Catabolism of Asialo-GM2 in Man and Mouse
SPECIFICITY OF HUMAN/MOUSE CHIMERIC GM2 ACTIVATOR PROTEINS

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Tay-Sachs disease is an inborn lysosomal disease characterized by excessive cerebral accumulation of GM2. The catabolism of GM2 to GM3 in man requires β-hexosaminidase A (HexA) and a protein cofactor, the GM2 activator. Thus, Tay-Sachs disease can be caused by the deficiency of either HexA or the GM2 activator. The same cofactor found in mouse shares 74.1% amino acid identity (67% nucleotide identity) with the human counterpart. Between the two activators, the mouse GM2 activator can effectively stimulate the hydrolysis of both GM2 and asialo-GM2 (GA2) by HexA and, to a lesser extent, also stimulate HexB to hydrolyze GA2, whereas the human activator is ineffective in stimulating the hydrolysis of GA2 (Yuzuki, J. A., Bertoni, C., Beccari, T., Orlacchio, A., Wu, Y.-Y., Li, S.-C., and Li, Y.-T. (1998) J. Biol. Chem. 273, 66–72). To understand the role of these two activators in stimulating the hydrolyses of GM2 and GA2, we have constructed human/mouse chimeric GM2 activators and studied their specificities. We have identified a narrow region (Asn108-Tyr111) in the mouse cDNA sequence that might be responsible for stimulating the hydrolysis of GA2. Replacement of the corresponding site in the human sequence with the specific mouse sequence converted the ineffective human activator into an effective chimeric protein for stimulating the hydrolysis of GA2. This chimeric activator protein, like the mouse protein, is also able to stimulate the hydrolysis of GA2 by HexB. The mouse model of human type B Tay-Sachs disease recently engineered by the targeted disruption of the Hexa gene showed less severe clinical manifestation than found in human patients. This has been considered to be the result of the catabolism of GM2 via converting it to GA2 and further hydrolysis of GA2 to lactosylceramide by HexB with the assistance of mouse GM2 activator protein. The chimeric activator protein that bears the characteristics of the mouse GM2 activator may therefore be able to induce an alternative catabolic pathway for GM2 in human type B Tay-Sachs patients.

In man, the degradation of the GM2 ganglioside requires

lysosomal β-hexosaminidase A (HexA) and a protein cofactor, the GM2 activator. The physiological importance of the GM2 activator is demonstrated by the severe clinical manifestations and neural accumulation of GM2 in type AB Tay-Sachs disease caused by the deficiency of this protein cofactor (1, 2). The recent studies of the mouse model of type B Tay-Sachs disease (Hexa<sup>-/-</sup>), generated via homologous recombination in embryonic stem cells, did not show the severe neurological symptoms characteristic in the same disease found in man (3–7). In these studies, the mild manifestations were initially attributed to the GM2-degrading activity of mouse HexB as reported by Burg et al. in 1983 (8). In contrast, we have shown that the highly purified mouse HexB was not able to convert GM2 to GM3, but was able to slowly catalyze the conversion of GA2 to LacCer in the presence of the mouse GM2 activator (mM2act) (9). In the same report, we also showed that mM2act was able to effectively stimulate the hydrolysis of GA2 catalyzed by either human or mouse HexA and that the human GM2 activator (hM2act) was not effective in stimulating the hydrolysis of GA2.

To better understand the role of hM2act and mM2act in the degradation of GM2 and GA2, we have constructed a series of human/mouse chimeric GM2 activators and studied their ability to stimulate the hydrolysis of GM2 and GA2 by human HexA. Since hM2act is not effective in stimulating the hydrolysis of GA2 by HexA, the specific human/mouse chimeras that elicit this activity should reveal the amino acids that are responsible for stimulating the hydrolysis of GA2.

