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THE PRO-PEPTIDE OF THE PROβ-POLYPEPTIDE CHAIN OF HUMAN β-HEXOSAMINIDASE IS NECESSARY FOR PROPER PROTEIN FOLDING AND EXIT FROM THE ENDOPLASMIC RETICULUM

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We examine the function of the proβ-peptide (residues 42-121) in the folding and intracellular transport of human β-hexosaminidase B (β-N-acetylhexosaminidase, EC 3.2.1.52). A construct was prepared that encoded an in frame deletion of residues 55-118. Expression of this construct in COS-1 cells produced a β-polypeptide chain that formed insoluble aggregates and remained trapped in the endoplasmic reticulum (ER). We conclude that the proβ-peptide may act as a type of intramolecular chaperone for the mature β-subunit.

As is the case for many lysosomal proteins the mature subunits of the human β-hexosaminidase (Hex) isozymes, Hex A (αβ) and Hex B (ββ) are synthesized as prepro-polypeptides. The prepro-α and prepro-β chains are encoded by the *HEXA* gene (chromosome 15) and *HEXB* gene (chromosome 5), respectively (reviewed in (1, 2)). The 60% homology in the deduced primary structures of the two polypeptides (3), as well as the conservation of 13 out of 14 intron-exon junctions within their respective genes (4) demonstrate that they share a common evolutionary origin. Thus, the function of aligned domains, *e.g.* the α and β pro-peptides, and conserved residues, *e.g.* αArg^{178} and βArg^{211} (5), within the two primary structures should be nearly identical. We have demonstrated this conservation of function experimentally (5-7).

The pre-sequences (residues α1-22 and β1-42) or signal peptides (Fig. 1) are removed upon entry of the nascent polypeptide into the ER. Proper folding and dimer formation also occur in the ER, after the removal of the signal peptide, and appear to be necessary conditions for the transport of the pro-Hex isozymes into the Golgi (8, 9) and ultimately the lysosome. Folding, and thus intracellular transport can be inhibited by mutations in either subunit (7, 10). Of the two isozymes only the Hex A is able to hydrolyze the terminal β-linked GalNAc residue from G_{M2} ganglioside. The absence of Hex A activity, as a result of mutations affecting either the α- or common β-subunit results in Tay-Sachs or Sandhoff disease, respectively, two forms of G_{M2} Gangliosidosis (1, 11).

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The function of the Hex pro-peptides (αLeu^{23}-His^{88} and βAla^{43}-Lys^{121}, Fig. 1) have not been determined. However, the pro-peptides of at least three other lysosomal enzymes have been studied. All three, mouse cathepsin L (12), yeast carboxypeptidase Y (13), and yeast subtilisin (reviewed in (14)), have been shown to be necessary for the proper folding of the mature polypeptide. These pro-peptides have been referred to as "intramolecular chaperones" (14). However, the pro-peptides of Hex differ from those previously investigated in that their presence does not inhibit the enzymatic activities of the pro-Hex isozymes (15, 16), nor are they entirely degraded during maturation in the lysosome. The peptides αLeu^{23}-Gly^{74} and βAla^{50}-Gly^{107} remain attached to their respective mature subunits by interchain disulfide bonds through a conserved Cys in each pro-sequence, *i.e.* αCys^{58} and βCys^{91} (Fig. 1) (17, 18). Aligned Cys are also present in the mouse polypeptides, but not in the Hex polypeptide from *Dictyostelium discoideum* (Fig. 1), suggesting that the retention of the pro-peptide may not be an evolutionarily conserved event.

In order to determine the function of the proβ-peptide, and by analogy the proα-peptide, we have constructed a βcDNA that encoded an in frame deletion of residues Ala^{55}-Phe^{118} (this would be similar to a deletion of Leu^{23}-Gly^{85} in the proα chain, Fig. 1). This construct was then expressed in COS-1 cells and compared to the protein produced by the wild-type βcDNA.

