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Production of pro-opiomelanocortin (POMC) by a vaccinia virus transient expression system and in vitro processing of the expressed prohormone by POMC-converting enzyme

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Pro-opiomelanocortin (POMC) was expressed in CV-1 (green monkey kidney) cells using a vaccinia virus transient expression system [(1986) Proc. Natl. Acad. Sci. USA 83, 8122]. The system involved infection of cells with a recombinant vaccinia virus carrying the T7 RNA polymerase gene and transfection with a plasmid containing the mouse POMC sequence flanked by the T7 RNA polymerase promoter at its 5'-end and the T7 RNA polymerase terminator at its 3'-end. Assay of the medium from transfected cells showed that 1–2 μg of immunoreactive ACTH was produced/10^6 cells. Analysis of the same medium by SDS-PAGE/Western blots revealed a band of 30–36 kDa, which was immunostained with both ACTH and β-endorphin antisera. Labeling the transfected cells with [^{3}H]Arg, followed by immunoprecipitation and SDS-PAGE showed the synthesis of a major peak of POMC, 33 kDa. Purified [^{3}H]POMC expressed by CV-1 cells was cleaved in vitro by bovine intermediate lobe secretory vesicle pro-opiomelanocortin-converting enzyme to ACTH intermediates (19–25 kDa), β-lipotropin and β-endorphin. Thus, this work has demonstrated a technique for expressing microgram quantities of prohormones in mammalian cells, suitable for use as substrates for prohormone-converting enzymes in vitro.

Adrenocorticotropin/endorphin prohormone; Prohormone processing; Proopiomelanocortin-converting enzyme; (CV-1 cell)

1. INTRODUCTION

Peptide hormones (e.g. insulin, vasopressin and ACTH) are synthesized from larger precursors known as prohormones [1–3]. These precursors are generally cleaved at pairs of basic amino acids and to a lesser extent at single basic residues, to yield the respective biologically active hormones [1–3]. The processing involves several proteolytic enzymes: endoproteases which cleave at paired or single basic residues, and exopeptidases (carboxypeptidase B-like and aminopeptidase B-like enzymes) which remove basic amino acids from the N- and C-terminus of peptides liberated from the prohormone by the endoproteases. Recent studies have suggested that in addition to having pairs of basic residues as signals for processing, the conformation of the prohormone may also contribute to the specificity of cleavage [1,2,4]. However, little is known about the conformation of prohormones, largely due to the lack of sufficient quantities of these molecules necessary for X-ray diffraction studies. Progress in testing the ability of various putative processing enzymes to cleave intact prohormones has also been hindered by the

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Abbreviations: POMC, pro-opiomelanocortin; ACTH, adrenocorticotropin; α-MSH, α-melanotropin or N^{α}-acetyl-ACTH$_{1-13}$ NH$_2$; LPH, lipotropin; β-END, β-endorphin; γ-MSH, γ-melanotropin; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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unavailability of radiolabeled prohormone substrates. Attempts have been made to produce large quantities of prohormones using bacterial expression systems [5]. However, although these systems offer high yields, the prohormones generated may not be appropriately post-translationally modified, e.g. glycosylated. Furthermore, the prohormones are often partially degraded within the bacterial cell and are difficult to purify.

In this communication, we report the use of a previously described vaccinia virus transient expression system [6] to produce the ACTH/endorphin prohormone, pro-opiomelanocortin, in CV-1 cells, an African green monkey kidney cell line. This expression system overcomes the problems encountered in bacterial systems. We further show that the expressed prohormone, which was secreted into the medium was easily recovered in pure form and was processed by bovine intermediate lobe secretory vesicle POMC-converting enzyme [7], to various hormone products. This enzyme has previously been shown to be an aspartic protease with an acidic pH optimum and specific for paired basic residues of POMC [7–10].