EXPERIMENTAL PROCEDURES

Materials

GM2 was isolated from the brain of a Tay-Sachs patient (10). GA2 was prepared from GM2 by mild acid hydrolysis (11). HexA (33.3 units/mg) from human liver was prepared as described previously (12). The following were purchased from the commercial sources indicated: precoated Silica Gel 60 TLC plates, Merck (Darmstadt, Germany); 4-methylumbelliferyl N-acetylgalactosaminide, Coomassie Brilliant Blue R-250, Trizma (Tris base), and glycine, Sigma; 4-methylumbelliferyl N-acetylgalactosaminide 6-sulfate, Research Development Corp. (Toronto, Canada); PM-10 ultrafiltration membrane (10,000 molecular weight cutoff), Amicon, Inc.; [<sup>32</sup>P]dCTP (3000 Ci/mmol), [<sup>35</sup>S]-dATP (1000 Ci/mmol), the multiprime DNA labeling system, the Sequenase sequencing kit, restriction endonucleases, DNA ligase, nitrocellulose membrane, and protein standards for molecular weights, Amersham Pharmacia Biotech; fmolTM DNA sequencing system, and Pfu DNA polymerase, Promega; LacCer and GM3, Matreya, Inc.; isoprropyl-1-thio-β-D-galactopyranoside, ampicillin, glutathione, yeast extract, and Tryptone, Difco; the T7 sequencing kit (Version 2.0), U. S. Biochemical Corp.; and Escherichia coli strain BL21(DE3), Novagen.

Construction of Human/Mouse Chimeric cDNAs by Exon Swapping

The numbering system for the deduced amino acids in the following constructs was based on the alignment of the nucleotide sequences of

PCR, polymerase chain reaction; bp, base pair(s)
hM2act and mM2act using the PC/GENE computer software program (Fig. 1). Three gaps in the human sequence and seven gaps in the mouse sequence were inserted to obtain the best alignment. Therefore, the numerical assignment for the amino acids in the human sequence differed from that in the mouse sequence by a factor of +4 amino acids. All the gaps inserted were contained within the first 27 amino acids of the human sequence at the propeptide region, which is removed by proteolysis during the maturation of GM2 activator protein (13). The insertion did not at all affect the construction of the chimeras since all of the cDNA constructs started from Ser2, the N-terminal amino acid of the mature human protein. The first set of cDNA constructs for the activator chimeras were generated from exon swapping (Fig. 2A). For the names of all constructs, the prefix "p" denotes plasmid that contains the designated cDNA.

ph2h3m4—This construct contains human exons 2 and 3 plus mouse exon 4. A T7-7 plasmid vector containing the cDNA (p513) encoding only the 162 amino acids of mature hM2act (14) was used as template to generate, by polymerase chain reaction (PCR), a 445-bp cDNA fragment. This human cDNA fragment encodes 9 amino acids of the pT7-7 expression vector (Met-Ala-Arg-Ile-Arg-Ala-Arg-Gly-Ser) plus human exon 4. The construct ph2h3m4 was digested with HindIII and ligated into ph2h3 at its HindIII sites.

ph2m3m4—This construct contains human exon 2, mouse exon 3, and mouse exon 4. The downstream primer (noncoding) was 5’-TAG-GGA-GA-3’ attached to the HindIII site within the pT7 vector region, and the upstream primer was the T7 primer (underlined). This cDNA segment was digested with BamHI and HindIII and ligated into pT7-7 vector at its HindIII sites. The two cDNA segments were subcloned into the pT7-7 expression vector at BamHI and HindIII sites.

pm2m3h4—This construct contains human exons 2 and mouse exons 3 and 4. A 177-bp DNA fragment encoding the 9 amino acids of the pT7-7 expression vector plus the first 50 amino acids of the mature hM2act sequence (13) encoded by exon 2 of the human sequence (also see Fig. 1) was generated by PCR amplification using p513 as template. The upstream primer was the T7 primer as described above. The downstream primer (noncoding) was 5’-TAG-TAG-GAG-TCT-TTTGTG-3’ with a built-in KpnI restriction site (underlined). This cDNA segment was digested with BamHI and KpnI. The remaining 164-bp cDNA fragment encoding mouse exon 4 was obtained by restriction digestion of pMact with KpnI and HindIII. The two cDNA segments were subcloned into the pT7-7 expression vector at BamHI and HindIII sites.

