THE ROLE OF GLYCOSYLATION ON THE BIOSYNTHESIS, DEGRADATION, AND SECRETION OF THE ACTH-β-LIPOTROPIN COMMON PRECURSOR AND ITS PEPTIDE PRODUCTS

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1. Introduction

The neurointermediate lobe of the African clawed frog *Xenopus laevis* synthesizes a common precursor to adrenocorticotropin (ACTH) and β-lipotropin (βLPH), which is also processed to α-melanocyte stimulating hormone (αMSH) and β-endorphin, the opiate-like peptide ([1], Y.P.L., in preparation). This precursor is a glycoprotein with an R_F of 0.46 (relative to cytochrome *c*) on acid–urea gels and mol. wt 32 000 on sodium dodecyl sulfate (SDS)–gels (Y.P.L., H.G., in preparation), and appears to be similar to that reported for the AtT-20 mouse pituitary tumor line [2–4]. Beeley [5] has proposed that the addition of carbohydrates at the β-turns of proteins would result in the masking of the turn conformations, and hence, could be an important feature in protecting the molecule from proteolysis. Glycosylation of secretory proteins has also been suggested to be a prerequisite for secretion, e.g., for thyroglobulin [6,7]. We have therefore examined the role of the carbohydrate on the ACTH–βLPH hormone precursor with respect to these two issues, using the drug, tunicamycin, an inhibitor of glycosylation. In this short communication, we present data in support of the hypothesis [5] showing that the lack of glycosylation of the ACTH–βLPH common precursor resulted in its rapid degradation, and formation of atypical processed peptides. The secretion of the processed peptides appeared to be unaffected by tunicamycin.

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2. Materials and methods

2.1. *Animals*

Adult African clawed frogs *(Xenopus laevis)* 40–70 g, were purchased from NASCO Biological Supplies, Fort Atkinson, WI and maintained in a black plastic aquarium at 22°C with 12 h dark–light cycles, for 15–20 days before use. This dark adaptation was to enhance the synthesis of ACTH and MSH in the neurointermediate lobe [1].

2.2. *Incubation of neurointermediate lobes in radioactive arginine*

Neurointermediate lobes of the pituitary were dissected from the animals and preincubated at 22°C in amphibian ringer (NaCl 112 mM, KCl 2 mM, $CaCl_2$ 2 mM, Hepes 15 mM, glucose 5 mg/ml; bovine serum albumin 1 mg/ml, ascorbic acid 1 mg/l, pH 7.35) for 6 h and then 'pulse' incubated for 30 min in amphibian ringer containing 17.5 μM [^{3}H]arginine, (New England Nuclear, Boston, MA; spec. act. = 28.5 Ci/mmol). After the pulse, the labeled lobes were either chased in amphibian ringer with 1 mM unlabeled arginine, for varying times and/or homogenized in 0.1N HCl. The chase medium was collected and HCl added to 0.1 N final conc. Proteins in the tissue homogenates and medium were then pre-

cipitated with cold 10% trichloroacetic acid (TCA) and the TCA precipitates analysed by acid–urea gel electrophoresis in [8,9].

2.3. *Treatment of the neurointermediate lobes with tunicamycin*

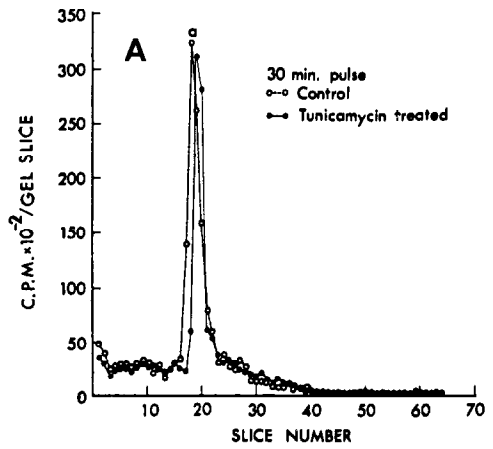
Neurointermediate lobes were preincubated for 6 h (minimum time for maximal effect of tunicamycin), pulse incubated, and chased as above except that 5 μg/ml of tunicamycin (a generous gift from Dr G. Tamura, Univ. Tokyo) was included throughout the procedure. Tunicamycin inhibits glycosylation of proteins by specifically inhibiting the synthesis of those sugar side chains which have *N*-acetylglucosamine linked to aspargine residues of glycoproteins, by preventing the formation of *N*-acetylglucosaminyl-dolichol phosphate intermediates [10–12]. Control experiments measuring [^{3}H]arginine and [^{3}H]glucosamine incorporation show that with tunicamycin treatment, the common precursor to ACTH and β-lipotropin (peak 'a' in fig.1A) was not inhibited in its synthesis, but was inhibited in its glycosylation by 93% ± 2.8. Tissues treated with tunicamycin were homogenized and analysed by acid–urea gel electrophoresis as described above.