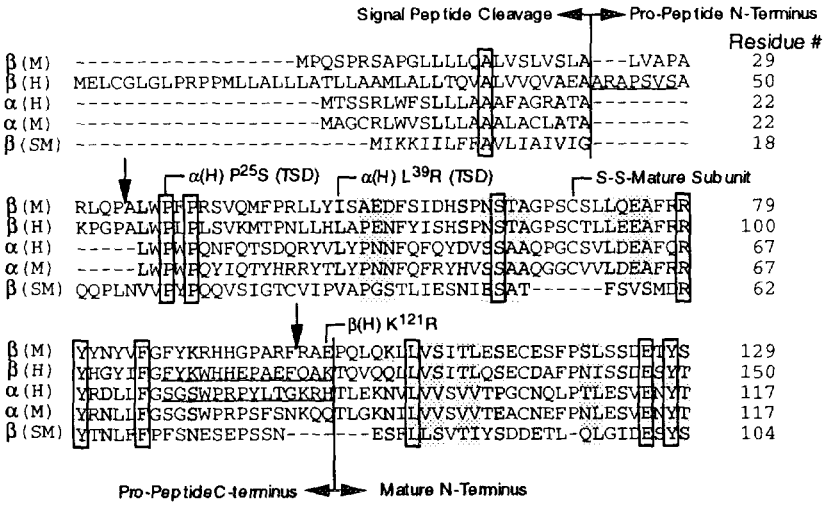

Figure 1. The aligned deduced amino acid sequences of the mouse and human preproβ chain, β(M) (32) and β(H) (3); the human and mouse preproα chain, α(H) (3, 33) and α(M) (34); and the preproβ chain from *Dictyostelium discoideum*, β(SM) (35). Boxed residues are identical in every example, whereas shaded residues have one or more conservative amino acid substitutions (the program GeneWorks 2.1 was used to generate these comparisons). The cleavage sites for the pre sequence (signal peptide) have been determined experimentally for the human polypeptides (25, 28) and have been calculated for the others (36). The mature amino-termini have also been experimentally determined for the human chains (24) and are estimated from the alignment for the others. Underlined residues are likely lost during maturation (18). Known missense mutations that have been associated with Tay Sachs disease are designated with "(TSD)". The residues that were deleted in our mutant cDNA are between the two vertical arrows (βAla^{55}-Phe^{118}).

MATERIALS AND METHODS

Removal of Pro-Sequence

General cloning procedures were as described by Maniatis (19). A NaeI (full digest of an unique restriction site after nucleotide 162) and an EcoRI (partial digest of the first of 2 sites after nucleotide 349) cDNA fragment was removed from the cDNA sequence encoding the preproβ-polypeptide of Hex contained within a pcD cloning and mammalian expression vector, pHexB43 (5). The EcoRI site was blunt-ended (removing nucleotides 350-353) and the vector re-ligated. The resulting mutant βcDNA encodes an in frame deletion of amino acids Ala^{55} to Phe^{118} in the pro-sequence of the β polypeptide chain. The only other change from the wild type nucleotide sequence is silent, *i.e.* the codon for Pro^{54} was converted from CCG to CCC. The mutation was verified by sequencing the mutation site using the Sanger dideoxy-chain termination method (20).

DNA Transfections

COS-1 cells were transfected using 10μg of pHexB43 containing either the wild type or the pro-sequence mutation. The constructs were co-transfected with 10μg of pBLCAT2 (21), a plasmid containing the chloramphenicol acetyltransferase gene driven by the HSV thymidine kinase promoter, to evaluate transfection efficiency from one cell culture dish to the other. Transient expression was carried out for 48hr and cells were harvested as described (5).

Activity Measurements and Western Blot Analysis

Cell lysates and media were analyzed for human Hex activity by a solid-state immunoprecipitation assay using 4-MUG (4-methylumbelliferone-β-N-acetylglucosamine) as a substrate. Briefly, 0.25-2μl of a total 20μl of cell lysate (obtained from one confluent 100 X 20mm tissue culture dish) diluted in 70μl PBS, or 500μl of cell culture medium was immunoprecipitated using an antiserum that specifically binds the human-β subunit (separating it from the endogenous monkey COS cell Hex) (5, 22). Western blotting was used to analyze the remaining COS lysate. Equal amounts of total protein (~15μl of lysate) were separated by SDS/PAGE (23). Proteins were transferred overnight to nitrocellulose. The Western blot was developed by the peroxidase anti-peroxidase procedure (Jackson Immunologicals) using a donkey-anti rabbit IgG as the bridging antibody and normal donkey serum as a blocking agent as previously described (5). Insoluble, mutant Hex protein (contained in the cell pellet) was recovered as follows: The pellet was washed (4x) with 500μl of PBS and the sample was vortexed and centrifuged after each wash. The pellet was extracted with 20μl of 50mM Tris pH 7.5 and 150mM NaCl, containing 1% SDS and 1% Triton-X-100. After vortexing and centrifugation, Laemmli sample buffer (23) with 25mM DTT (20μl) was added to the extract which was then boiled for 5min and loaded on the SDS/PAGE gel.

Immunofluorescence

For the examination of the intracellular location of Hex B protein by immunofluorescence, transfected COS-1 cells were cultured directly on gelatinized glass slides, and fixed in acetone at 4°C for 10 minutes. The sections were washed in PBS and then treated with the rabbit antibody to the β subunit of human Hex, diluted 1:3 in PBS and incubated for 30 minutes at room temp. The slides were then washed 3 times with PBS and then treated with fluorescein-conjugated goat anti-rabbit antiserum (Cappel, Cockranville, PA, USA) for 30 minutes at room temp. The slides were washed again 3 times with PBS. Normal rabbit serum was used as a negative control.