pm2m3m4—This construct contains human exon 2, mouse exon 3, and human exon 4. The cDNAs encoding human exon 2 and mouse exon 3 were obtained by restriction digestion of the construct ph2m3m4 using KpnI and HindIII to generate the pm2m4 fragment. The remaining cDNA of the 164-bp fragment encoding human exon 4 was generated by

PCR using p513 as template. The upstream primer was 5’-GAG-GGT-ACC-TAC-TCA-3’, which makes HindIII site. The downstream noncoding primer was the T7 primer within the p77 vector region and the downstream primer (noncoding) was 5’-GAG-TAG-GAG-TCT-TTTGTG-3’, corresponding to the 3’ untranslated region of the pT7-7 vector. The amplified human fragment (207 bp) was digested with KpnI and HindIII and ligated into pm2m4 at KpnI and HindIII sites.

pm2m3h4—a—This construct contains mouse exons 2 and 3 plus human exon 4. The construct pm2m3h4 was digested with PvuII and HindIII to yield the 324-bp cDNA fragment encoding Ser235-Glu238 of the mouse sequence followed by the h3 segment. This fragment was subcloned into mouse pMact that had been digested by PvuII and HindIII.

Construction of Chimeras from pm2m3h4 with a Modified m3 Segment

From the initial experiments, the m3 segment appeared to be important for eliciting the stimulatory activity for the enzymatic hydrolysis of GA2. Therefore, we subsequently modified the m3 segment in pm2m3h4 by including more human sequence and generated the following constructs (also see Fig. 2B).

pm2m3h4—This construct contains an extended human sequence (25 amino acids from Val82 to Cys106) at the N terminus of m3. The 236-bp fragment encoding Ser235-Cys236 of hM2act was excised from p513 by restriction digestion with BamHI and PvuII. The segment of mouse exon 3 encoding Ser193-Glu196 plus human exon 4 encoding Gly197-Ile198 was obtained by digestion of pm2m3h4 with PvuII and HindIII. The two fragments were ligated into the pT7-7 vector at its BamHI and HindIII sites.
**Expression and Purification of Chimeric Human/Mouse GM2 Activators**

Each construct was verified by sequencing the cDNA prior to transforming the competent *E. coli* BL21(DE3) cells. *E. coli* transformants were inoculated into 150 ml of LB medium containing 1 mg/ml ampicillin and incubated overnight at 37 °C. The overnight culture was diluted at a ratio of 1:33 with fresh LB/ampicillin medium (30 ml/1 liter) and grown for ~4 h at 37 °C. Expression of GM2 activator protein was then induced by addition of isopropyl-1-thio-β-galactopyranoside at a final concentration of 1 mM, and the culture was grown for an additional 6 h. The cells were harvested by centrifugation at 6000 rpm for 15 min using a GS3 rotor in a Sorvall RC5C centrifuge. Expression, refolding, and purification of the human/mouse GM2 activator chimeras were carried out as described previously for the refolding of hM2act (14).

**Enzyme assay**

Human HexA activity was determined using fluorogenic substrates (4-methylumbelliferyl N-acetylglucosaminide and 4-methylumbelliferyl N-acetylglucosaminide 6-sulfate) according to Potier et al. (15). An appropriate amount of HexA was incubated with 1.5 mM 4-methylumbelliferyl N-acetylglucosaminide or 4-methylumbelliferyl N-acetylglucosaminide 6-sulfate in 50 mM sodium citrate buffer (pH 5.0) in a total volume of 50 μl at 37 °C. After a preset time, 1.5 ml of 0.2 M sodium chloride buffer (pH 9.8) was added to stop the reaction. The released 4-methylumbelliferone was determined using a Sequoia-Turner Model 450 fluorometer. One unit of enzyme activity is defined as the amount that liberates 1 μmol of 4-methylumbelliferone/min at 37 °C. This fluorogenic assay was used only to standardize the amount of HexA for each experiment.

