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The amino-terminal sequences in the pro-α and -β polypeptides of human lysosomal β-hexosaminidase A and B are retained in the mature isozymes

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The α- and β-subunits of β-hexosaminidase (β-*N*-acetylhexosaminidase, EC 3.2.1.52) are synthesized in the rough endoplasmic reticulum as prepropolypeptides. After the loss of the signal peptide and formation of enzymatically active dimers, the pro-isoenzymes are transported through the Golgi and into the lysosome for proteolytic and glycolytic processing to their stable mature forms. Maturation includes the hydrolysis, and previously presumed loss, of small N-terminal peptides from each propolypeptide. A recent report characterizing the processing of the β-prepropolypeptide in β-hexosaminidase from a human fibroblast cell line [(1989) J. Biol. Chem. 264, 3380–3384] reported that the small pro-β peptide was retained through a disulfide bond in the mature subunit, and that it was glycosylated. We have confirmed this result in normal human tissue. However, we report a different N-terminal for the mature pro-β peptide. Furthermore, we have found that the pro-α peptide is similarly retained in the mature α-subunit through its single cysteine residue and that each pro-peptide undergoes C-terminal processing.

Lysosome; Structure; Biosynthesis; Posttranslational processing

1. INTRODUCTION

As typical glycoproteins, the components of β-hexosaminidase (Hex) are synthesized in the rough endoplasmic reticulum (RER) as prepropolypeptides. The presequence, the signal peptide, is cleaved shortly after entry into the RER resulting in the propolypeptides. After passage through the Golgi which must include the phosphorylation of one or more of the oligosaccharides for binding to the phosphomannosyl receptor (reviewed in [1]), the stable mature subunits are produced through proteolytic and glycolytic processing in the lysosome.

Utilizing purified isozymes from human placenta in our studies [2–6] or in vivo labeling of Hex in human fibroblasts by others [7–9], the details of the biosynthesis and posttranslational processing of the Hex subunits have largely been established. The single proteolytic event that occurs outside of the lysosome, signal peptide cleavage, results in N-termini at residues 23 and 43 for the pro-α and pro-β polypeptides, respectively [5,8] (fig.1). Specific internal cleavages and trimming of the proteins in the lysosome generate a 53 kDa α-chain and two β-polypeptides which we have called β_b (basic p*I*) and β_a (acidic p*I*) of similar molecular mass (26–30 kDa) [3,4] (fig.1). In the case of the pro-α chain, cleavage at the N-terminus results in an intermediate species beginning at residue 87 (Arg) [8]. Further trimming to residue 89 (Thr) or 90 (Leu) yields the N-terminus of the mature α-chain [4,8]. The N-termini of the mature β-peptides were mapped to residues 122 in the β_b chain and 315 or 316 in the β_a chain of Hex A or B, respectively [4,9]. This cleavage between the β_b and β_a chains

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involves the loss of four (in Hex A) or three (Hex B) residues between them [4].

Until recently, it was assumed that the residues between the signal peptide cleavage points and the mature N-termini of the α (α_p, residues 23–87) and β_b chains (β_p, residues 43–122) were lost. However, the deduced amino acid sequences of both of these peptides contain a single cysteine residue [10]. Furthermore, β_p contains a possible site for Asn-linked glycosylation [6]. In a recent report, Quon et al. [9] found that the β_p peptide (referred to as 'c' in the reference) was retained through a disulfide bond in the mature β-subunit from a human fibroblast cell line. These authors determined that β_p was glycosylated and had an N-terminus initially at residue 45 (Ala) that was further trimmed to residue 48 (Val) (fig.1). They were unable to find a corresponding α_p sequence. In this report, we have extended their findings to the demonstration of the presence of both α_p and β_p in the mature Hex isozymes from human placenta and defined their N- and C-termini.