RESULTS

Both endogenous (Table 1, "Free") and human (Table 1, "Bound") Hex activity was measured in lysates from a) untransfected COS cells, b) COS cells transfected with the wild type human βcDNA, and c) COS cells transfected with the pro-peptide deletion construct (Table 1).

Table 1: Specific Activity of Human β Hex B in Transfected COS Cells and Cell Culture Media

	Intracellular Specific Activity (nmol / hr / μg total protein)		Media
	Free (COS)	Bound (Human)	% Secretion (Human)
a) COS (-)	0.3	0.05	ND*
b) COS (+)	0.3	3.5	49.2
c) COS Pro deletion	0.3	0.05	ND

a Extract from untransfected COS cells, (-) control.
b Extract from COS cells transfected with wild type pHexB43, (+) control.
c Extract from COS cells transfected with the pro-sequence (Ala^{55} ->Phe^{118}) deletion.
* Not detectable (<2Xblank).

There was a 70-fold increase in human Hex activity when the positive control (Table 1, b) was compared with the negative control (Table 1, a); however the activity levels for the deletion construct (Table 1, c) were indistinguishable from those of the negative control. Hex activity that was not bound by our antiserum remained constant in all three lysates, confirming that the lack of Hex activity produced by the mutant construct was not caused by a change in the protein's antigenicity. There was also no increase in the Hex activity of the medium from cells transfected with the mutant cDNA, indicating that the mutation did not divert the enzyme to the secretory pathway.

Western blots were used to determine the fate of the inactive and/or unstable mutant Hex protein. The Western blot of the centrifuged lysates from the cells transfected with the wild type βcDNA gave the expected doublet band corresponding to a Mr of 28,000 (5) (the proβ polypeptide also undergoes an internal cleavage which produces the mature β_a (Mr=28,000) and β_b chains (24)) (Fig. 2, COS(+)). However like the non-transfected COS cell lysates (COS(-)), no immunoreactive band was found in the centrifuged lysates of cells transfected with the mutant construct (Fig. 2, PRO-lysate). When the cell pellets from the three lysates were extracted with detergent an immunoreactive band with a Mr corresponding to 56,000 appeared from the cells expressing the mutant construct (Fig. 2, PRO-pellet; the negative data from the positive and negative control cell pellets are not shown). The Mr is consistent with the removal of the pro-peptide, Mr=7,500, from the proβ polypeptide, Mr= 65,000 (25).

The cellular localization of the Hex expressed in transfected cells was determined by immunofluorescence microscopy. Whereas the wild type protein produced a distinct punctate pattern in keeping with a lysosomal localization (Fig. 3, panel A), the pro-peptide mutant produced a diffuse, finely granular pattern consistent with retention of the protein in the ER (Fig. 3, panel B).

DISCUSSION

We previously demonstrated that the over-expression in COS cells of αcDNAs (co-transfected with normal βcDNA) containing missense mutations that affect protein folding, results in an amplification of the residual Hex A activities and mature α-CRM (cross reacting

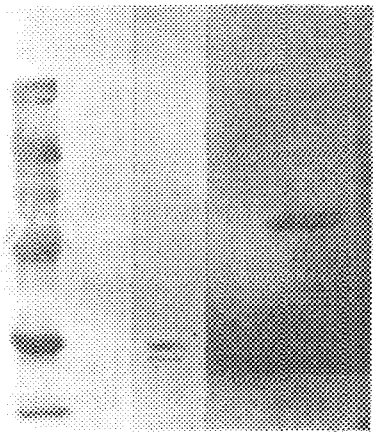

Figure 2. Western blot analysis of centrifuged lysates from non-transfected COS cells, COS(-); COS cells transfected with the wild type βcDNA, COS(+); and COS cells transfected with the mutant βcDNA construct encoding the in frame deletion of the pro-peptide, PRO-lysate. The pellet of the cells transfected with the mutant construct was then extracted with detergents and re-analyzed by Western blot, PRO-pellet. Pre-stained Mr standards (GIBCO BRL) are also shown, Std. (kDa = 215, 105, 68, 43, 28, 18).

