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EXAMINATION OF AN AREA IN β-HEXOSAMINIDASE B HOMOLOGOUS TO THAT IN CATHEPSIN D LINKED TO LYSOSOMAL TARGETING

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A lysine-rich area in the β subunit of β-hexosaminidase (β-N-acetylhexosaminidase, EC 3.2.1.52) homologous to residues 189-203 in Cathepsin D, previously proposed as being critical for efficient lysosomal targeting, was identified. *In vitro* mutagenesis of the Lys residues was followed by COS-1 cell expression of enzymatic activity. The intracellular mutant β-hexosaminidase B activity had a $T_{1/2}$ at 60° C similar to that of the wild type enzyme, indicating that this region is likely on the surface of the folded enzyme, as is the targeting domain of Cathepsin D. However, in the case of β-hexosaminidase B, mutation of the Lys residues did not affect lysosomal compartmentalization. These data suggest that the hunt for the common protein signal that results in proper intracellular transport of lysosomal enzymes will not be straightforward and that Lys residues may not be an absolute requirement of the signal.

β-Hexosaminidase is a lysosomal enzyme that catalyzes the hydrolysis of terminal β-hexosamines from various oligosaccharides, glycosaminoglycans and glycolipids. In normal human tissues, there are two major isozymes, hexosaminidase A (Hex A) a heterodimer (αβ) and hexosaminidase B a homodimer (ββ). Of the two isozymes, only the Hex A is able to hydrolyze 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).

The α- and β-subunits of Hex are synthesized as single prepro-polypeptides on polysomes attached to the rough endoplasmic reticulum (RER). Thus, they both must contain signal peptides which are cleaved on entry into the lumen of the RER to produce the pro-polypeptides. Targeting to the lysosome requires the synthesis of a mannose 6-phosphate recognition marker on a preexisting Asn-linked oligosaccharide by the sequential action of two enzymes. The first is a lysosomal enzyme-specific transferase, UDP-N-acetylglucosamine: glycoprotein N-acetylglucosamine-1-phosphotransferase (phosphotransferase), found in the salvage compartment and cis Golgi (2). The second enzyme, α-N-acetylglucosaminyl phosphodiesterase (3), removes

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the "blocking" GlcNAc residue to produce the mature ligand for mannose-6-phosphate receptor binding in the trans Golgi network (4). Final proteolytic and glycosidic processing occurs in the lysosome, giving rise to the mature forms of each subunit (5). The mature α-subunit is processed into two polypeptide chains α_p(7kDa) and α(56kDa) held together by disulphide bonds (6). The β-subunit is converted into three polypeptides, β_p(7-15 kDa), β_b(20-26kDa), β_a(28-30kDa) , also held together by disulphide bonds (6-8).

Since lysosomal enzymes share a common pathway with secretory proteins during the early stages of their biosynthesis, the ability of the phosphotransferase to recognize soluble proteins destined for the lysosome is critical for proper intracellular targeting. It has been shown that deglycosylated lysosomal enzymes but not secretory proteins are specific and potent inhibitors of the phosphorylation of intact lysosomal enzymes (9). This has led to the proposal that phosphotransferase recognizes a protein domain that is common to all lysosomal enzymes (reviewed in (10)).

To identify this protein determinant Kornfeld and colleagues (11) constructed a series of chimeric proteins. Cathepsin D is a lysosomal aspartyl protease while pepsinogen is a secretory aspartyl protease. It is generally believed that all such proteases evolved from a common ancestral gene and thus, retain a great deal of similarity in their three-dimensional structures. By exchanging aligned segments of Cathepsin D with those of pepsinogen two surface domains in close proximity to each other in native pepsinogen (and by extension in Cathepsin D) were identified that appeared to be necessary for efficient lysosomal incorporation of the chimers. Both of these domains, residues 188-230 and 265-348, were rich in Lys residues. However, it was demonstrated that only Lys_{203} in the former domain was a critical residue. Interestingly, we noted an area in the deduced sequence of Hex B that was homologous to residues 188-208 in Cathepsin D which included β-Lys_{476} which aligns with the critical Lys_{203} in Cathepsin D (Fig.1, double underlined). We thus tested the possibility that one or more of the Lys residues in this area of the β subunit could be involved in the recognition of Hex B by the phosphotransferase. We also included in our study another high-lysine area in the β sequence, Lys_{300} Gly Gln Lys.

