Identification of Domains in Human β-Hexosaminidase That Determine Substrate Specificity*

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The lysosomal β-hexosaminidases are dimers composed of α and β subunits. β-Hexosaminidase A (αβ) is a heterodimer, whereas hexosaminidase B (ββ) and S (αα) are homodimers. Although containing a high degree of amino acid identity, each subunit expresses a unique active site that can be distinguished by a differential ability to hydrolyze charged substrates. The site on the β-subunit primarily degrades neutral substrates, whereas the α-subunit site is, in addition, active against sulfated substrates. Isozyme specificity is also exhibited with glycolipid substrates. Among human isoforms, only β-hexosaminidase A together with the Ga2 activator can degrade the natural substrate, Ga2 ganglioside, at physiologically significant rates. To identify the domains of the human β-hexosaminidase subunits that determine substrate specificity, we have generated chimeric subunits containing both α- and β-subunit sequences. The chimeric constructs were expressed in HeLa cells to screen for activity and then selected constructs were produced in the baculovirus expression system to assess their ability to degrade Ga2 ganglioside in the presence of Ga2 activator protein. Generation of activity against the sulfated substrate required the substitution of two noncontiguous α-subunit sequences (amino acids 1–191 and 403–529) into analogous positions of the β-subunit. Chimeric constructs containing only one of these regions linked to the β-subunit sequence showed either neutral substrate activity only (amino acids 1–191) or lacked enzyme activity entirely (amino acids 403–529). Neither the chimeras nor the wild-type subunits displayed activator-dependent Ga2 hydrolyzing activity when expressed alone. However, one chimeric subunit containing α amino acids 1–191 fused with β amino acids 225 to 556, when co-expressed with the wild-type α-subunit, showed activity comparable with that of recombinant β-hexosaminidase A formed by the co-expression of the α- and β-subunits. This result indicates that the β-subunit amino acids 225-556 contribute an essential function in the Ga2-hydrolyzing activity of β-hexosaminidase A.

The human β-hexosaminidases (EC 3.2.1.52) are dimeric lysosomal enzymes composed of two subunits, α- and β, that share about 60% of their amino acid sequence (1, 2). The subunits are synthesized as precursors in the endoplasmic reticulum where amino-terminal signal peptides are removed, N-linked glycosylation occurs, disulfide bonds are formed, and the subunits are folded and assembled. The subunits dimerize and form three isozymes; β-hexosaminidase A (αβ), β-hexosaminidase B (ββ), and β-hexosaminidase S (αα). Dimerization of the subunits is required for acquisition of enzymatic activity. When properly folded and assembled the enzymes are transferred to the Golgi apparatus for synthesis of the mannose 6-phosphate recognition marker. Mannose 6-phosphate receptors then target the enzymes to lysosomes where the precursor subunits are proteolytically processed to their mature forms (for reviews see Refs. 3 and 4).

The β-hexosaminidases participate in the degradation of glycoproteins, glycolipids, and proteoglycans through the removal of terminal β-glycosidically linked N-acetylgalactosamine or N-acetylglactosamine residues. The capacity of this enzyme system to degrade this range of substrates is due, in part, to a unique active site with distinct specificities carried by each subunit (5). The β-subunit active site predominantly hydrolyzes neutral substrates, whereas the α-subunit active site can also react with negatively charged substrates. In addition, certain glycolipid substrates, such as Ga2 ganglioside, show a strict isozyme dependence for their degradation. Of the three isozymes, only the heterodimer β-hexosaminidase A, together with the Ga2 activator protein, is able to degrade Ga2 ganglioside significantly (6). The Ga2 activator protein functions by binding the ganglioside substrate and interacting with β-hexosaminidase A effecting release of the terminal N-acetylgalactosamine residue from the ganglioside (5, 7, 8).

The importance of this enzyme system is demonstrated by the consequences of mutations in the genes that encode the α-subunit, the β-subunit, or the Ga2 activator. Mutations in the HEXA gene cause Tay-Sachs disease as a result of a deficiency of the α-subunit and a resulting absence of β-hexosaminidase A and S. Mutations in the HEXB gene encoding the β-subunit cause Sandhoff's disease and result in the absence of β-hexosaminidase A and B. Defects in the GM2A gene result in Ga2 activator deficiency. In each of these disorders there is a massive accumulation of Ga2 ganglioside in neuronal lysosomes leading to severe neurodegeneration.

The goal of this work was to identify the domains that confer distinctive substrate specificity to the hexosaminidase...
Isozymes. Our approach was to create chimeric hexosaminidase subunits by interchanging analogous regions of the α- and β-subunits. We expected that some chimeric subunits would be enzymatically active due to the high degree of structural similarity shared between the two subunits. Using these chimeras, we have defined two noncontiguous sequences on the α-subunit that, substituted into the β-subunit, confer the ability to degrade a charged substrate. Further, we have localized the region of the β-subunit required for activator-dependent G_{M_2} ganglioside degradation by β-hexosaminidase A.

