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Differential glycosylation of N-POMC$^{1-77}$ regulates the production of γ_3-MSH by purified pro-opiomelanocortin converting enzyme

A possible mechanism for tissue-specific processing

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The amino terminus of bovine pro-opiomelanocortin (N-POMC$^{1-77}$) is partially processed in the intermediate lobe of the pituitary to N-POMC$^{1-49}$ and lys-γ_3-melanotropin. Two pools of N-POMC$^{1-77}$ were isolated which were differentially glycosylated at threonine^{45}, while N-POMC$^{1-49}$ isolated from bovine intermediate lobe extracts existed in a non-glycosylated form. This suggested that differential *O*-linked glycosylation of N-POMC$^{1-77}$ may regulate cleavage at the Arg^{49}–Lys^{50} processing site. We tested this hypothesis by incubating N-POMC$^{1-77}$ glycoforms with purified pro-opiomelanocortin converting enzyme. Only non-*O*-glycosylated N-POMC$^{1-77}$ and *O*-glycosylated N-POMC$^{1-77}$ with truncated oligosaccharide sidechains were sensitive to cleavage and generated predominantly lys-γ_3-melanotropin, identified by high-performance liquid chromatography. These data provide the first functional evidence to support a role for differential *O*-linked glycosylation in the regulation of the processing of the N-terminus of bovine POMC.

Neuropeptide; Maturation; Pro-opiomelanocortin processing; *O*-Linked glycosylation

1. INTRODUCTION

Most endocrine and neuroendocrine peptides are synthesized as larger precursors [1–3]. In many cases, the precursor protein contains multiple copies of a biologically active peptide, such as the enkephalin peptides in proenkephalin [4], or several unique peptides with distinct, although often synergistic actions as is seen in the pro-opiomelanocortin precursor (POMC, [5]). These biologically active peptides are often flanked by pairs of basic amino acids which are thought to act as recognition sites for cleavage by endoproteases. Characterization of biologically active peptides in the central and peripheral nervous systems and other peripheral tissues has indicated that not all paired basic residues are cleaved. Furthermore, it has been shown that the same pro-protein precursor can be processed at different pairs of basic residues in different areas of the brain or in peripheral tissues to release different peptide products [6–9]. To better understand the mechanisms involved in alternate processing of pro-hormones/neuropeptides, we have chosen to study the processing of the ACTH/endorphin precursor in the pituitary gland. In the anterior lobe of the pituitary gland, POMC is processed at paired basic residues predominantly to ACTH and β-LPH, while in the intermediate lobe and brain these two peptides are further processed to α-MSH, CLIP, γ-LPH and the endorphins [10]. The N-terminus of POMC is also processed differently in the two lobes. Isolation and composition analysis of the N-terminal peptides in the bovine pituitary demonstrated that N-POMC$^{1-77}$, N-POMC$^{1-49}$ and lys-γ_3-MSH were the major peptides in the intermediate lobe while the anterior lobe contained mainly N-POMC$^{1-77}$ [11–15]. A small proportion of lys-γ_3-MSH was found to be processed further to lys-γ_1-MSH in the intermediate lobe [13,16]. Detailed analysis of the N-terminal peptides revealed two forms of N-POMC$^{1-77}$ in the intermediate lobe; the major form (80%) was glycosylated at threonine^{45} (*O*-linked) and asparagine^{65} (*N*-linked) while the minor form was glycosylated only at asparagine^{65} [12]. However, only a single species of

Abbreviations: POMC, pro-opiomelanocortin; ACTH, adrenocorticotropin; MSH, melanocyte stimulating hormone; CLIP, corticotropin-like intermediate peptide; LPH, lipotropin; b, bovine; HPLC, high-performance liquid chromatography

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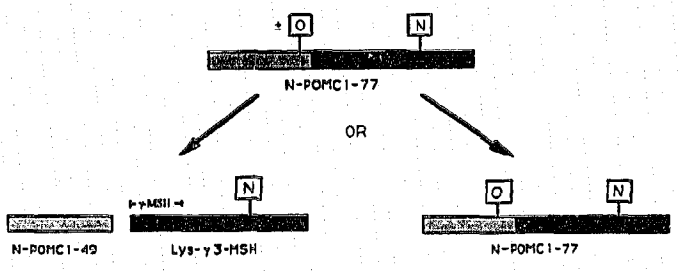

Fig. 1. Differential *O*-linked glycosylation of N-POMC$^{1-77}$ at threonine^{45} regulates the generation of N-POMC$^{1-49}$ and lys-γ_3-MSH.