MATERIALS AND METHODS

Oligonucleotide-Directed In Vitro Mutagenesis

General cloning procedures were as described by Maniatis (12). A 1.3 kb Eco RV/Pst I cDNA fragment of the β subunit from pHexB43, containing the cDNA sequence encoding the β subunits of Hex (13), was subcloned into bluescript (pBS-, Stratagene). Three mutant oligonucleotides were synthesized (HSC Biotechnology Service) for the mutagenesis: i) 5'-TTGGGGAAATGGCCAGAACGACCTCCTGAC-3' (30-mer), ii) 5'-GATTGGAGGAACT-ACTATAACGTGGAACCTC-3' (31-mer) and 5'ACTCAGAACCAGAACCAGCTGTTCATT-GGTGGA-3' (33-mer). They all contain (underlined in sequence) the necessary change to convert Lys→Asn in the deduced amino acid sequence of the β subunit. Conservative changes in the 33-mer oligonucleotide (dotted underlined) were made to create new restriction enzyme sites which were utilized in the screening process. The *in vitro* mutagenesis was performed as previously reported (14). The mutations were verified by nucleotide sequencing of approximately 900bp (including the mutation site). Mutant fragments were subcloned back into pHexB43; a pcD derived plasmid with a SV-40 early promoter for expression in mammalian cells (15).

Cell Culture and DNA transfections

COS-1 monkey kidney cells were maintained and transfected with 10μg each of the vectors pHexB43 (wild type or mutant) and pBLCAT2 (encoding the bacterial chloramphenicol

acetyltransferase (CAT)) to monitor transfection efficiency from one cell culture dish to another, as previously described (14, 16).

Analyses of the Human Hexosaminidase Expressed in COS Cells

In order to separate the endogenous COS-1 cell Hex activity from that of the transiently expressed human Hex, an immunoprecipitation and direct assay system (with 4-methyl umbelliferyl β-N-acetyl glucosamine (4-MUG) used as the Hex substrate) was employed for both the cell lysates and the media. This system utilizes a polyclonal rabbit anti-human hexosaminidase B-IgG prepared in our laboratory, as previously described (14, 16). The heat stability of wild type and mutant human Hex B contained in lysate samples was assessed by incubating cell lysates at 60° C in 0.3% HSA and citrate-phosphate buffer (pH 4.1). Aliquots (75μL) were removed at 0, 5, 10, 15, and 20 min intervals and assayed as above.

Western blots were performed as previously described (14). Lysates were analyzed using equal amounts of CAT activity (17) (25-75μg of total lysate protein).

RESULTS AND DISCUSSION

As previously reported (14), the specificity of the solid state assay system for human Hex B synthesized from pHexB43 results in an at least 25-fold increase in the activity of Hex immobilized by the protein A-Sepharose per μg of protein from transfected cell lysate as compared to untransfected COS cell lysate (Table 1; compare bound COS (+) to bound COS (-)).

The specific activity of the immobilized mutant Hex B synthesized from each of seven independently transfected mutant constructs, was similar to the wild type positive control (Table 1; Bound, compare COS constructs 1-7 with COS (+)). The activity in the unbound fraction (Table 1, Free) which represents the endogenous COS enzyme, failed to show any elevation in specific

Table 1. Analyses of Human β-Hexosaminidase B from Transfected COS-1 Cell Lysates and Cell Culture Media

	Hexosaminidase Specific Activity (nmol 4MU/ hr/ μg total lysate protien)			Stability 60° C
	Free (Endogenous)	Bound (Human)	% Secreted (Human)	$T_{1/2}$ (min.) (Human)
COS (+)[a]	0.34±0.03	1.0±0.07	34.6 ± 1.0	18.9
COS (-)[b]	0.26±0.03	0.04 ± 0.02	-	-
COS Constructs[c]				
1) $N_{300}GQN_{303}$	0.28±0.1	1.0 ± 0.07	46.5 ± 2.6	11.1
2) $N_{462}YYN_{465}$	0.37±0.09	2.0 ± 0.9	33.3 ± 7.7	9.3
3) $N_{476}QN_{478}Q$	0.35±0.05	1.4 ± 0.3	53.3 ± 11.9	21.4
4) NGQN/NYYN	0.30±0.02	1.1 ± 0.2	54.8 ± 8.7	8.9
5) NGQN/NQNQ	0.36±0.03	1.3 ± 0.2	52.1 ± 12.8	15.0
6) NYYN/NQNQ	0.54±0.19	1.2 ± 0.1	48.4 ± 14.1	10.0
7) NGQN/NYYN/ NQNQ	0.50±0.12	0.7 ± 0	57.9 ± 9.6	7.8

[a] Extract from COS cells transfected with pHexB43, positive control.

[b] Extract from untransfected COS cells, negative control.

[c] Extract from three independent transfections of COS cells with constructs containing encoding the Lys (K)→Asn (N) mutants indicated (see Fig. 1).

activity indicating that the mutations did not affect the antigenicity of the proteins. Human Hex activity measurements of the transfected cell culture media failed to show a significant percent increase of mutant Hex secreted by the cells, as compared to media from cells transfected with the wild type construct (Table 1; compare % secretion of COS constructs 1-7 to COS (+)). These results demonstrate that these lysine-rich regions are not required for intracellular retention of the enzyme.

