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An α -subunit loop structure is required for GM2 activator protein binding by β -hexosaminidase A

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Abstract

The α - and/or β -subunits of human β -hexosaminidase A ($\alpha\beta$) and B ($\beta\beta$) are ~60% identical. In vivo only β -hexosaminidase A can utilize GM2 ganglioside as a substrate, but requires the GM2 activator protein to bind GM2 ganglioside and then interact with the enzyme, placing the terminal GalNAc residue in the active site of the α -subunit. A model for this interaction suggests that two loop structures, present only in the α -subunit, may be critical to this binding. Three amino acids in one of these loops are not encoded in the *HEXB* gene, while four from the other are removed posttranslationally from the pro- β -subunit. Natural substrate assays with forms of hexosaminidase A containing mutant α -subunits demonstrate that only the site that is removed from the β -subunit during its maturation is critical for the interaction. Our data suggest an unexpected biological role for such proteolytic processing events.

Keywords

GM2 gangliosidosis; GM2 ganglioside; Tay–Sachs; Lysosome; Storage disease; Posttranslational processing; Protein–protein interaction; Saposin

The hydrolysis of the terminal, β -linked GalNAc residue from GalNAc β (1–4)-[NANA α (2–3)-]Gal β (1–4)-Glc-ceramide (GM2 ganglioside, GM2) requires the correct synthesis, intracellular transport, and interactions of three gene products. Two of these are the evolutionarily related α -(encoded by the *HEXA* gene) and β -subunits (encoded by the *HEXB* gene) of heterodimeric β -hexosaminidase A (Hex A). The third is the GM2 activator protein (GM2AP, encoded by the *GM2A* gene), which acts as a substrate-specific co-factor for Hex A. GM2AP first removes GM2 from its membranous environment forming a soluble 1:1 complex, which then binds to Hex A in such a manner as to place the terminal GalNAc residue in the correct position in the active site of the α -subunit for hydrolysis, producing the active quaternary complex. After hydrolysis the quaternary complex dissociates, the GM2AP releases GM3 ganglioside at the membrane and replaces it with a new molecule of

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GM2 (reviewed in [1]). Thus, mutations affecting any of these three genes can prevent the turnover of GM2, resulting in its storage, primarily in neuronal lysosomes, and disease. Deficiency of the α -subunit results in Tay–Sachs disease; of the β -subunit, Sandhoff disease; and of the GM2AP, the AB variant form of GM2 gangliosidosis. A model for the quaternary complex has recently been published [2]. It is based on Hex A, modeled on the experimentally deduced three dimensional structure of the Hex B ($\beta\beta$) isozyme, and the three dimensional structure of GM2AP [3].

Dimerization of the α and/or β -subunits is necessary for activity, resulting in three Hex isozymes. The mechanism for this activation has been derived from the crystal structure of Hex B [2]. As well, despite their ~60% sequence identities and the ability of both the α and β -active sites to hydrolyze many of the same neutral substrates, e.g., 4-methylumbelliferyl- β -*N*-acetylglucosaminide (MUG), only the α -active site is able to efficiently hydrolyze negatively charged artificial substrates, e.g., 4-methylumbelliferyl- β -*N*-acetylglucosaminide-6-sulfate (MUGS). Thus, each isozyme has its own characteristic MUG to MUGS ratios; Hex A ($\alpha\beta$) 3–4/1, Hex B ($\beta\beta$) 150–300/1, and Hex S ($\alpha\alpha$) 1–1.5/1 [4].

