)	(0.005)	
pEFNEO-αβ2	fusion 2	0.41	0.16	2.5
pREP4-α	Hex S	1.0	0.80	1.2
pEFNEO-β & pREP4-α	Hex B & Hex A[c]	31	4.0	7.7
pEFNEO-β	Hex B	70	0.22	320
pEFNEO-β*	inactive Hex B ($Arg^{211}Lys$)	0.03	0.00	
pEFNEO-β* & pREP-α	inactive Hex B* & partially active Hex A* (Hou et al., 1996a)	7.0	3.5	2.0

[a] The nmol of MU h^{-1} (μg of cell lysate protein)$^{-1}$, negative control levels from untransfected CHO cells, in parentheses, were subtracted from the levels presented in the table for cells transfected with the various cDNA constructs. Total recoveries of Hex units in both the bound and unbound fractions were 80−90% of that originally measured in the lysate. [b] The ratio of units of MUG versus units of MUGS hydrolyzed. [c] The MUG/MUGS ratio for Hex A, separated by ion-exchange from Hex B and S, is 4.0 (Hou et al., 1996a).

the Western blot data, the mutant cDNA encoding the $Ser^{129}Tyr$ substitution produced Hex activity only slightly higher than the background when assayed with the MUG substrate (data not shown). Since there was little or no activity toward the α-specific MUGS substrate, natural substrate (G_{M2}) hydrolysis was not tested.

Another unique characteristic of the Hex B isozyme is its increased thermostability (Robinson & Stirling, 1968). Thus, the rate of heat denaturation at 45° and 60 °C of fusion 1 was compared with those of wild-type Hex B and Hex A. There was only a slight difference in the heat stability between Hex B and the fusion 1 protein at 60 °C; both were stable at 45 °C. Thus, both isozymes had half-lives greater than that those of Hex A at either temperature (Table 2). Therefore, the N-terminal domain of the α subunit is not responsible for either its ability to hydrolyze negatively charged substrates or its decreased thermal stability.

Fusion 2. Since the substitution of the N-terminal domain of the β chain with the aligned section of the α (Figure 1) had little effect on either the substrate specificity or the heat stability of the chimeric protein (as compared to the wt Hex B isozyme, Tables 1 and 2), we increased the percent of α chain sequence in the fusion 2 protein. The additional section of the α chain that was added, $\alpha Val^{132}-\alpha Pro^{283}$ replacing $\beta Val^{165}-Lys^{315}$, is the most highly conserved region found in 15 deduced Hex sequences (α and/or β subunits) from various species including bacteria (Tse et al., 1996).

The two-step PCR process used to produce this chimera proved to give more consistent results than did the original one-step procedure (data not shown). The fusion fragment sequence was confirmed by nucleotide sequencing and subcloned into the mammalian expression vector pEFNEO (Anson et al., 1992) which was then used to permanently transfect CHO cells. Clonal populations of CHO cells that survived growth in selection medium were assayed for Hex activity with the MUG and MUGS substrates. The majority of the clones showed only slightly increased levels of activity toward either substrate over that found in nontransfected cells (data not shown).

After selection of apparently "high producing" clones, immunoprecipitation followed by the solid-state human Hex-specific assay was used to increase the human Hex "signal" over the endogenous CHO Hex "noise" in the lysates. This procedure resulted in the transfected cell lysates producing 4- or 32-fold higher levels of immunoprecipitable Hex activity than did lysates from untransfected cells with the MUG or MUGS substrates, respectively (Table 3, data from the highest producing clone). Thus, unlike Hex B and fusion 1, fusion 2 has the ability to hydrolyze negatively charged substrates.

The levels of activity immunoprecipitated from lysates of cells transfected with pEFNEO-β* [producing inactive but otherwise normal Hex B dimers (Brown & Mahuran, 1991; Hou et al., 1996a)] were nearly identical to those of the nontransfected lysates (Table 3). These data indicate that like human Hex expression in monkey COS cells, not even very low levels of CHO−human Hex interspecies dimers form using this expression system. These data confirm that the additional α sequences in the fusion 2 protein convey to it the substrate specificity of Hex A, Hex A*, or Hex S. Finally, the K_{ms} for fusion 2 binding of the MUG and MUGS substrates were determined. These more closely resembled those determined for Hex A or Hex A* than those for Hex S (Table 2).