**Enzymatic Hydrolysis of GM2 and GA2**

For the hydrolysis of GM2 and GA2, the reaction mixture contained 3 nmol of substrate in 40 μl of 10 mM sodium acetate buffer (pH 5.0). The reactions were initiated by adding 20 milliliters of human HexA and terminated by adding 40 μl of ethanol. The mixtures were dried under vacuum using a SpeedVac, redissolved in 20 μl of chloroform/methanol (2:1, v/v), and applied to a TLC plate. The plate was developed with chloroform/methanol/water (60:35:8, v/v/v), sprayed with diphenylamine reagent (16), and heated at 115 °C for 15–20 min to visualize the glycolipids. The quantitative analysis of the glycolipid bands on the TLC plate was carried out using a Scan Jet 2C/ADF scanner (Hewlett-Packard Co.) and the NIH Image 1.41 program.

**Western Blot analysis**

The recombinant human/mouse chimeric GM2 activators were analyzed by SDS-polyacrylamide gel electrophoresis using 15% gel (17). Proteins were electrophoretically transferred onto a nitrocellulose membrane in 20 mM Tris and 150 mM glycine buffer (pH 8.0) containing 20% methanol at 70 V for 1 h using a Bio-Rad transfer apparatus. The nitrocellulose membrane was first soaked with 1% milk powder and then overlaid with rabbit anti-hM2act antibodies (produced by Cocalico Biological, Inc.) as the primary antibody followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000) as the secondary antibody. The membrane was then developed in 3.4 mM 4-chloro-1-naphthol containing 0.01% hydrogen peroxide to produce purple bands.

**RESULTS**

**Alignment of the Deduced Amino Acid Sequences of hM2act and mM2act**

The nucleotide sequences of hM2act and mM2act downstream from the initiation codon were analyzed using the PC/GENE program (Fig. 1). With 10 gap insertions in exon 1 (three in the human sequence and seven in the mouse sequence), the two sequences obtained the best alignment and showed 67% identity in their nucleotide. The gaps are up to three in the sequence compared to the mature form of hM2act (13). A higher degree of similarity (74.1% identical and 9.9% similar) was found in the deduced amino acid sequences when they were compared as mature proteins from Ser32 to the C terminus of the human sequence (Fig. 1). The comparison based on the mature protein is appropriate in this study since...
all the chimeras were expressed only as the mature proteins.

Stimulatory Activities of the Parent hM2act and mM2act for the Hydrolysis of GM2 and GA2—Although hM2act and mM2act share a very high degree of homology and both are active in stimulating the enzymatic hydrolysis of GM2, their stimulatory activities for the hydrolysis of GA2 are distinctly different. Fig. 3A shows the time course of GM2 hydrolysis by human HexA in the presence of either hM2act or mM2act. Under the same condition, 1 μg each of hM2act and mM2act showed comparable stimulatory activity for the hydrolysis of GM2 throughout the 10-, 20-, and 30-min incubations. This indicates that these two activator proteins have similar potency in stimulating the hydrolysis of GM2. However, when the hydrolysis of GA2 (Fig. 3B) was examined in the presence of the same amount of each activator as that used in Fig. 3A, mM2act showed a much more pronounced stimulatory activity than that exerted by hM2act. This result corroborates our previous report (9) that mM2act was able to effectively stimulate the hydrolysis of GA2 and that hM2act was ineffective in stimulating this reaction. Image scanning of the TLC plate showed that the stimulatory activity of hM2act for GA2 hydrolysis was only ~15–18% of that of mM2act. Based on this difference, we reasoned that any human/mouse chimeric activator that can alter the stimulatory activity of the chimeric proteins for the hydrolysis of GM2. These results indicate that the exchange of the corresponding exon segments between the human and mouse sequences did not alter the stimulatory activity of the chimeric proteins for the hydrolysis of GM2. These results were not unexpected since both hM2act and mM2act could effectively stimulate this reaction. We were intrigued, however, by the differences in the ability of these chimeras to stimulate the hydrolysis of GA2. This strategy was proven to be effective. Alternatively, one can start from the mouse sequence and substitute it with certain parts of the human sequence. This strategy would result in chimeras with diminished stimulatory activity for GA2 hydrolysis. Because the stimulatory activity of the activator protein can also be attenuated by factors such as poor protein refolding, the second approach might not provide clear results.