GM2 is also negatively charged, but unlike MUGS, Hex B has no residual activity towards this molecule. The specificity of the α -active site for negatively charged substrates has been explained from the molecular model of Hex A [2], which is supported by previously published in vitro mutagenesis and expression studies [5]. In α , a site consisting of α Asn⁴²³Arg⁴²⁴ (aligns with β Asp⁴⁵³Leu⁴⁵⁴) is responsible for binding both the 6-sulfate group in MUGS and the sialic acid moiety in GM2 [2,6]. The absolute specificity of the α -active site for GM2 is explained by the specificity with which the GM2/GM2AP complex interacts with Hex A [2]. Biologically this specificity likely prevents the possibility of the GalNAc and sialic acid residues of GM2 being mistakenly placed in the β -active site of Hex A or B, where hydrolysis and recycling of GM2AP would be as inefficient as MUGS hydrolysis.

The subunits of Hex, like many lysosomal enzymes, undergo numerous posttranslational modifications. These include the attachment of multiple Asn-linked oligosaccharides and their phosphorylation at selected mannose residues in the endoplasmic reticulum and *cis* Golgi [7,8], and proteolytic cleavage events that occur in the late endosome/lysosome [9–11]. The former modifications have been shown to be necessary for proper folding of the subunits in the endoplasmic reticulum [7] and intracellular transport of the assembled isozymes from the *trans* Golgi to late endosomes [12], respectively. The latter events have been assumed to have no biological significance as they appear to occur at exposed loop structures [13] and would therefore be susceptible to proteolysis once the protein had entered the lysosomal environment. Additionally, there appear to be no differences in the kinetic parameters of mature and pro-forms of the Hex isozymes when measured with artificial substrates [14]. However, the model of the active quaternary complex suggested that two unique loop structures in the α -subunit may confer the binding specificity of GM2AP with Hex A. The aligned primary structures of the α - and β -subunits [2] indicate that one of these loop structures (α 393–406, β 425–435) lacks three amino acids, corresponding to α I-P-V₃₉₈, in the wild type (WT) β -subunit. The other loop structure is completely preserved in both the pro- α -(277–289) and pro- β -(309–321) subunits, but four of

its amino acids are removed from the β -subunit's loop (R-Q-N-K₃₁₅, corresponding to α G-S-E-P₂₈₃) during maturation in the lysosome, producing the β_a and β_b polypeptide chains (held together by a disulfide in the mature subunit) [11]. In this report we examine the roles of these two α -loop structures in forming the active quaternary complex.

Materials and methods

DNA construction and mutagenesis

To create the pREP4- α constructs encoding the mutations α - GSEP (280–283), α - IPV (396–398), and the double deletion α - GSEP (280–283) -IPV(396–398), a 3-step PCR was used. The previously reported pREP4- α [4] construct was used as a template for steps 1 and 2 of the PCRs. Briefly, in step 1, amplification was achieved by a forward primer containing the appropriate nucleotide change and a 3' end oligo. In step 2, amplification was performed by a reverse primer containing the appropriate nucleotide change and a 5' end oligo. The products from the first and second PCRs were purified and utilized as templates along with 3' and 5' end oligo to produce the full length α -fragment containing the appropriate mutation.

For the α - GSEP (280–283) construct, the first PCR was performed along with oligo-3 and -2 (described in Table 1), and the second PCR round was achieved by using oligo-4 and -1. For the α - IPV (396–398) construct the first PCR was performed using oligo-5 and -2, and the second PCR was performed along with oligo-6 and -1. The products of the first and second PCRs were isolated by agarose gel electrophoresis and purified with the QIA Quick Gel Extraction kit (Qiagen). They were then utilized as a template for the third PCR, including a 3' and a 5' end oligo. The 5' and 3' end oligonucleotides each carried a restriction site (*Xho*I and *Bam*HI) to facilitate the ligation of the final PCR product containing the derived mutation into the expression vector, pREP4.

For the α - GSEP (280–283) -IPV (396–398) mutation, the α - GSEP (280–283) construct was used as a template and the first step PCR amplification was achieved using oligo-5 and -2. The second step PCR was performed along with oligo-6 and -1. The products of the first and second PCRs were isolated from agarose gel and used as a template for the third PCR, including 3' and 5' end oligo. The third step PCR product was purified, digested with *Xho*I and *Bam*HI, and subcloned into pREP4.