The activity levels of the fusion 2 protein were far less than those found for CHO cells cotransfected with pREP4-α and either pEFNEO-β or pEFNEO-β* (Table 3: "Hex B & Hex A" and "Inactive Hex B* & partially active Hex A*"), or transfected with pEFNEO-β alone (Hex B, Table 3). Interestingly, when compared to either α and β, or α and β* cotransfected cells, lower levels of MUGS activity were also observed in cells transfected with the pREP4-α vector alone (Table 3). These data lead to the investigation of the intracellular location of the various Hex isozymes. Western blotting was done in order to confirm the synthesis of the fusion 2 protein. Because the α sequence replaced the β sequence responsible for the cleavage event in the lysosome that produces the mature β_b and β_a chains (Figure 1), only

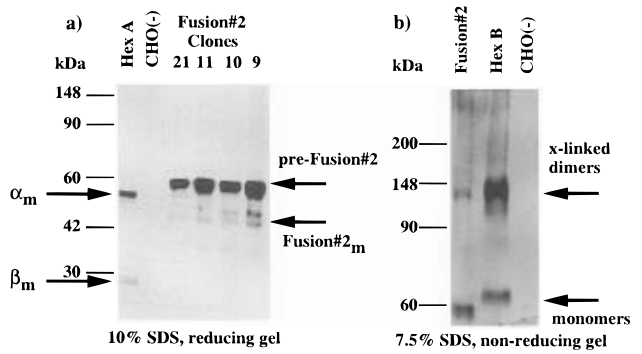

FIGURE 3: (a) Western blot of reduced and denatured CHO cell lysates using an anti-Hex A IgG (strongly reacts with the α_p and α_m chains of the α subunit, and the β_p segment of the β subunit; only reacts weakly with the β_a chain) from cells cotransfected with α and β cDNAs, Hex A; nontransfected cells, CHO(−); and four clones expressing the fusion 2 protein (9, 10, 11, and 21). Positions of the α_m (56 kDa) and β_m (β_a, 30 kDa) chains are indicated on the left with arrows. Positions of the fusion 2 precursor (pre-fusion 2) and mature fusion 2 polypeptides are indicated by arrows on the right. (b) Western blot of chemically cross-linked, nonreduced (reduction would remove the cross-links) and denatured CHO cell lysates from cells expressing the fusion 2 protein, fusion 2; cells transfected with a βcDNA construct, Hex B; and nontransfected cells, CHO(−). The regions of the gel corresponding to the cross-linked dimers and monomers are indicated by arrows at the right. The difference in molecular mass between the Hex B bands and those from the fusion 2 protein (~2 kDa) is caused by the presence of an N-linked oligosaccharide on the β_p chain in the former which has been replaced by a similar sized but unglycosylated α_p chain in the latter.

two polypeptides should be generated in the lysosome, α_p and the $\alpha-\beta_a$ fusion. In addition, there are three potential glycosylation sites in the $\alpha-\beta_a$ chain (apparent $M_r \cong 2000$ each). Thus, the apparent M_r for the reduced pro-form of the fusion 2 protein (no cleavage of the unglycosylated α_p chain, *i.e.*, $\alpha_p-\alpha-\beta_a$) should be about 63 000, and the apparent M_r of the reduced $\alpha-\beta_a$ fusion alone about 55 000. A major immunologically reactive band was detected by Western blot analysis of transfected cell lysates which migrated more slowly than the α_m chain of wt Hex A, 56 kDa (from pREP4-α and pEFNEO-β cotransfected CHO cell lysates, Table 3), indicating that the majority of the fusion 2 protein was in the precursor form (Figure 3a). A minor doublet was also seen which migrated slightly faster than the α_m chain. These bands likely represent the mature fusion 2 polypeptide ± the oligosaccharide at βAsn^{327} (Figures 1 and 3a, clone 9). The loss of this oligosaccharide through a single lysosomal processing step often produces a doublet for the β_a chain in transfected cells (McInnes et al., 1992; O'Dowd et al., 1988). Thus, the low levels of Hex activity found in cells expressing fusion 2 are consistent with the low levels of mature fusion 2 protein detected by Western blotting, suggesting the precursor forms are inactive and/or monomeric.