Taking advantage of the fact that the cDNAs of hM2act and mM2act share a high degree of homology and identical intron/exon junctions (19, 20), we first constructed four chimeric constructs (ph₂₃m₄, ph₂₃h₄, ph₃₅h₅, and pm₂₃h₄) according to the four possible exchanges of exons (Fig. 2). All four proteins expressed by these chimeric constructs were fully active in stimulating the hydrolysis of GM2 (Fig. 4A, lanes 4–7). These results strongly suggest the possibility that the peptide segment encoded by mouse exon 3 (m₃) may be responsible for exerting the stimulatory activity on GA2 hydrolysis.
The m3 region and to generate two additional chimeras, proteins with a modified m3 segment.

GA2 (B) by human HexA in the presence of chimeric activator 1

mM2act to stimulate the hydrolysis of GA2 (Fig. 5B).

ph2m3h4-b had practically no stimulatory activity for the GA2 hydrolysis of GM2.

struct without “p” was used to designate the expressed protein.

indicated) of the activator at 37 °C for 30 min (GM2 hydrolysis) or 3 h (GA2 hydrolysis). The detailed assay conditions are described under

result, we proceeded to further narrow down the region between

Characterization of Chimeric Activator Proteins Expressed by ph2m3h4-a and ph2m3h4-b—Since the m3 segment of mM2act appeared to be important for the hydrolysis of GA2, we carried out further analysis of this region. The presence of a PvuII site in both the human and mouse sequences at the equivalent position enabled us to replace the h3 sequence with a section of the m3 region and to generate two additional chimeras, ph2m3h4-a and ph2m3h4-b (Fig. 2B). In addition to having human exons 2 and 4 in both chimeras, the middle segment of ph2m3h4-a contained human Val12-Cys106 followed by mouse Ser103-Glu138, whereas ph2m3h4-b contained mouse Val78-Cys106 followed by human Thr107-Glu142 (Fig. 2B). Again, both proteins expressed by ph2m3h4-a and ph2m3h4-b were fully active in stimulating the hydrolysis of GM2 (Fig. 5A, lanes 4 and 5). However, only ph2m3h4-a showed the characteristic of mM2act to stimulate the hydrolysis of GA2 (Fig. 5B, lane 4). Judging from the substrate (GA2) remaining and the product (LacCer) formed, hM2act had only a basal activity and ph2m3h4-b had practically no stimulatory activity for the GA2 hydrolysis (Fig. 5B, lanes 3 and 5). These results indicate that Ser103-Glu138 of the mouse sequence (see Fig. 1) may be responsible for eliciting the stimulatory activity for the hydrolysis of GA2. In this region, only 7 amino acids are different between the mouse and human sequences (Asn106 and Ile107, NI) in the mouse sequence are very