EXPERIMENTAL PROCEDURES

Construction of Chimeric Enzymes—The human β-hexosaminidase α- and β-subunit cDNA were subcloned into pBluescript II KS (Stratagene) as described (9) to produce pBS containing primer used to make the α-subunit. The open reading frame of the α-subunit cDNA sequence were amplified with a primer containing an NcoI (cDNA site) and a XbaI site (5’-GGGGGCTCGAGGGCTCAGGTCTGT-3’). The α-subunit fragment was cut with NcoI and XbaI and subcloned into the NcoI/XbaI-digested pBS. To make the βα1 enzyme construct, nucleotides 1206–1590 of the α-subunit cDNA sequence were amplified by primers containing a Ncol site (restriction sites underlined), 5’-CCCCCATGGCATCCAGAGTGTCCAGGATGC-3’ and an XbaI site, 5’-GGCTTAGACCGGCGGCTAGCAGAAGCTCGACGCTTGG-3’. The resulting fragment was cut with Ncol and XbaI and subcloned into the Ncol/XbaI-digested pBS. To make the βaα1 enzyme construct, nucleotides 1080–1590 of the α-subunit cDNA sequence were amplified by primers containing a Bsu36I site (5’-CCCCCTGAGGAGCTGGAACTGC-3’) and an XhoI site (5’-GGGGCGCTCGAGGGGCGCCATGCTTGGTGCAATCTCTGGCTCAG-3’). The resulting fragment was digested with Bsu36I and XhoI and purified by low melting point agarose gel electrophoresis analysis. The Coomassie Blue-stained protein bandsof concanavalin A-purified recombinant hexosaminidase were excised from the gel and subjected to in-gel trypsin digestion, followed by 2-dimensional gel electrophoresis and subcloned into Bsu36I- and XhoI-digested pBS. To make the αβ2 construct, nucleotides 1080–1590 of the α-subunit cDNA sequence were amplified by primers containing a Bsu36I site (5’-AGGCAAGGTCTTGACGCTTAGGAGCTGGAGCAGTTGGCTCAG-3’) and the XhoI site containing primer used to make βα1. The resulting α-subunit cDNA fragment was then digested with HindIII and XhoI and subcloned into HindIII- and XhoI-digested (polylinker site) pBS. To make the αβ3 and βα2 constructs, nucleotides 1519–1590 of the α-subunit cDNA sequence were amplified with a primer containing a PstI site (5’-GGGGGGCTCGAGGGGCGCCATGCTTGGTGCAATCTCAG-3’) and the XhoI site containing primer used to make the αβ2 and βα1 constructs. The polymerase chain reaction fragment was then digested with PstI and XhoI, gel purified, and subcloned into PstI (cDNA site) and XhoI (polylinker site) digested pBSα1 with NcoI.

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Large scale expression of β-hexosaminidase and chimeric enzyme constructs was accomplished in High-Five cells (Invitrogen) by Kemn Biotechnologies Inc. For co-infection studies, the optimal multiplicity of infection for co-expression of chimeric and wild-type cDNA constructs was determined by isoelectric focusing of the High-Five cell conditioned medium (described below). The cell pellets were incubated at the appropriate multiplicity of infection in 5 ml of serum-free Grace’s medium for 4 h on a rocking platform. After the incubation, the cells were washed and incubated with 5 ml of serum-free medium for 3 days.

Purification of β-Hexosaminidase and Chimeric Enzymes—Enzymes were obtained from the serum-free culture medium of infected cells. The enzymes were partially purified on a column of concanaval A-Sepharose (Pharmacia Biotech Inc.) (13). Column fractions containing enzyme activity were pooled prior to dialysis with 0.5 M NaCl and 10 mM sodium phosphate, pH 7.5, 10 mM sodium citrate, pH 4.5, 0.05 mM EDTA, and stored at 4°C until use. Purified chimeric enzymes were dialyzed against 50 mM sodium phosphate, pH 7.5, and 100 mM sodium citrate, pH 4.5, and MU-GlcNAc-6-SO4 (0.1–1.0 mM in 50 mM sodium citrate buffer, pH 4.5) and MU-GlcNAc-6-SO4 hydroryzing activity. Viral stocks were generated by large scale infection of 2 × 10^9 SF9 cells in suspension culture and titrated in High-Five cells (Invitrogen) using the Quick-titer kit from Kemp Biotechnologies Inc.
RESULTS