Since dimer formation has been shown to be a prerequisite for both the transport of the Hex isozymes out of the ER and the expression of enzymatic activity (Proia et al., 1984), we assessed the level of dimer formation attained by the fusion 2 protein. The assessment was obtained by introducing a cross-linking reagent (cleavable if reduced) into the lysates of CHO cells transfected with either pEFNEO-$\alpha\beta$2 or pEFNEO-β. We have previously shown that only dimeric forms of the β subunit are detectable in wild-type βcDNA-transfected cells (Brown et al., 1989). Thus, the latter sample

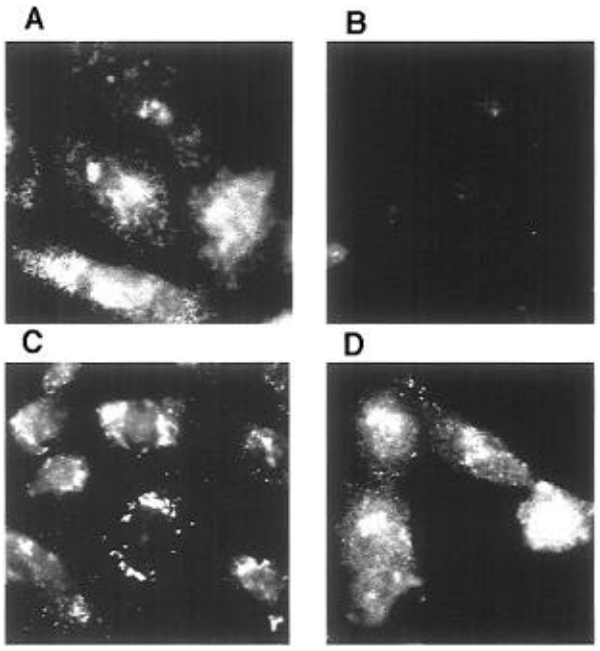

FIGURE 4: Immunofluorescence microscopy of CHO cells permanently transfected with pEFNEO-$\alpha\beta$2 (fusion 2), (A), nothing (negative control), (B), pEFNEO-β and pREP4-α (Hex A and Hex B), (C), or pREP4-α (Hex S) (D).

serves as a control for the efficiency of the cross-linking reaction (never 100%). The results demonstrated that the fusion 2 subunits are not as efficiently formed into dimers as were the wild-type β subunit controls (Figure 3b).

Next we directly examined the intracellular locations of the various Hex isozymes by immunofluorescence microscopy using the rabbit anti-Hex A antibody. Cells cotransfected with both pREP4-α and pEFNEO-β were found to have a staining pattern consistent with the majority of the α and β chains being localized to the lysosome (Figure 4). Interestingly, cells transfected with either pREP4-α or pEFNEO-$\alpha\beta$2 alone produced more diffuse patterns with fewer distinct spots, indicating that a large population of the two proteins (Hex S and Hex fusion 2) resides in the ER, with a corresponding decreased level of lysosomal incorporation (Figure 4). Taken along with the results from the Western blot and the Hex activity measurements (above), these data suggest that both the human proα and fusion 2 proteins are not able to efficiently form active dimers, a prerequisite for transport out of the ER.