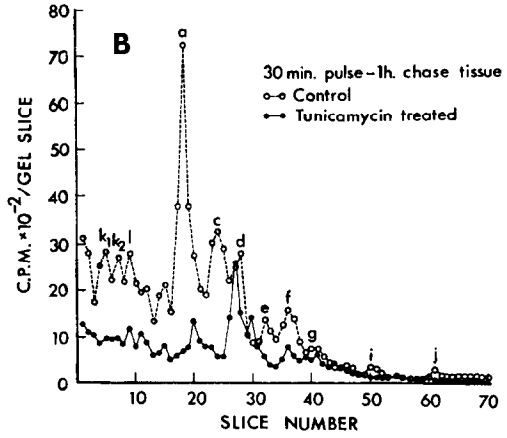
3. Results

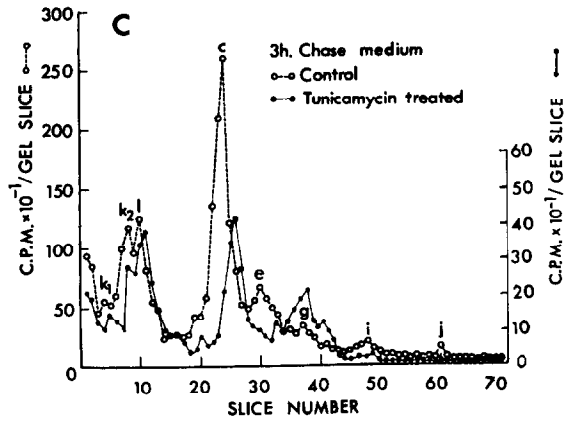
Figure 1A shows the effect of tunicamycin on the synthesis of the ACTH–βLPH common precursor shown as peak 'a' on the control acid–urea gel profile (open circles). Synthesis of the precursor is compared in untreated (control, open circles) and tunicamycin treated lobes (closed circles), after a 30 min pulse labeling with [^{3}H]arginine. The results suggest that there is virtually no inhibition of precursor (peak 'a') synthesis after tunicamycin treatment except that the unglycosylated form migrated faster on the gel. The radioactivity in the unglycosylated peak 'a' was found to be 92.5% ± 12 (n = 3) of control value. Since the acid–urea gel separates primarily on the basis of size, the faster mobility of peak 'a' with tunicamycin treatment is consistent with the expectation that the unglycosylated precursor has a lower molecular weight without the sugars attached. The unglycosylated peak 'a' was otherwise indistinguishable from the normally glycosylated peak 'a' with respect to immunoreactivity with ACTH and β endorphin antisera (Y.P.L., H.G., in preparation).

After a 30 min pulse followed by a 1 h chase, (fig.1B, open circles) the precursor peak 'a' underwent processing to 3 forms of immunoreactive ACTH: two glycoproteins (peak 'c', R_F 0.65, mol. wt 21 000, and peak 'e', R_F 0.83, mol. wt 13 000) and peptide peak 'i' (R_F 1.3, mol. wt 4300) which coran with $ACTH_{1-39}$. Two other peptides, immunoreactive αMSH (peak 'j') and immunoreactive βLPH (peak 'g') were also processed from the precursor (Y.P.L., in preparation). While in the control lobes (fig.1B, open circles) there was a considerable amount of peak 'a', after 1 h chase, in the tunicamycin-treated lobes, there was a significant disappearance of peak 'a' without the conservation of radioactivity in the tissue in the form of processed product peaks (fig.1B, closed circles). However, some processing of the precursor which appeared to escape degradation did occur, yielding a peak which migrated slightly faster than peak 'c' in acid–urea gels, but the presence of other processed products (peaks 'e', 'g', 'i' and 'j') were not detected. The major processed product in the tunicamycin treated lobes, i.e., the peak that migrated ahead of peak 'c' in the control profile (open circles) may represent an unglycosylated form of peak 'c'.