The α- and β-subunits of β-hexosaminidase share about 60% of their amino acid sequence (1, 2) (Fig. 1). Chimeric hexosaminidase subunits were constructed by interchanging cDNA sequences encoding analogous segments of the α- and β-subunits (Figs. 1 and 2). The constructs were positioned adjacent to the T7 polymerase promoter in the pBluescript vector so that expression of the constructs could be driven by T7 polymerase after their transfection into HeLa cells. In the representative experiment shown in Fig. 3, the extracts from the transfected HeLa cells were first assessed by Western blot analysis to ensure protein expression (Fig. 3A) and then for enzyme activity using the neutral (MU-GlcNAc) and the charged (MU-GlcNAc-6-SO4) synthetic substrates. Polypeptides, reactive with anti-β-hexosaminidase A, were expressed from each chimeric construct that were similar (60–70 kDa) but not identical in electrophoretic mobility to the wild-type subunit precursors. The wild-type enzymes demonstrated the expected specificity: β-hexosaminidase S, produced by expressing the wild-type α-subunit, cleaved both the MU-GlcNAc and MU-GlcNAc-6-SO4 substrates. By contrast, β-hexosaminidase B, produced from expressing the β-subunit, showed a marked preference for the neutral MU-GlcNAc substrate and, correspondingly, a very low ratio of sulfated to neutral substrate cleaved. Chimeric construct, αβ1, containing the amino-terminal residues 1–191 of the α-subunit fused to the carboxyl-terminal amino acids 225–556 of the β-subunit, was active against the MU-GlcNAc with relatively little activity against the MU-GlcNAc-6-SO4 substrate in a manner similar to wild-type β-subunit. A number of other αβ constructs were prepared containing both larger and smaller segments of the α-subunit at the amino terminus, but they were all catalytically inactive (not shown). Also inactive was construct βa1, which consisted of amino-terminal β-subunit sequence up to amino acid 431 linked to α-subunit sequence from amino acid 403 to the carboxyl terminus (Fig. 1 and 2).

In an attempt to prepare an active chimera with MU-GlcNAc-6-SO4 activity, we modified construct αβ1 by replacing the β-subunit carboxyl-terminal portion with the analogous α-subunit sequence 403–529 so that this construct contained the same amino-terminal segment as αβ (active only against MU-GlcNAc) and the same carboxyl-terminal segment as βa1 (inactive).
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unsatisfactory because of a high background GM2 ganglioside degrading activity inherent in the human and monkey cells. We, therefore, chose the baculovirus expression system because of the potential for very high levels of protein production and because of very low background activity. We analyzed two representative chimeric enzymes, αβ1α and αβ1β, in this manner. Apparent Km and Vmax values for MU-GlcNAc and MU-GlcNAc-SO4 were first determined for the chimeric enzymes and for recombinant β-hexosaminidase S and B produced by expression of the wild-type α- and β-subunit, respectively (Table I). Like β-hexosaminidase B, chimeric construct αβ1 displayed a lower Kmax with MU-GlcNAc compared with MU-GlcNAc-SO4. By contrast, β-hexosaminidase S and the chimeric construct, αβ1α, both of which display activity against the sulfated substrate, showed a lower Kmax with MU-GlcNAc-SO4 relative to MU-GlcNAc. All enzymes developed the greatest maximal activity against the sulfated substrate and had a ratio of sulfated to neutral substrate cleaved similar to the wild-type α-subunit. This result indicates that the sulfated regions are responsible for their characteristic specificity. In addition to their very similar, although differences obviously exist that are responsible for their characteristic specificity.

The Km and Vmax values of recombinant β-hexosaminidase isozymes and chimeras expressed in the baculovirus system and partially purified toward the synthetic substrates MU-GlcNAc and MU-GlcNAc-SO4 are shown in Table I. For an explanation see Fig. 2.

### Table I

| Enzyme          | MU-GlcNAc | MU-GlcNAc-SO4 |
|-----------------|-----------|---------------|
| β-Hexosaminidase S (wild-type α-subunit) | 1.74 1.2 × 10⁻⁴ | 0.71 6.8 × 10⁻⁵ |
| β-Hexosaminidase B (wild-type β-subunit) | 0.57 2.2 × 10⁻⁴ | 1.2 1.4 × 10⁻⁶ |
| αβ1α            | 0.58 3.6 × 10⁻⁴ | 9.3 5.7 × 10⁻⁶ |
| αβ1β            | 0.73 6.2 × 10⁻⁵ | 0.25 2.0 × 10⁻⁵ |

*For an explanation see Fig. 2.*

None of the constructs (α-subunit, β-subunit, αβ1α, or αβ1β) produced significant activator-dependent GM2 ganglioside degrading activity when expressed alone (Fig. 4). In contrast, co-expression of the wild-type α- and β-subunits, to produce β-hexosaminidase A yielded significant activator-dependent GM2 ganglioside hydrolyzing activity. We next co-expressed the chimeric subunits with each wild-type subunit in order to assemble heterologous subunits. The assembly of the various subunit combinations was verified by isoelectric focusing (not shown). However, only the combination of αβ1β and the wild-type α-subunit resulted in activity comparable with the co-expression of wild-type α- and β-subunits. All other combinations were not effective in the activator-dependent GM2 ganglioside degradation assay. This result indicates that the β-subunit amino acids 225-556 contribute an essential function in combination with the wild-type α-subunit for effective activator-dependent degradation of GM2 ganglioside.