N-POMC$^{1-49}$ was isolated which was unglycosylated [11] (Fig. 1). This observation has recently been confirmed by others [17]. This suggested that only N-POMC$^{1-77}$ which lacked the *O*-linked glycosylation of threonine^{45} was capable of being processed to N-POMC$^{1-49}$ and various forms of γ-MSH [18]. In the present study, we tested this hypothesis by incubating enriched species of principally glycosylated or non-glycosylated N-POMC$^{1-77}$ with purified pro-opiomelanocortin converting enzyme which we have previously shown to cleave N-POMC$^{1-77}$ (as a mixed glycoform substrate) at paired basic residues [19]. We present data to support a role for *O*-linked glycosylation in the regulation of cleavage of pro-opiomelanocortin.

2. MATERIALS AND METHODS

2.1. *Isolation of O-linked and non-O-linked N-POMC*$^{1-77}$

N-POMC$^{1-77}$ was prepared from bovine posterior pituitaries essentially as described previously using a combination of reversed-phase and ion-exchange batch extraction procedures followed by reversed-phase HPLC [12,13]. While homogeneous in terms of peptide content, the chromatographic behavior of this material clearly indicated a considerable degree of heterogeneity. Analysis of peptides derived from N-POMC$^{1-77}$ following hydrolysis with trypsin revealed that this heterogeneity was due to the nature and extent of glycosylation. N-POMC$^{1-77}$ was completely *N*-glycosylated at asparagine residue 65 as shown by analysis of the tryptic fragment 65–77. Analysis of the tryptic fragment 23–49 which bears the *O*-linked site at residue 45 demonstrated two forms of this peptide, either glycosylated or non-glycosylated at threonine residue 45. The N-POMC$^{1-77}$ glycoforms were further separated by reversed-phase HPLC on a μBondapak C_{18} column which was eluted with a linear gradient over 2 h from 8–48% acetonitrile containing 0.01 M ammonium bicarbonate pH 8 at a flow rate of 1.5 ml per min.

2.2. *Analysis of neutral and sialic acid sugars*

The *O*-linked glycoforms of N-POMC$^{1-77}$ were trypsinized and the tryptic fragments 23–49 were analyzed for sialic acid, and neutral and amino sugars after hydrolysis with constant boiling 6 M HCl or 2 M trifluoroacetic acid respectively, a HPAE-PAD high pH anion-exchange system with pulsed amperometric detection (Dionex Corp, Sunnyvale, CA) was used to separate and quantitate the various sugars [20].

2.3. *Enzyme incubations*

Pro-opiomelanocortin converting enzyme was purified from bovine intermediate lobe secretory vesicles as described previously [19]. 1 nmol of either the *O*-glycosylated or non-*O*-glycosylated substrate was digested with ~100 ng of purified pro-opiomelanocortin converting enzyme in 0.1 M sodium citrate/HCl, pH 4.5 in a final volume of 200 μl of 37°C. At various time intervals (see results), 20 μl aliquots were removed and acidified with an equal volume of 50 mM acetic acid. Samples were quantitated by radioimmunoassay, either directly using antibody MC7 raised against γ_1-MSH and which recognized γ_3-MSH and lys-γ_3-MSH [19], or following separation of the digestion products by reversed phase HPLC. Samples were separated on a Bio-Rad Hi-Pore RP-318 column (5 × 250 mm) using a gradient from 0–20% Solvent B in 10 min and then 20–45% Solvent B in 80 min at a flow rate of 1 ml/min. Solvent A was 0.1% trifluoroacetic acid and Solvent B was 80% acetonitrile in 0.1% trifluoroacetic acid. 0.5 ml fractions were collected for radioimmunoassay.

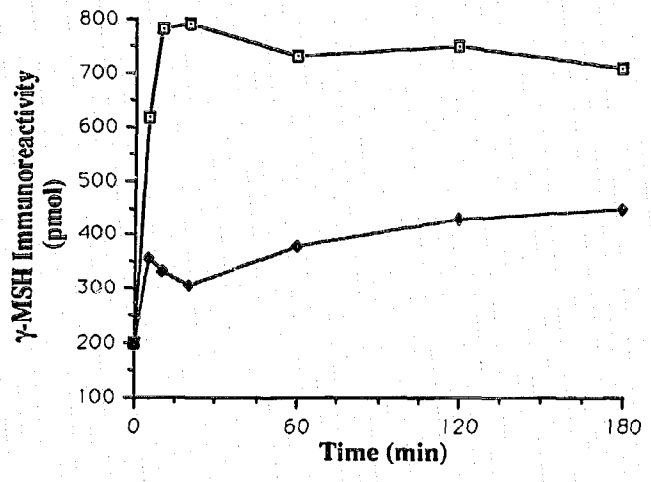

Fig. 2. Generation of γ-MSH immunoreactivity following incubation of N-POMC$^{1-77}$ enriched in either the *O*-linked (–◆–) or non-*O*-linked (□) threonine^{45} glycoform with pro-opiomelanocortin converting enzyme. Each time point represents the total amount of γ-MSH immunoreactivity in the original incubation volume (200 μl).