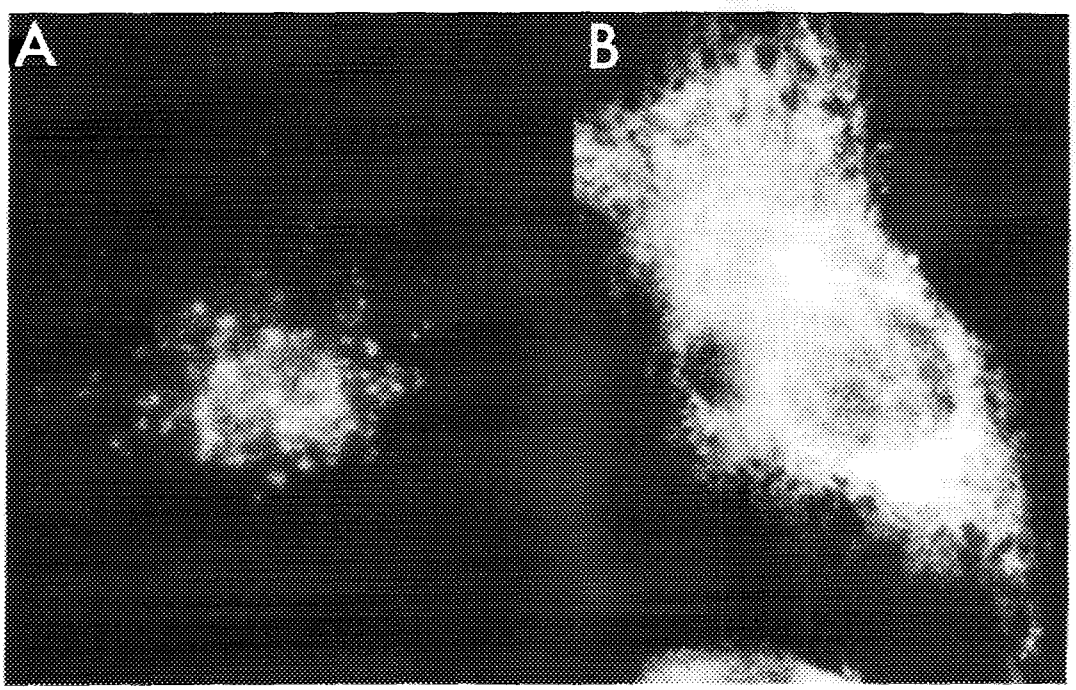

Figure 3. Immunofluorescence microscopy of COS-1 cells transfected with the wild type βcDNA (panel A) and a mutant cDNA (panel B) encoding the in frame deletion of the pro-peptide.

material), as compared to the levels found in the patient fibroblasts (7). Furthermore, when we tested one of these α-mutations, αGly^{269}Ser, by introducing it at the homologous position in the β-chain, βGly^{301}Ser. We found that this substitution had a less deleterious effect on the residual Hex B activity and mature β-CRM (65% of wild type Hex B, as compared to 46% of wild type Hex A). These data are consistent with the inherently more stable nature of the β subunit of Hex (7). Thus, the observations made in this report using the same over-expression system, of no detectable Hex B activity (even with our 70:1 positive to negative control ratio, Table 1) or mature β-CRM (Fig. 2), emphasize the severity of the deletion mutation's effect on the ability of the β polypeptide to fold properly.

The pro-peptides' involvement in the initial folding of the proα and β polypeptide chains of Hex has been suggested by the occurrence of two different missense mutations in the proα sequences of two patients with Tay-Sachs disease (26, 27), *i.e.* Pro^{25}Ser (only two residues from the amino-terminus of the proα chain (28)) and Leu^{39}Arg (Fig. 1). There was <2.5% residual Hex A in patient cells containing the former substitution (the 2^{d} allele was Met^{1}Thr which eliminated the site of protein translation initiation). Whereas a soluble proα chain was detected immunologically, there was no detectable mature α chain suggesting that the protein was trapped in the ER. The latter missense mutation (the 2^{d} allele was the exon 11 insertion (29)) produced only low levels of immunologically detectable proα chain, again suggesting a failure of the protein to be transported out of the ER. Unfortunately the direct correlation between these substitutions and the deleterious effects on ER transport observed in patient cells was not made through expression of the appropriately mutagenized cDNA. However, these two residues are conserved in both the mouse and human α and β cDNAs, as well as in the cDNA encoding Hex in slime mold, *Dictyostelium discoideum* (Fig. 1). The conservation of these residues through evolution is strong evidence for their importance in producing a functional protein.

A third missense mutation in the proβ sequence, Lys^{121}Arg, was found to have no effect on the transport of functional Hex A or Hex B in patient samples or in the expression of the mutant βcDNA in COS cells (30, 31). This residue is also not evolutionarily conserved (Fig. 1).

Our data demonstrate that the removal of the pro-peptide from the proβ polypeptide of human Hex A and B prevents the protein from folding and exiting the ER. Because of the evolutionary relationship of the *HEXA* and *HEXB* genes, we conclude that the same purpose is served by the pro-peptide of the proα-polypeptide chain of Hex A. These data lend support to the linkage between the two α-mutations described above and the failure of the proα chain in the patients' cells to exit the ER, resulting in Tay-Sachs disease.

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