**Discussion**

The α- and β-subunits of β-hexosaminidase are the product of a gene duplication event (1), and as a result, they show a high degree of structural and functional similarity. It is also likely that the structure of the active site in each subunit is very similar, although differences obviously exist that are responsible for their characteristic specificity. In addition to their active sites, the subunits express other distinctive functions related to the binding of the GM3, ganglioside activator complex (6). The structural similarity of the subunits has enabled the creation of a series of enzymatically active chimeric polypeptides and has allowed us to attribute the functional differences of the subunits to discrete polypeptide segments.

Only the α-subunit active site, expressed in β-hexosaminidase...
Clearly, a three-dimensional structure of β-hexosaminidase, when available, will greatly facilitate the interpretation of these results.

Although the active sites on β-hexosaminidase subunits have been well characterized kinetically (5), there is only minimal information concerning their structure. Recently, however, Glu-355 in the β-subunit was photoaffinity labeled with an active site-directed inhibitor implicating Glu-355 and the amino acids in the immediate vicinity as binding or active site residues (Fig. 1) (18). This evolutionarily conserved area (Fig. 1) may be involved in a catalytic mechanism shared between the α- and β-subunits and other hexosaminidases (18), so it is not surprising that the region is excluded from the segments that confer MU-GlcNAc-6-SO₄ specificity, a unique property of the α-subunit. A second amino acid implicated in the catalytic activity of β-hexosaminidase is Arg-178 of the α-subunit, the residue mutated in the B1-variant of Tay-Sachs disease (19, 20). Mutagenesis of the corresponding amino acid in the β-subunit impairs catalytic activity again, suggesting a shared function of the this residue between subunits. Clearly Arg-178 and surrounding residues alone are not sufficient for MU-GlcNAc-6-SO₄ activity because chimera αβ1, which contains the amino-terminal 191 amino acids of the α-subunit, is only active against MU-GlcNAc.

The degradation of G₂-M₂ ganglioside by β-hexosaminidase A requires the G₂-M₂ activator protein (reviewed in Ref. 6). The activator binds the ganglioside in a 1:1 complex and the complex interacts directly with the enzyme allowing the α-subunit active site to remove the terminal GalNAc moiety from the tetrasaccharide moiety of G₂-M₂ ganglioside. The α-subunit possesses a binding site for the activator-ganglioside complex. However, the β-subunit must also contribute to this interaction, because only β-hexosaminidase A of the three isozymes can carry out the reaction at physiological rates. We found that neither chimeric subunits αβ1 nor αβ21, when expressed alone, demonstrated activator-dependent ganglioside degradation activity. In this regard, they were no more effective than the wild-type subunits expressed singly. However, when these chimeric subunits were expressed in combination with each wild-type subunit, we found that αβ1 together with the α-subunit caused the activator-dependent degradation of the ganglioside about as well as the co-expression of the wild-type α- and β-subunits. This result shows that the specific function of the β-subunit in activator-dependent ganglioside degradation resides in the carboxyl-terminal two-thirds of the precursor polypeptide (amino acids 225–556). The result also indicates that the amino-terminal one-third (amino acids 1–224) does not provide a β-subunit-specific function because it can be replaced by the corresponding segment from the β-subunit. In addition, the chimeric enzymes also shed some light on the important regions of the α-subunit in activator-dependent ganglioside degradation. The inability of αβ1 to substitute for the α-subunit in a heterodimer with the β-subunit indicates that amino acids 192–402 of the α-subunit, which are missing from αβ21, impart an essential function for ganglioside hydrolysis.

We have established a structure-function relationship of discrete regions of the β-hexosaminidase subunits with the ability to degrade particular substrates. By substituting smaller segments into these chimeras and through site-directed mutagenesis within these regions, it should be possible to define individual residues that participate in determining the specificity of the hexosaminidase isozymes. Ultimately, a complete understanding of the interaction of various substrates with β-hexosaminidase will require a combination of approaches including chimera analysis and affinity labeling together with a three-dimensional structure of the enzyme.
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