Since the fusion 2 protein has the ability to hydrolyze negatively charged MUGS, like Hex A, Hex A*, and Hex S (Hou et al., 1996a) (Table 3), its ability to hydrolyze the negatively charged natural substrate of Hex A, G_{M2} ganglioside/G_{M2} activator complex, was tested. In this experiment, equal numbers of units of MUGS activity (rather than protein mass) from (a) nontransfected CHO cells (CHO-Hex A), (b) human fibroblast lysate from a Sandhoff patient shown to be homozygous for a partial *HEXB* gene deletion (Neote et al., 1990b) (human Hex S), (c) CHO cell transfected with wt pREP4-α and pEFNEO-β (human-Hex A), and (d) CHO cell transfected with pEFNEO-$\alpha\beta$2 (human-derived Hex fusion 2) were used along with 2 μg of purified human G_{M2} activator protein produced in bacteria (Klima et al., 1993). As previously shown (Hou et al., 1996a), endogenous CHO cell Hex A and human Hex S do not interact productively with the G_{M2}−human activator protein complex, and there

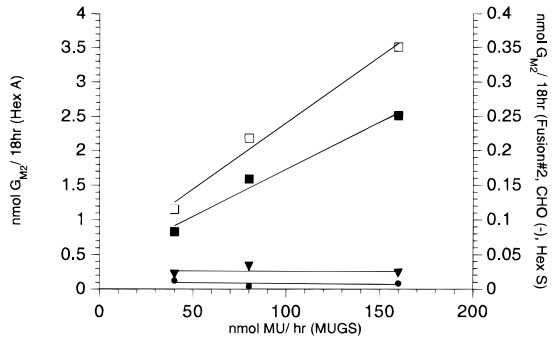

FIGURE 5: Hydrolysis of G_{M2} ganglioside in the presence of human G_{M2} activator protein by CHO cell lysates containing equal numbers of MUGS activity units (*x*-axis) from (a) nontransfected cells [endogenous CHO-Hex A, CHO(−)] (closed triangles plotted on the right *y*-axis), or cells permanently transfected with (b) pEFNEO-β and pREP4-α (Hex A) (open squares plotted on the left *y* axis), or (c) pEFNEO-$\alpha\beta$2 (fusion 2), (closed squares plotted on the right *y*-axis). These were compared to equal numbers of MUGS activity units (*x*-axis) from human fibroblast lysates from a patient shown to be homozygous for a partial deletion of the *HEXB* gene (human Hex S) (closed circles plotted on the right *y*-axis). The best-fit slopes of these lines (divided by 18 to equalize the hydrolysis times) are reported in Table 2.

was no detectable increase in G_{M2} hydrolysis with increased units of MUGS activity added to the reaction mix (Figure 5, Table 2). Whereas assays using Hex A (Figure 5) or Hex A* (Hou et al., 1996a) produced a G_{M2}/MUGS hydrolysis ratio of $\sim 1 \times 10^{-3}$, assays using the fusion 2 protein produced a G_{M2}/MUGS hydrolysis ratio of $\sim 0.1 \times 10^{-3}$ (Figure 5, Table 2). Thus, G_{M2} ganglioside in the presence of the human activator is a substrate for the fusion 2 Hex. Therefore, this chimeric enzyme contains a significant portion of both the α subunit's active site and its G_{M2} activator/G_{M2} ganglioside complex binding site. It also contains at least part of the previously undefined domain in the β subunit necessary to force a productive interaction of the α-active site in the heterodimer with the bound G_{M2} activator/G_{M2} ganglioside complex.

The heat stability of fusion 2 was tested at 45 °C and compared to fusion 1 and Hex A, B, and S. The protein had a $T_{1/2}$ about twice that of Hex S, but only 50% that of Hex A. At this temperature, fusion 1 and Hex B are stable (Table 2). Thus, the heat stability of the Hex isozymes is as follows: Hex B $\cong$ fusion 1 > Hex A > fusion 2 > Hex S. These data indicate that the middle segment of the α subunit is responsible for both its ability to hydrolyze negatively charged substrates and its decreased thermal stability.