All of the PCRs were performed in 100 μ L. Each of the reaction mixtures contained 50 ng plasmid DNA, 0.2 mM each of dNTPs, 100 ng of each primer, 1.5 mM MgCl₂, and 1 U KOD Hi-Fi DNA polymerase (Novagen). The cycling steps used were as follows: 30 cycles each consisting of denaturation at 98 °C for 30 s, annealing at 60 °C for 20 s, and extension at 74 °C for 20 s, and a final extension of 10 min at 74 °C, in a Perkin-Elmer-Cetus thermal cycler.

Cell culture and DNA transfections

Transformed Tay-Sachs neural glial cells (TSD-NG, from R.A. Gravel, University of Calgary) were grown in α -minimum essential medium (α -MEM) with 15% fetal calf serum (FCS) and antibiotics at 37 °C in 5% CO₂. Transfections were performed according to the

Superfect reference manual from Qiagen. The neural glial cells were seeded overnight in 100-mm tissue culture dishes and grown until they were about 80% confluent. For each dish of cells being transfected, 10 µg of DNA was mixed with 60 µL of Superfect in 300 µL of serum-free α -MEM. The mixture was allowed to incubate for 10 min at room temperature to form the DNA–Superfect complexes. Three milliliters of α -MEM containing serum was added and the mixture was added dropwise to the culture dishes. The dishes were incubated at 37 °C for 2 h, after which the cells were washed and then fed with α -MEM containing 15% FCS for 48 h. Following this, the cells were fed with α -MEM plus 15% FCS and 1 µL/mL of hygromycin B every 48 h. After drug selection for 1 month, the mixed colonies were trypsinized and the cells were passaged into larger dishes for further growth and analyses. Hygromycin B was present in the growth media of all stable transfectants.

Hex activity assay

Hex activity was measured using MUG (Hex A, B, and S activity) and MUGS (Hex A and S activity) substrates as previously reported using 1.6 mM MUG or MUGS substrate incubated at pH 4.2 in citrate phosphate buffer at 37 °C for 15 min [4].

Removal of endogenous GM2AP and separation of the Hex A isozyme by ion-exchange chromatography

Cell pellets collected from the stably transfected neural glial cells containing either the WT or the mutant cells were kept at –20 °C until use. Cells were lysed by multiple freeze–thaw cycles in 10 mM phosphate buffer, pH 6. Following centrifugation, the endogenous GM2AP was removed from the lysate by passage through a 1 mL octyl–Sephacrose hydrophobic column preequilibrated in the lysis buffer. The lysate (~10 mg total protein for WT, ~20 mg total protein for the mutant) was then applied to a 2 mL column of DEAE–Sephacrose CL-6B (Pharmacia) also pre-equilibrated with the lysis buffer. The column was washed with the buffer and fractions were eluted using a NaCl gradient (from 0 to 250 mM NaCl). Fractions were assayed for MUG and MUGS specific activity. Appropriate fractions, i.e., high activity with a MUG/MUGS ratio of 3–4 (Hex A), were pooled and concentrated using HSA-pretreated Centricon YM-10 spin concentrator.

Thermal stability study

An aliquot of each WT or mutant Hex A, separated by DEAE chromatography, was added to 700 µL of preheated citrate phosphate buffer (pH 4.1) with 0.3% human serum albumin. Heat denaturation was performed at 45 °C with aliquots (100 µL) being removed at intervals of 0, 15, 30, 45, 60, 75, and 90 min and placed on ice for enzyme assay. Additionally, the WT or mutant Hex A from transfected neural glial cells (TSD-NG) was assayed with MUGS after incubation at 37 °C for 18 h (conditions that mimicked the natural substrate assay).