3. RESULTS AND DISCUSSION

Previous studies had demonstrated that the N-terminal fragment of bovine pro-opiomelanocortin (N-POMC$^{1-77}$) from the neurointermediate lobe of the pituitary was differentially glycosylated at threonine residue 45 [12]. Using reverse-phase HPLC (see section 2), purified N-POMC$^{1-77}$ was further resolved as a broad peak over approximately 45 fractions. The leading edge of this peak (i.e. the first 15 fractions, Pool A) contained N-POMC$^{1-77}$ which was essentially 100% *O*-glycosylated at threonine^{45} and the trailing edge (i.e. the last 15 fractions, Pool B) contained N-POMC$^{1-77}$ which contained ~20% *O*-linked oligosaccharide. The extent of *O*-linked glycosylation was determined by HPLC separation and analysis of the glucosamine and galactosamine content in the tryptic fragment (residues 23–49) which contained the *O*-linked glycosylation site. Resolution of the *O*-linked glycosylated tryptic peptide (rather than intact N-POMC$^{1-77}$) into several forms by HPLC suggested considerable heterogeneity within the oligosaccharide structure of these two pools. While the low levels of peptide made analysis difficult, we estimate

that approximately 80% of the *O*-glycosylated N-POMC$^{1-77}$ in pool A contained a simple oligosaccharide structure composed of galactosamine and glucosamine. The remaining 20% was composed of truncated oligosaccharide structures. The presence of truncated *O*-linked oligosaccharide structures in pool A was substantiated as follows. The leading and trailing edges of the u.v.peak containing the 23–49 tryptic fragments from pool A were subjected to neutral, amino and sialic acid sugar anaysis. The following compositions were found (mol sugar/mol peptide): leading edge: Gal Nac 2.5, Glc Nac 1.7, Gal 2.6, Man 4.2, Sialic acid 0.8, trailing edge: Gal Nac 1.5, Glc Nac 1.1, Gal 1.7, Man 3.2, Sialic acid 0.3, indicating the presence of N-POMC$^{1-77}$ forms with truncated *O*-linked oligosaccarides. These truncated structures were probably generated during the initial tissue extraction by acid hydrolysis of glycosidic linkages. The fine structural carbohydrate analyses are not critical to the present study and will be presented elsewhere (A. Dell and H.P.J. Bennett, in preparation), but the somewhat simplistic divisions of the *O*-linked N-POMC$^{1-77}$ glycoforms are necessary for interpretation of the processing results. Thus, for incubations with purified pro-opiomelanocortin converting enzyme we had two substrate pools: Pool A consisted of 100% *O*-glycosylated N-POMC$^{1-77}$ of which 20% was comprised of molecules with truncated sugars; and pool B consisted of 80% non-*O*-glycosylated N-POMC$^{1-77}$. N-POMC$^{1-77}$ glycoform substrates isolated from parallel purifications were used for incubations with pro-opiomelanocortin converting enzyme and for trypsinization followed by amino and neutral sugar analysis.

Incubation of the non-*O*-glycosylated enriched pool B N-POMC$^{1-77}$ with bovine pro-opiomelanocortin converting enzyme resulted in the generation of γ-MSH immunoreactive material (Fig. 2). The initial production of γ-MSH immunoreactive material was very rapid as demonstrated by the 'zero' time point which more accurately represents a ~5 s incubation. By 20 min, γ-MSH immunoreactivity had reached a maximum of 790 pmol. This represented 79% of the total moles of added substrate. In a second experiment, 81% (812 pmol) of pool B N-POMC$^{1-77}$ was converted to γ-MSH immunoreactive material. As pool B N-POMC$^{1-77}$ contained ~20% *O*-glycosylated N-POMC$^{1-77}$ these results are consistent with the hypothesis that pro-opiomelanocortin converting enzyme is unable to cleave *O*-glycosylated N-POMC$^{1-77}$. Incubation of the *O*-glycosylated pool A N-POMC$^{1-77}$ substrate with pro-opiomelanocortin converting enzyme resulted in the generation of γ-MSH immunoreactive material. After a 20 min incubation, the amount of γ-MSH immunoreactivity generated from *O*-glycosylated enriched N-POMC$^{1-77}$ was ~305 pmol (Fig. 2) and ~195 pmol (data not shown) in two separate experiments. As pool A N-POMC$^{1-77}$ was shown to be 100% *O*-glycosylated, our hypothesis would predict that there should be no γ-MSH immunoreactivity generated by pro-opiomelanocortin converting enzyme. However, pool A contains a heterogeneous mixture of *O*-linked N-POMC$^{1-77}$ glycoforms and we attribute the conversion of between 20–30% of the substrate to the presence of truncated *O*-linked oligosaccharide structures which represented approximately 20% of this pool.