Characterization of the Proteins Expressed by ph2m3h4-a-SH, ph2m3h4-a-NI, and ph2m3h4-a-NI—Based on the above results, we proceeded to further narrow down the region between Ser103 and Glu138 of the mouse sequence. Using the specific primers and PCR, we generated the construct ph2m3h4-a-SH, which contained human exon 2 followed by a segment of human exon 3 up to Cys106 and then the mouse sequence from Ser103 to Pro112 and, again, back to the human sequence from Gly122 until the C terminus of the activator protein (Fig. 2B). This construct was similar to ph2m3h4-a except that Ser120 and His126 of the mouse sequence were changed to Pro124 and Arg130 respectively, to match the human sequence. The protein obtained from this construct was fully active for the stimulation of GM2 hydrolysis (Fig. 5A, lane 6) and also had ~70% activity for the hydrolysis of GA2 as compared with that of mM2act (Fig. 5B, lanes 6 and 11). It was therefore necessary to further clarify the involvement of Ser120 and His126 of the mouse sequence in the stimulation of GA2 hydrolysis. We subsequently constructed two chimeras, ph2m3h4-a-NI and ph2m3m4-a-NI (Fig. 2B). The construct ph2m3h4-a-NI was basically identical to ph2m3h4-a-SH except for the substitution of Asn106 and Ile107 (NI denotes the one-letter code of the 2 amino acids) in the mouse sequence with the corresponding human His110 and Phe111 residues. Another chimera, ph2m3m4-a-NI, contained the human sequence from the N terminus up to Asp113 followed by the mouse sequence from Leu110 until the C terminus (Fig. 2B). As expected, the proteins obtained from these two constructs had the stimulatory activity for the hydrolysis of GM2 (Fig. 5A, lanes 7 and 8). However, both of them were very weak in stimulating the hydrolysis of GA2 (Fig. 5B, lanes 7 and 8). Only using an increased amount of protein could we detect some hydrolysis of GA2 (Fig. 5B, lanes 9 and 10). Comparison of the stimulatory activities of ph2m3h4-a, ph2m3h4-a-NI, and ph2m3m4-a-NI (Fig. 5B, lanes 4, 7, and 8) clearly suggested that within the m3 region, at least 2 amino acids (Asn106 and Ile107, NI) in the mouse sequence are very important for eliciting the stimulatory activity for the hydrolysis of GA2. Both proteins obtained from ph2m3m4-a-NI (with the mouse Ser120 and His126 residues) and ph2m3h4-a-NI (without the mouse Ser120 and His126 residues) showed very poor activity in stimulating the hydrolysis of GA2. This indicates no or little involvement of Ser120 and His126 in exerting the stimulatory activity for the hydrolysis of GA2.

Characterization of the Chimeric Proteins Expressed by p513ML, p513MLT, and p513HMFMLT—To further clarify the involvement of Asn106, His126, Ser120, and His126 in the effect for expressing the stimulatory effect on GA2 hydrolysis, it was necessary to generate three additional chimeras: p513ML, p513MLT, and p513HMFMLT. The region of amino acid sequences between Lys96 and His137 of these chimeras together with those of mM2act (p513) and mM2act are listed in Fig. 6. The construct p513ML has 98.76% of the human sequence except for Met117 and Leu118 (ML), which were substituted with the corresponding Glu113 and Tyr114 residues of the mouse sequence.
sequence. The chimera p513MLT contains the same changes as in p513ML plus the substitution of Thr^{121} of the human sequence with Pro to match the Pro^{117} of the mouse sequence (Fig. 6). The third construct, p513HFMLT contains the crucial Asn^{106} and Ile^{107} residues of the mouse sequence. All proteins obtained from these three chimeras were able to stimulate the hydrolysis of GM2 by HexA (Fig. 7). These results indicate that human HexA is capable of hydrolyzing both GM2 and GA2 and that mM2act plays a specific role in assisting the enzyme to hydrolyze GA2. Since mM2act and m2act share a very high degree of homology, it is reasonable to search for the amino acids that are responsible for the differences in their ability to stimulate the hydrolysis of GA2. If such amino acids can be identified, it should be possible to construct a human/mouse chimera by replacing the specific amino acids in the human sequence with the mouse sequence, thereby converting the ineffective h2m2act into an effective protein capable of stimulating the enzymatic hydrolysis of GA2. Through this engineering, h2m2act can acquire an extra stimulatory activity for the hydrolysis of GA2, in addition to its native role for stimulating the degradation of GM2.