2. MATERIALS AND METHODS

2.1. *Separation of the polypeptide components of mature Hex A and B*

Samples containing ≅3 mg of purified placental Hex A and 0.5 mg of Hex B [2] were precipitated with 4 vol. of acetone, dried and dissolved in 250 μl of a buffer containing 0.625 M Tris/HCl, pH 6.8, and 3% SDS (Laemmli sample buffer [10]). Each sample was incubated at 60°C for 20 min, centrifuged and injected into high performance liquid chromatography sieve columns (two linked TSK-4000 molecular sieve columns, 7.5 mm × 300 mm; HPLC-M_r) [11], eluted at 0.5 ml/min with a 0.1 M phosphate buffer, pH 6.0, containing 0.1% SDS. Protein was detected by constantly monitoring the column effluent at A_{280}. The peaks (≅2 ml), containing the nonreduced β-subunit from Hex B or the nonreduced α- and β-subunits from Hex A, were collected by hand. Three portions of each peak (≅80 μg/portion) were retained for analysis by SDS-PAGE (below). The remaining samples from each isoenzyme were freeze-dried and dissolved in 250 μl of 50 mM DTT, incubated at 60°C for 20 min, and reapplied to the HPLC-M_r columns. The small protein peak eluting at 45 min, between the peak containing the β_a and β_b chains (41.5 min) and the V_{total} of the columns (60 min), corresponding to a molecular mass of <14.5 kDa was collected from each isozyme. The SDS was removed from the sample by ion-pair extraction [4]. The amino acid composition of the remaining Hex B component and 10% of the sample from Hex A was determined using the PICOTAG method [12]. The partial N-terminal sequence (the residues in ten sequenator 'turns') of the remaining sample from Hex A was determined as previously described [13].

2.2. *Polyacrylamide gel electrophoresis*

PAGE-SDS was carried out on a Biorad minigel apparatus using the method of Laemmli [10] with an 18% running gel. Two 'analytical' nonreduced samples from the HPLC-M_r (≅80 μg) were dialyzed against sample buffer diluted 1:4. The samples were freeze-dried and redissolved in 25 μl of water (nonreduced) or water containing 50 mM DTT (reduced), incubated at 60°C for 20 min, mixed with 5 μl of tracking dye in 75% glycerol, and applied to the SDS-slab gel. Proteins were stained with Coomassie blue or silver reagent [14].

The third Hex A derived analytical aliquot from the HPLC-M_r was treated with 2 U of glycopeptidase F and the third Hex B-derived sample was treated with 0.01 U of endoglycosidase H (Boehringer-Mannheim) as previously described [4,15]. The samples were then analyzed by SDS-PAGE.

3. RESULTS AND DISCUSSION

The separation of nonreduced and reduced samples of purified human placental Hex A and B by HPLC-M_r eluted with 0.1% SDS, resulted in the detection of a previously uncharacterized minor protein peak found exclusively in each of the reduced samples. This peak eluted in the extreme lower M_r range of the columns (fig.2). We had previously separated reduced and alkylated α- and β-chains by HPLC-M_r using 5 M guanidine-HCl and isolated the chains by ethanol precipitation [4,6]. It appears that these small peptides were either not precipitated by this procedure or that they eluted with the large V_{total} peak and were not collected.

Partial N-terminal sequence analysis of the protein contained in the new peaks from reduced samples of placental Hex A, confirmed the presence of the β_p sequence reported by Quon et al. [9]. However, the placental peptide sequence began at residue 50, Ala (fig.1), instead of 45 and/or 48 as reported for the fibroblast peptide. Surprisingly, the partial N-terminal sequence revealed a second peptide through the presence of a second residue in each sequenator turn. This additional peptide sequence corresponds exactly to residues 23 (Leu)–33 (Thr) in the deduced α-sequence and is designated α_p (fig.1). Thus both the α_p and the β_p sequences are retained in the mature Hex A through disulfide bonds, and while the β_p peptide undergoes the loss of 7 residues from its initial N-terminus at the signal peptide cleavage site, the N-terminus of the α_p chain is not further processed. (Since, unlike the mature β-subunit, there are no 'a' and 'b' polypeptides in the α-subunit, we have chosen a nomenclature utiliz-