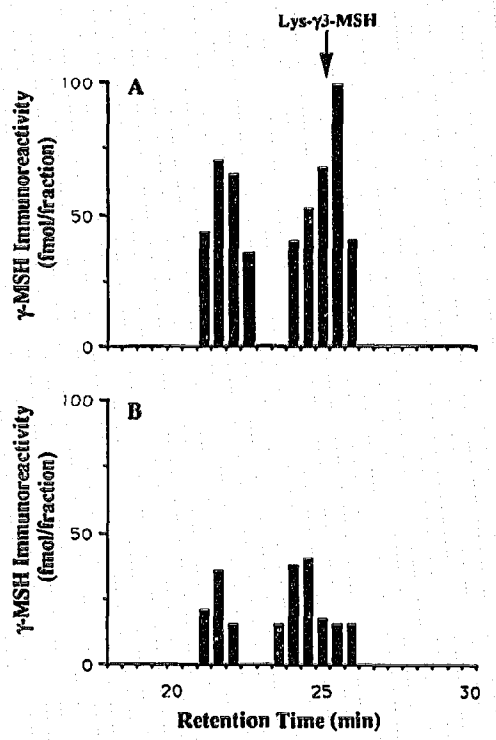

Fig. 3. HPLC identification of γ-MSH immunoreactive products generated from bovine N-POMC$^{1-77}$ by purified pro-opiomelanocortin converting enzyme. HPLC profiles of α-MSH immunoreactive products generated from *O*-glycosylated (A) and non-*O*-glycosylated (B) N-POMC$^{1-77}$. The elution position of lys-γ_3-MSH is shown.

Identification of the γ-MSH immunoreactive products was determined following HPLC. Both pools of N-POMC$^{1-77}$ were processed to identical products. The major immunoreactive peak had a retention time identical to human lys-γ_3-MSH standard (Fig. 3). An earlier eluting peak, with a retention time of 22 min may represent an oxidized lys-γ_3-MSH species. γ-MSH immunoreactive material with a similar retention time, eluting just before the human lys-γ_3-MSH peptide standard has been seen in acid extracts of rat pituitary (Estivariz, unpublished observation) and following incubations of the mixed glycoform substrate with pro-opiomelanocortin converting enzyme [21]. No immunoreactivity was seen with a retention time which corresponded to γ_2-MSH in agreement with our earlier work [19].

These data strongly support a role of *O*-linked glycosylation in the regulation of cleavage of the N-terminus of the prohormone precursor, pro-opiomelanocortin, in bovine intermediate pituitary. The results suggest a more complicated mechanism than our initial

hypothesis, with the degree of *O*-linked glycosylation appearing to affect the processing of N-POMC$^{1-77}$ to γ_3-MSH. However, as the truncated *O*-linked glycosylated forms were probably generated during the purification of the substrate, the biological mechanism, in vivo, may still conform with our hypothesis. The existence of glycosylated and non-glycosylated forms of N-POMC$^{1-74}$ in rat and mouse intermediate lobe along with partial cleavage of this fragment to a non-glycosylated N-POMC$^{1-49}$ suggests a similar mechanism may be operating in these species [18]. The precise mechanism by which the *O*-linked glycosylation at threonine^{45} blocks processing at the Arg^{49}–Lys^{50} bond is unclear. Negatively charged terminal sialic acid residues may interact with positively charged basic residues to form a stearic block of the cleavage site. A stearic mechanism is supported by the observation of processing of N-POMC$^{1-77}$ which bears truncated oligosaccharide structures. Interestingly, the Arg^{49}–Lys^{50} bond remains sensitive to cleavage by trypsin which suggests that the substrate binding pocket for pro-opiomelanocortin converting enzyme must be significantly different from trypsin-like proteases.

In summary, the findings indicate that *O*-linked glycosylation appears to be a mechanism underlying tissue-specific processing of pro-opiomelanocortin in the intermediate lobe and possibly also the anterior lobe of the pituitary. It will be interesting to see whether differential *O*-linked glycosylation represents a mechanism for effecting tissue- and brain-specific processing of other prohormones and proneuropeptides.

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