The alignment of the amino acid sequences of h2m2act and m2act from amino acid 32 to the C terminus showed 74.1% identity (Fig. 1). In addition, the characterization of the genomic structures of h2m2act (19) and m2act (20) revealed that the splice junctions of the two genes are completely conserved. This suggests that the inframes of the two species may contribute similarly to the processes of making the final mature proteins. Therefore, it is logical to use exon swapping of the human and mouse sequences to minimize the alteration of the structural integrity of the protein. By SDS-polyacylamide gel electrophoresis under nonreducing conditions, all chimeric proteins showed one major band with Coomassie Brilliant Blue staining. All proteins cross-reacted with both anti-h2m2act (18) and anti-m2act (data not shown) antibodies in Western blot analysis. Although not quantitative, these results indicate that the chimeric proteins do preserve the necessary structural features to be recognized by the antibodies.

The initial results from the chimeras obtained by exon swapping (Fig. 3) supported our rationale concerning the possibility of locating the active domain in m2act responsible for the stimulatory activity for GA2 hydrolysis. Our first set of results clearly indicated that the active domain was between Ser^{103} and Ile^{107} of the mouse sequence except for the 5 amino acids (His^{110}, Phe^{111}, Met^{117}, Leu^{118}, and Thr^{121}) being replaced by the corresponding Asn^{106}, Ile^{107}, Glu^{113}, Tyr^{114}, and Pro^{117} residues of the mouse sequence. All proteins obtained from these three chimeras were able to stimulate the hydrolysis of GM2 by HexA (Fig. 7A, lanes 5–7). However, only the protein expressed by p513HFMLT showed a full activity like m2act in stimulating the hydrolysis of GA2 (Fig. 7B, lane 5). The protein expressed by ph_{m,m_{m,a}} was included here for comparison because this protein showed the best activity among those examined in the previous experiments (Figs. 5B, lane 4; 7B, lane 4). These results underscore the importance of Asn^{106} and Ile^{107} in eliciting the stimulatory activity for the hydrolysis of GA2 by HexA. Although both the proteins obtained from the constructs p513MLT and p513ML were weak in stimulating the hydrolysis of GA2 (Fig. 7B, lanes 6 and 7), the former had better activity than the latter. Only with a prolonged incubation of 16 h (Fig. 7B, lanes 9 and 10) did these two proteins show detectable activity for this reaction.

Characterization of the Protein Expressed by p513MLAla^{132}—During the attempt to produce the chimera p513ML by PCR, we accidentally obtained a construct that has the same sequence as p513ML, but that also contains a 3-bp insertion for an extra Ala at position 132. This construct is therefore called p513ML-Ala^{132}. Interestingly, this mutant was able to stimulate neither the hydrolysis of GM2 (Fig. 7A, lane 8) nor the hydrolysis of GA2 (Fig. 7B, lane 8). Even with a prolonged incubation, the protein expressed by p513ML-Ala^{132} was completely inactive in stimulating the hydrolysis of GM2 (Fig. 7A, lane 9) and GA2 (Fig. 7B, lane 11). This protein was used as a negative control for all incubations.

DISCUSSION

We have previously shown that although human HexA is capable of hydrolyzing GM2 in the presence of either h2m2act or m2act, it can only effectively degrade GA2 in the presence of m2act (9). These results indicate that human HexA is capable of hydrolyzing both GM2 and GA2 and that m2act plays a specific role in assisting the enzyme to hydrolyze GA2. Since m2act and m2act share a very high degree of homology, it is reasonable to search for the amino acids that are responsible for the differences in their ability to stimulate the hydrolysis of GA2. If such amino acids can be identified, it should be possible to construct a human/mouse chimera by replacing the specific amino acids in the human sequence with the mouse sequence, thereby converting the ineffective h2m2act into an effective protein capable of stimulating the enzymatic hydrolysis of GA2. Through this engineering, h2m2act can acquire an extra stimulatory activity for the hydrolysis of GA2, in addition to its native role for stimulating the degradation of GM2.
tein to stimulate the hydrolysis of GA2. The fact that the protein expressed by p513HFMLT was as active as mM2act in stimulating the hydrolysis of GA2 (Fig. 7B, lanes 5 and 12) indicated that the region from Ser126 to the C terminus of the mouse sequence was not crucial for the stimulatory activity of GA2 hydrolysis. From the diminished activity of the protein expressed by pm3h4-a-SH for the hydrolysis of GA2, we further concluded that the presence of Pro124 next to Cys125 in the human sequence might form a conformation unfavorable for GA2 hydrolysis. The protein expressed by p513HFMLT has only 5 out of 162 amino acids (3.2%) different compared with mM2act. However, this protein has the full activity compared with that of mM2act in stimulating the hydrolysis of GA2. These results clearly indicate that the region between Asn106 and Pro117 of the mouse sequence is extremely important for the stimulatory activity for GA2 hydrolysis.