DISCUSSION

Linking biochemical functions and characteristics to protein domains through *in vitro* mutagenesis and mammalian cell expression is now a common technique. However, in the case of Hex, as well as many other proteins that are synthesized in the ER, the common approach of making random changes to its primary structure as a means of probing for functional domains is thwarted by the quality control system present in the ER (Edgington, 1992; Hurtley & Helenius, 1989; Pelham, 1989). In an attempt to circumvent this problem, we constructed $\alpha-\beta$ chimeric proteins joined at adjacently aligned sequences (Figure 1). This approach proved to be at least partially successful. It was found that a significant part of the fusion 2 protein and likely some of the fusion 1 protein were retained in the ER. However, the degree of sensitivity of the Hex system to random amino acid substitutions was emphasized by the failure of a mutant form of the fusion 1 protein, containing an additional αSer^{129}Tyr substitution, to be transported out of the ER even in transfected COS-1 cells.

Unlike the transient transfection of αcDNA in the COS cell system where efficient expression of Hex S has been shown to be possible (Brown & Mahuran, 1993), we were only able to detect low levels of MUGS (Hex S) activity in transfected CHO cells. However, high levels of MUGS activity were observed when both α and β cDNAs were cotransfected into CHO cells (Table 3). In this respect, permanently transfected CHO cells behave more like fibroblasts from Sandhoff patients where the majority of α chains are retained and degraded in the ER [reviewed in Mahuran (1991)]. We confirmed the similar fates of the α subunit and fusion 2 proteins in transfected CHO cells by immunofluorescence microscopy (Figure 4). Chemical cross-linking studies also showed that fewer fusion 2 dimers were formed (monomers are not active), as compared to wt β subunit dimers, in lysates from transfected CHO cell (Figure 3b, fusion 2 as compared to Hex B). We have used this technique previously to demonstrate that in CHO cells expressing a β subunit containing a Asp^{208}Asn substitution, no $\beta\beta$ dimers were formed. These data were also consistent with the lack of any human Hex B activity and the detection of only pro βAsp^{208}Asn chains in untreated cell lysates (Tse et al., 1996). Thus, the α sequences contained in the fusion 2 protein may be responsible for the increased retention of both the wild-type α chain and the fusion 2 protein in the ER.

At low levels of expression such as those we report for fusion 2 and Hex S, the question of whether or not interspecies heterodimers, *e.g.*, human-$\alpha-$CHO-β, CHO-$\alpha-$human-β, or CHO-$\beta-$human-β, are able to form must be considered. Such heterodimers would be expected to immunoprecipitate in our assay because of their single human-derived subunit. A careful examination of the data in Table 3 demonstrates that there is no such heterodimer formation because of the following: (a) We have shown that the ratios of MUG/MUGS hydrolysis for isozymes partially purified by ion-exchange chromatography are Hex B ~300:1, Hex A ~4:1, Hex A* ~2:1, and Hex S ~1:1 (Hou et al., 1996a). When normal human αcDNA (pRep4-α) is expressed and immunoprecipitated from CHO cell lysates, the MUG/MUGS ratio is ~1:1 (Table 3). Thus, no human-$\alpha-$CHO-β heterodimers are formed. (b) When a cDNA insert encoding a nearly inactive (MUG V_{max} = 0.25% of wt Hex B) but otherwise normal β* subunit, pEFNEO-β* (Brown & Mahuran, 1991; Hou et al., 1996a), is expressed in CHO cells, the immunoprecipitated protein does not produce activities significantly above those of the negative control cells with either substrate. Detectable amounts of MUG and/or MUGS activity would be expected if β* subunits could recruit potentially active CHO cell α and/or β subunits, as they do when human α subunits are present, *i.e.*, when pEFNEO-β* is cotransfected with pREP4-α (Table 3). Thus, while both inactive $\beta^*-\beta^*$ dimers and active human-$\alpha-\beta$* dimers form in transfected CHO cells (Hou et al., 1996a), no CHO-$\beta-$human-β* or CHO-$\alpha-$human-β* active, interspecies dimers are able to form. (c) The MUG/MUGS ratio for fusion 2 is

very similar to that of Hex A* (Table 3), as are the K_m values for MUG and MUGS (Table 2). Thus, there is likely no fusion 2−CHO-β dimer present. (d) Finally, like the α-active site in human Hex A, CHO cell Hex A can hydrolyze MUGS, but unlike its human counterpart it cannot hydrolyze G_{M2} ganglioside when it is complexed with the human activator protein; *i.e.*, no interspecies CHO-Hex A−G_{M2}/human activator complex can form (Figure 5, Table 2). Since the G_{M2}/activator complex is bound by elements of the α subunit of Hex A [reviewed in Furst and Sandhoff (1992) and Sandhoff et al., (1989)], the retention of this species-specific ability to utilize the G_{M2}/human activator complex as a substrate by fusion 2 indicates that fusion 2 contains the section of the human α subunit responsible for both complex binding and the hydrolysis of negatively charged substrates (G_{M2} and MUGS).