Figure 1C (open circles) shows the peptides normally released by the neurointermediate lobe. The 3 forms of immunoreactive ACTH (peaks 'c', 'e' and 'i') as well as αMSH (peak 'j') and βLPH (peak 'g') were released. In the presence of tunicamycin, atypical peptides (a presumed unglycosylated peak 'c' and a peak comigrating with peak 'g' in the control lobes), appear to be released (fig.1C, closed circles). Peaks 'k_1', 'k_2' and 'l' (fig.1B,1C, open circles) are other large glycoproteins synthesized and released by the neurointermediate lobe and are not related to the processing of the common precursor peak 'a' (Y.P.L., H.G., in preparation). Note also in the release profile (fig.1C) the difference in ratio of peak 'c' relative to peaks 'k_1', 'k_2' and 'l' in the control (open circles) and tunicamycin treated lobes (closed circles) suggesting the instability of the presumed unglycosylated form of peak 'c'. We have shown that the release of peptides is normally under inhibitory control by dopamine [1]. The pathway of release of the atypical peptides in tunicamycin treated lobes (seen in fig.1C) is also inhibited by dopamine (data not shown). Thus, it







appears that the lack of glycosylation of precursor has no effect on the secretion mechanism per se.

4. Discussion

Using the drug tunicamycin, which inhibits the lipid-linked pathway for the core glycosylation of glycoproteins [10–12], it was shown that the biosynthesis of the unglycosylated common precursor to ACTH and βLPH was not inhibited (fig.1A). However, during the chase period, it was found that there was an enhanced proteolysis of the unglycosylated precursor in comparison to the normally glycosylated form in control tissue (fig.1B). Moreover, the unglycosylated precursor which did escape degradation was processed to a set of atypical peptides which was secreted by the lobe (fig.1B,1C) via a dopamine-inhibited pathway similar to normal lobes. This data

Fig.1. Acid–urea gel labeling profiles of TCA-precipitable peptides synthesized and released by neurointermediate lobes after pulse labeling in the presence and absence of tunicamycin.

(A) Lobes were preincubated for 6 h in amphibian ringer with and without tunicamycin and then pulse labeled for 30 min in [^{3}H]arginine ± tunicamycin. (o----o) Without tunicamycin (control); (•----•) with tunicamycin treatment. The ACTH–βLPH precursor is denoted by peak 'a'.

(B) Lobes were preincubated for 6 h in amphibian ringer (± tunicamycin), pulse-labeled for 30 min in [^{3}H]arginine (± tunicamycin) and then chased for 1 h (± tunicamycin). (o----o) Without tunicamyin (control), (•----•) with tunicamycin treatment. Peak 'a' is the ACTH–βLPH precursor and peaks 'c–g', 'i' and 'j' are processed peptides. Peaks 'c', 'e' and 'i' are immunoreactive forms of ACTH, peak 'g' has been identified as βLPH and peak 'j' as αMSH (Y.P.L. in preparation, see text).

(C) Lobes were preincubated for 6 h in amphibian ringer (± tunicamycin), pulse-labeled for 30 min in [^{3}H]arginine (± tunicamycin) then chased for 3 h (± tunicamycin). The chase media were collected for analysis of secreted peptides. 3 h was the optimum release time determined [1]. (o----o) Without tunicamycin; (•----•) with tunicamycin treatment. The processed peptides peaks 'c', 'e', 'g', 'i' and 'j' are released in control lobes while the atypical peptides in tunicamycin treated lobes are also released. In (A–C), the ordinate shows the cpm/gel slice and the abscissa the slice number.

represents the first demonstration that the glycosylation of a prohormone appears to be important for its protection from non-specific proteolysis, and provides experimental support for Beeley's hypothesis [5]. Other studies have shown that when membrane proteins such as CSP (fibrinectin) and the hemagglutinin precursor of influenza virus are prevented from glycosylation by tunicamycin, they are also increasingly degraded by intracellular proteases [13,14]. It is possible that the carbohydrate may also confer specific conformational properties to the ACTH–βLPH precursor so as to direct programmed limited proteolysis (processing).

The secretion of other glycoproteins CSP (fibrinectin) and procollagen does not appear to be inhibited by tunicamycin [13], consistent with the observations of this study. Thus contrary to previous proposals [6,7], glycosylation of secretory glycoproteins is not a prerequisite for their secretion.

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