The targeting domains in Cathepsin D are reported to be on the surface of the protein (11). It has been suggested that apart from their hydrophilic properties, individual surface residues add little to the overall stability of a folded protein (reviewed in (18)). Thus, to determine the likelihood of the lysine-rich areas we selected for this study residing in surface domains of Hex B, we tested the heat stability of the enzymes synthesized by our mutant constructs. The human Hex B activity in lysates from cells transfected with wild type pHexB43 had a $T_{1/2}$ at 60° C similar to that of purified placental Hex B (data not shown). The mutant Hex B activities synthesized from all the constructs except COS 3) which originally contained β-Lys_{476} (Fig. 1) were more heat labile than the wild type enzyme (Table 1; $T_{1/2}$, 3) N_{476}QNQ). The construct COS 7) where all 6 Lys residues were mutated to Asn was the least stable, but still retained a $T_{1/2} \cong 40\%$ of wild type (Table 1). Overall, these results indicate that the individual Lys→Asn substitutions have only a slight effect on the mutant enzyme's heat stability. These data are consistent with their location on the surface of the folded protein.

The proper intracellular localization of the mutant proteins was confirmed by Western Blot analysis. The β subunits of the intracellular Hex B synthesized by each of the mutant constructs were processed to their mature lysosomal form, confirming correct targeting (Fig. 2) (the rabbit anti-human hexosaminidase B-IgG, generated in our laboratory, preferentially recognizes the more antigenic 28 kDa mature β_a polypeptide chain (14)). Thus, the mutations of Lys→Asn at each of the lysine-rich sites does not interfere with lysosomal incorporation.

Despite the similarities in primary sequence and surface location with the functional area in Cathepsin D, intracellular and extracellular measurements of human Hex B activity indicated that the mutations we introduced into lysine-rich sites and particularly β-Lys_{476} caused no significant increase in secretion. Western blot analysis demonstrated that the 65kDa pro-β chain had been processed to its mature 28kDa lysosomal form (Fig. 2). Thus, these Lys residues do not seem to play a role in the phosphotransferase recognition and lysosomal targeting of Hex B. This study demonstrates that identification of the common protein domain among lysosomal enzymes recognized by the phosphotransferase will not be straightforward and may not even require the presence of a Lys residue.

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Hex B    RKYYKVEPLDFGGTQKQKQLF    461/481
          ::::          : :
Cat D    SKYYKGSLSYLNVTRKAYWQV    188/208
         : :: :::     ::   :::
PG       SSYYTGSLNWVPVTVEGYWQI    221/241
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Fig. 1. Aligned amino acid sequence of the lysosomal enzymes β-hexosaminidase B (Hex B), Cathepsin D (Cat D) and the secretory enzyme, pepsinogen (PG).

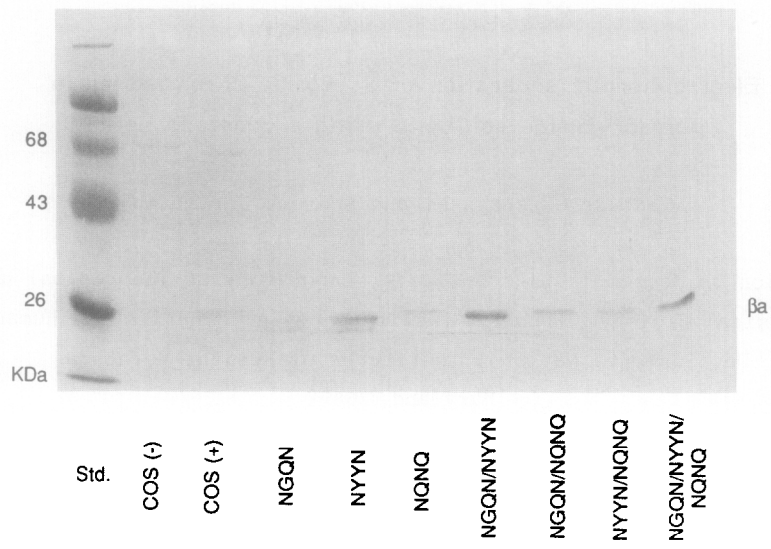

Fig. 2. Western blots of equal amounts of normalized protein from transfected COS cell lysate. COS cells were transfected independently with the normal pHexB43 plasmid, COS (+), and the seven various mutant pHexB43 plasmids encoding the Lys (K)→Asn (N) substitution, $N_{(300)}$GQN, $N_{(462)}$YYN, $N_{(476)}$QNQ, NGQN/NYYN, NGQN/NQNQ, NYYN/NQNQ, and NGQN/NYYN/NQNQ (see Fig. 1). Untransfected COS cells were used as the negative control, COS (-). Prestained protein Mr standards can be seen at the left with the relevant Mr indicated. The position of the immunoreactive band corresponding to the mature β_a polypeptide chain of hexosaminidase is shown on the right (14).

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