The data in this report link various protein domains within the α and β subunits of Hex with some of the biochemical properties associated with each of the Hex isozymes. Since the two α and β polypeptides are evolutionarily related (Proia, 1988), one hypothesis could be that common functions are linked to highly homologous protein domains and the areas of least homology confer their functional differences. This is an attractive hypothesis since the overall 60% amino acid identity between the two subunits is not distributed evenly throughout their primary sequences. To test this hypothesis, we exchanged the sequences between the α and β chains that comprise the least homologous domains within their aligned sequences, joined at the beginning of a stretch of 15 invariant residues so as not to affect any unique secondary structure of the proα chain, fusion 1 (Figure 1). The region of exchange contained the signal sequence of the preproα chain, the entire α_p chain, and a small segment of the mature α N-terminus (Figure 1) which accounts for all of exons 1and 2, and most of exon 3 (all but 6 codons) (Neote et al., 1988; Proia, 1988; Proia & Soravia, 1987) (Figure 1). Our results demonstrated that this chimeric protein had both the thermal stability and the substrate specificity of Hex B. Thus, this nonhomologous α N-terminal domain does not appear to confer any of the unique α properties to the otherwise β-derived fusion 1 protein.

As the nonhomologous N-terminal regions seem to be functionally irrelevant [except for initial folding (Sagherian et al., 1994) and perhaps for the rate of transport out of the ER], determinants that create the novel biochemical characteristics of the α and β subunits might be located only in a few short stretches of nonhomologous sequences within the much more homologous middle and C-terminal regions of the two chains. Of these two sections, the middle section, exons 5−6, is the most conserved in 15 deduced primary structures of Hex from various organisms/species (Tse et al., 1996). We have also shown that exon 5 contains two residues involved in substrate hydrolysis which are invariant in all 15 aligned Hex sequences, *i.e.*, $\alpha Arg^{178}/\beta Arg^{211}$ and $\alpha Asp^{163}/\beta Asp^{196}$ (Tse et al., 1996). However, a recent report suggests that a residue in exon 8 may be involved in substrate binding. In this study, a photoaffinity label in the aglycon region of a substrate analog was transferred to βGlu^{355} (Liessem et al., 1995). This residue is invariant in 10 of the 15 aligned Hex sequences. Since no *in vitro* mutagenesis, expression, and kinetic studies were reported, the relationship between this residue and the active site of Hex remains to be proven. The fusion 2 construct contained all of exons 1−7 and 15 codons from exon 8 of the *HEXA* gene (α sequence), with the rest of the coding sequence from the *HEXB* gene (β sequence). This area was chosen because the remaining β sequence encodes all of the mature β_a chain (Figure 1). However, the normal β_a/β_b cleavage does not occur in fusion 2 (Figure 3) because the β_p-β_b-Arg-Gln-Asn-Lys^{315}-β_a cleavage sites (underlined) have been replaced by the aligned α sequence (α_p-αGly-Ser-Glu-Pro^{283}-β_a) which is not cleaved (Figure 1). We have previously shown that the β_a/β_b cleavage site is located in a hydrophilic β-turn structure, and that the deletion of the entire βArg-Gln-Asn-Lys^{315} sequence, or its substitution by the aligned α sequence, prevents posttranslational cleavage. However, neither of these modifications affects the substrate specificity; *i.e.*, each mutant Hex B hydrolyzed MUG but not MUGS, and only the deletion construct produces a less heat-stable Hex isozyme ($T_{1/2}$ at 60 °C = 4.6 min) (Sagherian et al., 1993). Thus, the fusion point did not interrupt a domain critical to folding of the β chain.