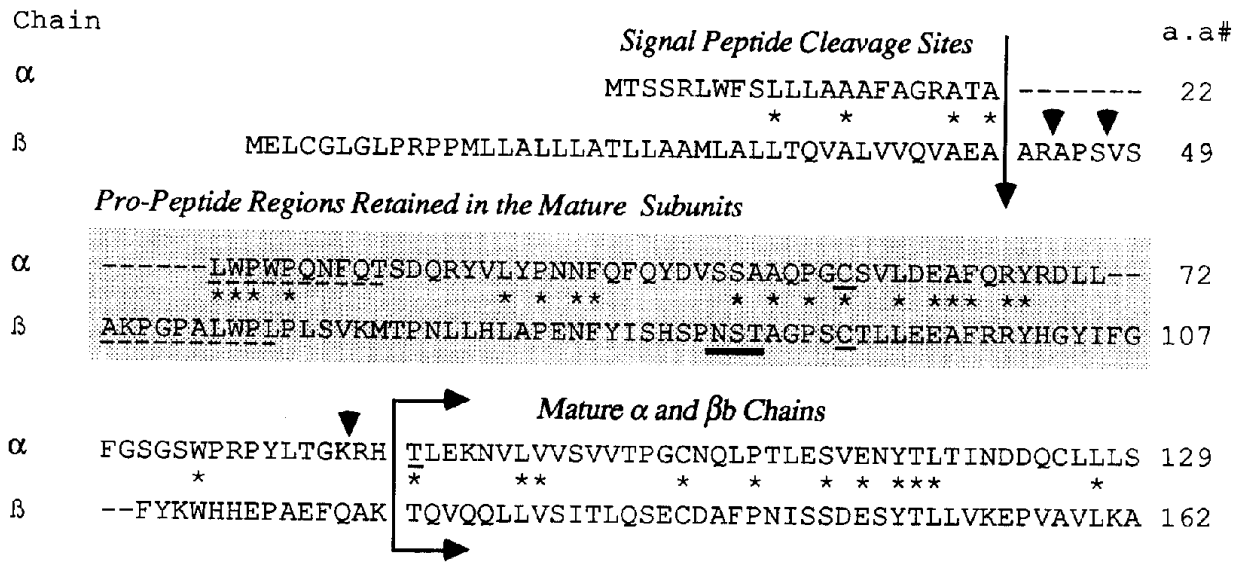

Fig.1. Deduced primary structures of the N-terminal section of the prepro-α and -β polypeptide chains of Hex [11]. The site of signal peptide cleavage is marked by a vertical arrow [5,8]. Arrowheads represent sites of hydrolysis giving rise to stable intermediates [8,9]. Shadowed sequences are the deduced α_p and β_p peptides. While the partial N-terminal sequences (dotted underline) were unequivocally identified in this report, the C-termini were calculated from amino acid composition data and could vary by ± 2 residues. The *N*-linked glycosylation site in the β_p peptide and the Cys residues in both peptides are underlined. Homologies between the α- and β-sequences are indicated by asterisks. Amino acids between residues 42–50 and 107–122 in the β-sequence, and residues 74–89 in the α are not present in the mature isozymes, presumably due to N-terminal and/or C-terminal processing (note the lack of sequence homology in these areas). Residue 90 (Leu) rather than 89 (Thr) was found to be the exclusive mature N-terminus in fibroblasts [8], while it and residue 89 were found in equal proportions as the N-terminal residue in mature α-chains from human placental Hex A [4].

ing the subscript 'p' for both the α and β propeptides, rather than the 'c' nomenclature used by Quon et al. [9]. Thus, the detailed structure of mature Hex A is $[(\alpha_p\alpha)(\beta_p\beta_b\beta_a)]$ and that of Hex B is $2(\beta_p\beta_b\beta_a)$. For simplicity we suggest the general use of the pro-isozymes structures, i.e. $\alpha\beta$ and $\beta\beta$, respectively.)

The amino acid analysis of purified β_p from Hex B and the α_p-β_p mixture from Hex A (not shown), indicated that both peptides undergo C-terminal processing. With the knowledge that residues 50 and 122 are the sites of the N-termini of the mature β_p and β_b chains, respectively, it was possible to deduce the most likely C-terminus of the β_p chain from the amino acid composition of the purified peptide in comparison to the total deduced sequence of this region of the protein deduced from the cDNA. This assigned the C-terminus of the β_p chain of Hex B to residue 107 (Gly, fig.2).

Further, the C-terminus of the α_p component of the α_p-β_p HPLC pool from Hex A was determined in relation to the His content of the sample. Since there is no His residue in the deduced α_p sequence, the amount of His in the mixed sample was directly proportional to the β_p component. After subtraction of the calculated β_p fraction from the amino acid composition of the pool, the ratios of the α_p amino acids were compared with the deduced α_p sequence. The peptide that best fit both sets of data, given an N-terminus at residue 23, would end at residue 74 (Gly, fig.2), aligning exactly with the putative C-terminus of β_p. Thus, α_p contains 52 amino acids while the β_p is composed of 58 residues.

Fig.3 A and B shows the SDS-PAGE pattern of reduced and nonreduced samples of Hex A (silver stained) and Hex B (Coomassie blue stained), respectively. Like the β_p peptide from fibroblasts [9] the placental peptide, from samples reduced prior to application, runs as a doublet (fig.3, β_p). However, it is converted to a 'fuzzy' singlet on treatment with either glycopeptidase F (fig.3 A, A-R + Glyco F) or endoglycosidase H (not shown). This indicates that the single putative glycosylation site in the β_p chain contains a high mannose-type oligosaccharide.