A recent study (21) reported the existence of four pairs of putative disulfide bonds in hM2act. They were Cys19–Cys183, Cys99–Cys106, Cys112–Cys138, and Cys125–Cys136. The latter two pairs of disulfide bonds were suggested to form a clamp to stabilize the region in hM2act that is equivalent to the mouse region between Asn106 and Pro117. Our results indicate that this region in the mouse sequence is important for the stimulation of GA2 hydrolysis. The m3 segment is rich in Cys residues that are completely conserved between hM2act and mM2act. A Cys138 mutation in hM2act has been characterized in a human type AB Tay-Sachs patient by Schroeder et al. (22) and Xie et al. (23). In this patient, the mutation of thymidine to cytidine at position 412 of the full-length hM2act cDNA (19) caused the substitution of Cys138 with Arg and abolished the activator’s stimulatory activity for GM2 hydrolysis. In a recent study, Xie et al. (24) further characterized the C138R substitution in hM2act. They suggested that the mutation specifically affected the domain in the activator protein that was responsible for the recognition of the activator-ganglioside complex by HexA. This suggestion agrees with our observation that the same region in the mouse sequence seems to be crucial for HexA to hydrolyze GA2. Xie et al. also suggested that the mutation might cause localized changes in the mutant protein without major changes in the secondary or tertiary structure of the protein. The fact that all of our chimeras showed good activities in stimulating the hydrolysis of GM2 indicates that the conformation of the chimeric proteins must remain very close to that of the native protein. Moreover, all of the chimeras in this study did not involve any Cys substitution; thus, no direct disturbance was introduced into their disulfide bond formation. The specific action of the mouse sequence between Asn106 and Pro117 for GA2 hydrolysis cannot be fully understood until the revelation of the crystal structures of hM2act, mM2act, and the chimeric protein expressed by p513HFMLT.

It is remarkable that the substitution of a very small region (5 amino acids) in the human activator sequence with the specific mouse sequence can create a mutant protein (p513HFMLT, 96.8% human and 3.2% mouse) effective in stimulating the degradation of both GM2 and GA2 carried out by human HexA. We have further examined the stimulatory activities of hM2act, mM2act, and the chimeric protein expressed by p513HFMLT in the hydrolysis of GM2 and GA2 carried out by human HexB. It is known that human HexB is not able to catalyze the hydrolysis of GM2. We also observed that human HexB was not able to hydrolyze GM2 in the presence of any one of the three activators. However, human HexB could hydrolyze GA2 in the presence of either mM2act or the protein produced by p513HFMLT, but not in the presence of hM2act. Although the hydrolysis of GA2 by HexB is relatively slow, at a rate ~5% of that by human HexA, this activity could be important in the classical Tay-Sachs patients who are devoid of HexA and have only HexB. This importance has been illustrated in the milder clinical conditions in the murine model for type B Tay-Sachs disease (targeted disruption of the Hexa gene) (3–7) whose activator is effective in stimulating HexB to hydrolyze GA2. In human Tay-Sachs patients, their hM2act activators are not able to stimulate HexB to hydrolyze GA2. Therefore, based on our results, the chimeric protein expressed by p513HFMLT should be theoretically able to induce an alternative catabolic pathway for GM2 in human through the degradation of GA2. Thus, the chimeric activator protein may be ultimately useful for type B Tay-Sachs patients who can be benefited from the induction of the alternative catabolic pathway for GM2.

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