Despite the lower than expected yield of the fusion 2 protein and its associated Hex activities (Table 3), we were still able to characterize it biochemically through our human Hex-selective solid-state assay systems (Brown & Mahuran, 1993) (Table 2). In several respects, fusion 2 resembled Hex S. Neither was efficiently transported from the ER to the lysosome of transfected CHO cells (Figure 4), but both could bind and hydrolyze the α-specific MUGS substrate with similar K_{ms}. However, fusion 2's K_m for the MUG substrate was similar to that of Hex A*, rather than Hex S (Table 2), indicating that the elements of the β subunit that moderate the kinetics of the α subunit in the Hex A and Hex A* heterodimers (Hou et al., 1996a) are contained in the β subunit's C-terminal segment. This conclusion was strengthened by the most striking difference between biochemical properties of fusion 2 and Hex S; fusion 2 was able to hydrolyze G_{M2} ganglioside in the presence of the human G_{M2} activator at 10% the specific activity of Hex A or Hex A* (Figure 5, Table 2).

Our data from the biochemical characterization of fusions 1 and 2 localize the area responsible for the α subunit's decreased thermal stability and its ability to specifically bind negatively charged substrates to residues α132−283 (Figure 1). This region is encoded by exons 5−7 in *HEXA* and must, as well, contain part or all of the domain responsible for binding the activator/G_{M2} complex. Our data also identify the C-terminus of the β subunit as the area in the Hex A heterodimer responsible for correctly orienting the G_{M2} ganglioside/G_{M2} activator complex for hydrolysis by the α-active site (Figure 5). This area also appears to be responsible for the kinetic differences we have previously reported for the active site of the α subunit when present in its heterodimeric Hex A* form, versus its homodimeric Hex S form (Tables 2 and 3) (Hou et al., 1996a). Having these effects on a domain in α suggests that this area in the β subunit is involved in α subunit−β subunit interactions.

REFERENCES

Anson, D. S., Taylor, J. A., Bielicki, J., Harper, G. S., Peters, C., Gibson, G. J., & Hopwood, J. J. (1992) *Biochem. J. 284*, 789−794.

Bayleran, J., Hechtman, P., & Saray, W. (1984) *Clin. Chim. Acta 143*, 73−89.