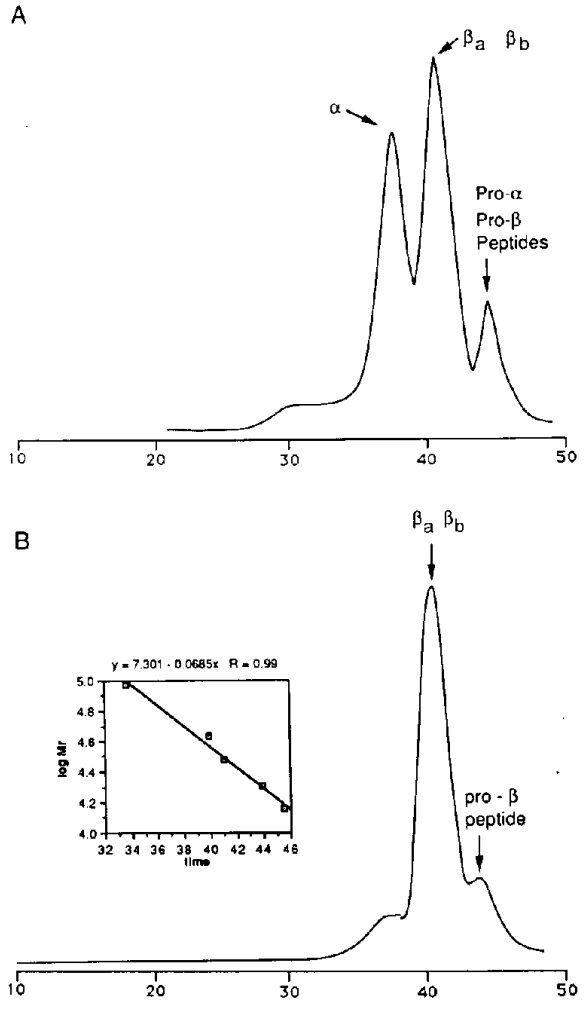

Fig.2. HPLC-M_r separation of reduced samples of Hex A (panel A) and Hex B (panel B). The identity of each protein (A_{280}) peak is indicated. A standard M_r curve is shown as an inset to panel B.

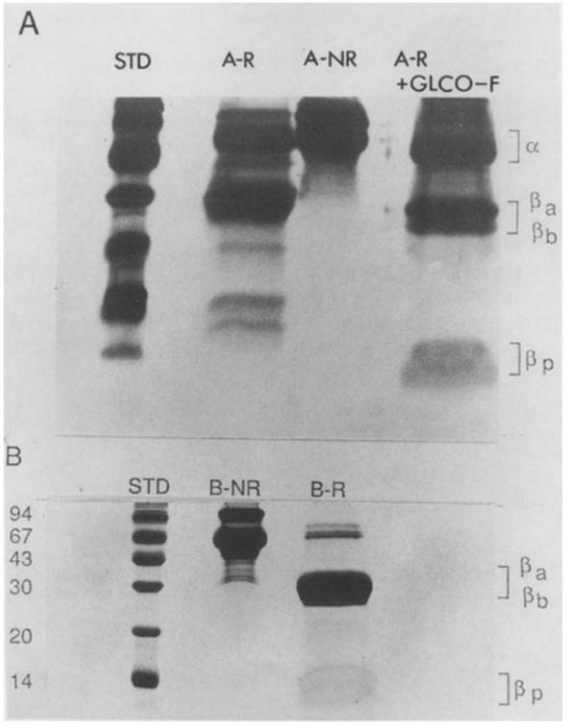

Fig.3. Molecular mass standards from the Pharmacia 'low molecular mass standard kit' (Std.) are shown at the side of each gel. (Panel A) SDS-PAGE separation of nonreduced Hex A (A-R), reduced Hex A (A-NR), and reduced Hex A treated with glycopeptidase F (A-R + Glyco F), ≅80 μg of protein/lane, visualized with silver reagent. The 'fuzzy' deglycosylated β_p band is indicated. (Panel B) SDS-PAGE of nonreduced Hex B (B-NR) and reduced Hex B (B-R), ≅80 μg of protein/lane, visualized with Coomassie blue. The glycosylated β_p doublet (consistent with that found in fibroblasts [9]) is indicated.

It is evident from fig.3 why this peptide has been previously overlooked. The β_p bands are barely visible in contrast to extremely pronounced mature β_a and β_b bands even when the mini-slab gel is overloaded (80 μg/lane). Even at this level of detection, a band corresponding to α_p in the Hex A sample cannot be seen (fig.3, panel A). Since this peptide was not detected in the gels produced by Quon et al. [9] who were visualizing labeled protein, it is likely that the α_p peptide cannot be fixed within the gel.

It is not known whether the oligosaccharide on the β_p peptide from fibroblasts or placenta is phosphorylated. As such, the possibility of a physiological role for β_p in the intracellular transport of the β-subunit cannot be entirely ruled out. Similarly, any biological function related to either of the protein domains in α_p or β_p in the native Hex isoenzymes remains to be determined.

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