Kinetic analysis

The K_m values were determined for the WT or mutant forms of Hex A by assaying each at five different MUGS concentrations (0.05–2.5 mM). The data were analyzed using computerized non-linear least squares curve fitting program for the Macintosh (KaleidaGraph 3.0) based on the Michaelis–Menten equation, $V_i = V_{max} \cdot$

$[S]/(K_m + [S])$. This approach also allows the calculation of standard errors for each parameter.

GM2 hydrolysis assay and kinetic analyses

The affinity of WT and mutant forms of Hex A for the GM2AP–GM2 ganglioside complex was determined by incubating 20 nmol [^3H -GalNAc]GM2 ganglioside (200 μM final concentration), contained in negatively charged liposomes for enhanced activity [15], with varying amounts of recombinant activator protein (0–2.3 μg , isolated and refolded from *Escherichia coli* [16,17]) at 37 °C for 18 h in 20 mM citrate buffer (pH 4.1) containing 0.5% human serum albumin, with ~2000 U (nmol of MUG hydrolyzed/h) of Hex A (produced from transfected glial cells (final volume of 100 μL)). After 18 h incubation, the reaction was stopped by the addition of 1 mL chloroform and 1 mL of 12 mM citrate phosphate buffer containing 13.6 mM GlcNAc. The hydrolyzed product, [^3H]GalNAc, was separated from the remaining negatively charged GM2 substrate by passage through positively charged ion exchange minicolumn [0.6 ml AG3X4 (acetate form) resin (Bio-Rad)] and quantitated by liquid scintillation counting. In all cases, less than 10% of the total GM2 was hydrolyzed.

We have previously reported the K_m and $V_{\max}$ values for Hex A, isolated from Tay–Sachs glial cells transfected with WT human αCDNA , incubated with varying amounts of GM2 (0–430 μM) and 2 μg GM2AP [6]. However, in this publication an incorrect specific activity for the [^3H]GM2 substrate was used to calculate these values, resulting in both being reduced by 3.6-fold. The corrected values indicate a K_m of $140 \pm 40 \mu\text{M}$ GM2 and a $V_{\max}$ of $14 \pm 4 \text{ fmol GM2/h/U of Hex A (nmol MUG/h)}$.

Results and discussion

Three deletion mutations were made in the αCDNA that removed the codons for residues 280–283 (α - GSEP, loop 1), 396–398 (α - IPV, loop 2), and both 280–283 and 396–398 (α - IPV-GSEP). Residues corresponding to α 396–398 are not coded for in the *HEXB* gene; whereas residues α 280–283 align with residues β 312–315 (RQNK) which are removed during its maturation in the lysosome because of its basic amino acids [13]. All three constructs expressed α -subunits that were able to form active dimers with the endogenous β -subunits in the Tay–Sachs cell line, because acidic isozymes could be separated by ion exchange chromatography from each transfected line that produced the characteristic MUG to MUGS ratios of ~3 (data not shown). K_m values for the MUGS substrate were the same as for the WT isozyme (Fig. 1, Table 2). Heat denaturation studies demonstrated that they are also as stable as WT Hex A (Table 2). Thus, these deletions had no effect on the stability of the Hex A isozyme or its ability to bind and hydrolyze an artificial substrate.

Whereas both α -loop structures may be involved in the formation of the quaternary complex [2], the binding specificity of Hex A with the GM2AP–GM2 complex appears to be dictated by the retention in the α -subunit of its GSEP₂₈₃ sequence during lysosomal maturation. Our kinetic examination of the three mutant forms of Hex A demonstrated that; while removal of the α IPV₃₉₈ sequence had no detectable effect on natural substrate hydrolysis, no hydrolysis of GM2 could be detected, even at the highest concentrations of GM2AP used in the assays, in either of the two forms of Hex A lacking α GSEP₂₈₃ (Fig. 2, Table 2). Since the β -active

site cannot efficiently hydrolyze negatively charged substrates like GM2, our data suggest that the removal of the aligned sequence in the β -subunit may serve a novel biological role, i.e., the prevention of non-productive binding of the GM2AP–GM2 complex to Hex B and/or to the wrong face of the Hex A heterodimer.