Bikker, H., Meyer, M. F., Merk, A. C., de Vijlder, J. J., & Bolhuis, P. A. (1988) *Nucleic Acids Res. 16*, 8198−8198.
Brown, C. A., & Mahuran, D. J. (1991) *J. Biol. Chem. 266*, 15855−15862.
Brown, C. A., & Mahuran, D. J. (1993) *Am. J. Hum. Genet. 53*, 497−508.
Brown, C. A., Neote, K., Leung, A., Gravel, R. A., & Mahuran, D. J. (1989) *J. Biol. Chem. 264*, 21705−21710.
Edgington, S. M. (1992) *Biotechnology 10*, 1413−1420.
Furst, W., & Sandhoff, K. (1992) *Biochim. Biophys. Acta 1126*, 1−16.
Gravel, R. A., Clarke, J. T. R., Kaback, M. M., Mahuran, D., Sandhoff, K., & Suzuki, K. (1995) in *The Metabolic Basis of Inherited Disease* (Scriver, C. V., Beaudet, A. L., Sly, W. S., & Valle, D., Eds.) pp 2839−2879, McGraw-Hill, New York.
Hasilik, A., & Neufeld, E. F. (1980a) *J. Biol. Chem. 255*, 4937−4945.
Hasilik, A., & Neufeld, E. F. (1980b) *J. Biol. Chem. 255*, 4946−4960.
Hou, Y., Tse, R., & Mahuran, D. J. (1996a) *Biochemistry 35*, 3963−3969.
Hou, Y., Vavougios, G., Hinek, A., Wu, K. K., Hechtman, P., Kaplan, F., & Mahuran, D. J. (1996b) *Am. J. Hum. Genet. 59*, 52−58.
Hurtley, S. M., & Helenius, A. (1989) *Annu. Rev. Cell Biol. 5*, 277−307.
Ikonne, J. V., Rattazzi, M. C., & Desnick, R. J. (1975) *Am. J. Hum. Genet. 27*, 639−650.
Klima, H., Klein, A., Van Echten, G., Schwarzmann, G., Suzuki, K., & Sandhoff, K. (1993) *Biochem. J. 292*, 571−576.
Korneluk, R. G., Mahuran, D. J., Neote, K., Klavins, M. H., O'Dowd, B. F., Tropak, M., Willard, H. F., Anderson, M.-J., Lowden, J. A., & Gravel, R. A. (1986) *J. Biol. Chem. 261*, 8407−8413.
Kresse, H., Fuchs, W., Glossl, J., Holtfrerich, D., & Gilberg, W. (1981) *J. Biol. Chem. 256*, 12926−12932.
Kytzia, H.-J., & Sandhoff, K. (1985) *J. Biol. Chem. 260*, 7568−7572.
Liessem, B., Glombitza, G. J., Knoll, F., Lehmann, J., Kellermann, J., Lottspeich, F., & Sandhoff, K. (1995) *J. Biol. Chem. 270*, 23693−23699.
Mahuran, D. J. (1991) *Biochim. Biophys. Acta 1096*, 87−94.
Mahuran, D., Novak, A., & Lowden, J. A. (1985) *Isozymes: Curr. Top. Biol. Med. Res. 12*, 229−288.
McInnes, B., Potier, M., Wakamatsu, N., Melancon, S. B., Klavins, M. H., Tsuji, S., & Mahuran, D. J. (1992) *J. Clin. Invest. 90*, 306−314.
Meier, E. M., Schwarzmann, G., Fürst, W., & Sandhoff, K. (1991) *J. Biol. Chem. 266*, 1879−1887.
Nakai, H., Byers, M. G., Nowak, N. J., & Shows, T. B. (1991) *Cytogenet. Cell Genet. 56*, 164.
Neote, K., Bapat, B., Dumbrille-Ross, A., Troxel, C., Schuster, S. M., Mahuran, D. J., & Gravel, R. A. (1988) *Genomics 3*, 279−286.
Neote, K., Brown, C. A., Mahuran, D. J., & Gravel, R. A. (1990a) *J. Biol. Chem. 265*, 20799−20806.
Neote, K., McInnes, B., Mahuran, D. J., & Gravel, R. A. (1990b) *J. Clin. Invest. 86*, 1524−1531.
O'Dowd, B. F., Cumming, D., Gravel, R. A., & Mahuran, D. (1988) *Biochemistry 27*, 5216−5226.
Pelham, R. B. (1989) *Annu. Rev. Cell Biol. 5*, 1−23.
Proia, R. L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1883−1887.
Proia, R. L., & Soravia, E. (1987) *J. Biol. Chem. 262*, 5677−5681.
Proia, R. L., d'Azzo, A., & Neufeld, F. (1984) *J. Biol. Chem. 259*, 3350−3354.
Robinson, D., & Stirling, J. L. (1968) *Biochem. J. 107*, 321−327.
Sagherian, C., Poroszlay, S., Vavougios, G., & Mahuran, D. J. (1993) *Biochem. Cell Biol. 71*, 340−347.
Sagherian, C., Thorner, P., & Mahuran, D. (1994) *Biochem. Biophys. Res. Commun. 204*, 135−141.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Vol. 1−3, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sandhoff, K., Conzelmann, E., Neufeld, E. F., Kaback, M. M., & Suzuki, K. (1989) in *The Metabolic Basis of Inherited Disease* (Scriver, C. V., Beaudet, A. L., Sly, W. S., & Valle, D., Eds.) pp 1807−1839, McGraw-Hill, New York.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Tse, R., Vavougios, G., Hou, Y., & Mahuran, D. J. (1996) *Biochemistry 35*, 7599−7607.
Yon, J., & Fried, M. (1989) *Nucleic Acids Res. 17*, 4895−4895.

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