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Fig. 1.

Kinetic examination of Hex A containing the WT α -subunits (solid triangles, data points; solid line, best fit curve); α -subunits with loop 1, GSEP₂₈₃, deleted (solid diamonds, data points; large dashed line, best fit curve), α -subunits with loop 2, IPV₃₉₈, deleted (solid squares, data points; medium dashed line, best fit curve), or α -subunits with both loops deleted (solid circles, data points; small dashed line, best fit curve) using various amounts of MUGS (mM) as substrate (see Table 2 for values).

Fig. 2.

Kinetic examination of Hex A containing the WT α -subunits (solid triangles, data points; solid line, best fit curve); α -subunits with loop 1, GSEP₂₈₃, deleted (solid diamonds, data points; large dashed line, best fit curve), α -subunits with loop 2, IPV₃₉₈, deleted (solid squares, data points; medium dashed line, best fit curve), or α -subunits with both loops deleted (solid circles, data points; small dashed line, best fit curve) using 200 μ M GM2 and various amounts of GM2AP (μ g/mL) as the substrate and substrate-specific cofactor, respectively (see Table 2 for values).

Table 1

Oligonucleotides utilized to mutate α-cDNA

Number	Oligonucleotide	Use
1	5'-GCCGCTCGAGGTCGCCGCTCACCTGACC-3'	5'-primer
2	5'-GCCAGGGATCCCCCTCGGTGGCTGGGGC-TCAGGTCTG-3'	3'-primer
3	5'-CCTTGCTACTCTTCTGGCACCTTTG-3'	-280 GSEP ₂₈₃
4	5'-CAAAGGTGCCAGAGAGTAGCAAGG-3'	-280 GSEP ₂₈₃
5	5'-GGCGAGAGGATACTATATGAAGG-3'	-396 IPV ₃₉₈
6	5'-CCTTCATATAGTTATCTCTCGCC-3'	-396 IPV ₃₉₈

Oligo-1 and -2 serve as the general 5' and 3' end primers to create the mutations by a 3-step PCR procedure. They also contain the underlined restriction sites to facilitate subcloning the resulting PCR fragment into pREP4 (see Materials and methods).

Table 2

Kinetic analysis of WT and mutant form of Hex A

Hex A	MUGS		GM2AP		
	$T_{1/2}^a$	% initial act. ^b	K_m^c	K_m^d	V_{max}^e
WT	191 ± 8	76 ± 4	0.32 ± 0.04	12 ± 3	15 ± 2
α- GSEP ₂₈₃ (loop 1)	188 ± 6	72 ± 3	0.27 ± 0.04	N/D ^f	0
α- IPV ₃₉₈ (loop 2)	187 ± 5	71 ± 4	0.31 ± 0.01	10 ± 2	12 ± 1
α- GSEP ₂₈₃ - IPV ₃₉₈	187 ± 4	70 ± 3	0.25 ± 0.01	N/D	0

^aTime at 45 °C necessary to reduce Hex A activity by 50%, the standard deviation (±) was calculated from the best-fit line generated by plotting incubation times versus log(% remaining activity) (data not shown).

^bPercentage remaining activity after 18 h at 37 °C, the standard deviation (±) was calculated from $n = 3$ assays.

^cmM, the standard error (±) was calculated by the Kaleidagraph 3.0 model fitting Macro (directly fitting the initial velocity versus substrate (MUGS)/cofactor (GM2AP) concentration data to the Michaelis–Menten equation) R values for each curve-fit (Figs. 1 and 2) were 0.993.

^dµg/mL (GM2AP).

^efmol (GM3) h⁻¹ U⁻¹ (MUG), the standard error (±) was calculated as in “c.”

^fNot detectable.