2. MATERIALS AND METHODS

2.1. *Plasmid construction*

Restriction enzymes were purchased from Bethesda Research Laboratories (Bethesda, MD) and New England Biolabs (Beverly, MA) and were used according to instructions provided. The Klenow fragment of DNA polymerase I and bacterial alkaline phosphatase were obtained from New England Biolabs and Bethesda Research Laboratories, respectively. A 946 bp cDNA fragment containing the entire coding region of the mouse pro-opiomelanocortin (POMC) was isolated from pmcPOMC (a generous gift from E. Herbert, Portland, OR) and inserted into the pAR2529 vector such that the POMC sequence was flanked by the T7 RNA polymerase promoter at its 5′-end, and the T7 RNA polymerase terminator at its 3′-end. The resulting plasmid was called pKA2. A control plasmid containing the POMC sequence oppositely oriented with respect to the T7 was called pKA5. Plasmids were prepared by centrifugation in CsCl and ethidium bromide as described elsewhere [11].

2.2. *Cells*

CV-1 (green monkey kidney) cells were grown in Dulbecco's minimal essential medium (DMEM) with 10% fetal calf serum (FCS, purchased from Gibco, Grand Island, NY) under 10% CO_2, and used when the cells were 50–80% confluent.

2.3. *Transient expression of POMC in CV-1 cells*

CV-1 cells were plated at 5×10^5 cells per 25 cm^2 flask and incubated overnight in 5 ml of MEM/10% FCS. Medium was removed, and cells were inoculated with 1 ml of viral suspension at a multiplicity of infection (m.o.i.) of 30 for 30 min at 37°C with occasional rocking. The virus used was either wild type vaccinia or vTF7-3, a vaccinia recombinant containing the T7 RNA polymerase gene [6]. The viral inoculum was removed, cells were washed with phosphate buffered saline, and 1 ml of $CaPO_4$ precipitate containing 10 μg DNA (plasmid construct pKA2 or pKA5) was layered onto the cells. The cells were then incubated at room temperature for 30 min with occasional rocking. At 1 h post-inoculation, MEM/2.5% FCS (4 ml) was added and the cells were incubated at 37°C. At 5 h post-infection, the medium was removed, and 2 ml of MEM/2.5% FCS were added to the cells, and incubation continued overnight for 12 to 16 h at 37°C. By that time, the cells had lysed, and the medium was collected, centrifuged at $12000 \times g$ for 5 min and the supernatant analyzed for immunoreactive ACTH and synthesis of POMC by radioimmunoassay and Western blots.

2.4. *Radioimmunoassay of ACTH*

The supernatant of the medium of transfected CV-1 cells (see section 2.3) was radioimmunoassayed for ACTH as described previously [12], except that the ACTH antiserum used was DP4 (final dilution 1:90000). This antiserum binds to intact POMC and ACTH-related products and does not cross-react with α-MSH or ACTH(1–10). The primary antigenic site of the antibody is directed to the region of ACTH between residues 11–39.

2.5. *Western blot identification of POMC synthesized by transfected CV-1 cells*

The supernatant of medium from CV-1 cells (section 2.3) was treated with cold 10% trichloroacetic acid (TCA) for 1 h at 4°C. The precipitated proteins were air dried, washed 5 times with acetone and dried again. The precipitate was then resuspended in 0.125 M Tris-HCl-SDS buffer (pH 6.8) containing 0.1% β-mercaptoethanol and 0.002% bromophenol blue and electrophoresed in a 13.5% polyacrylamide slab gel (SDS-PAGE) using the Laemmli procedure [13]. Electroblotting and immunostaining procedures (Western blot) of the separated proteins were as described previously [12]. The antisera used for the Western blots were DP4 (final dilution 1:750) for ACTH and DP3 (final dilution 1:750) for β-endorphin. The DP3 antiserum is directed towards the C-terminus of β-endorphin and binds to intact POMC as well as to β-lipotropin and β-endorphin.

2.6. *Radiolabeling and purification of [^{3}H]POMC from transfected CV-1 cells*

CV-1 cells plated at 10^6 cells/75 cm^2 flask were infected two days later with the recombinant vaccinia virus vTF7-3 and then transfected with the plasmid pKA2 as described in section 2.3 with minor modifications as follows. At 5 h post-infection, 4 ml of MEM (minus the arginine), containing 0.25% FCS/0.1% BSA and 3.0 mCi [^{3}H]arginine (spec. act. 59.0 Ci/mmol, Dupont-New England Nuclear, Boston, MA), was added to the cells. After 18–20 h incubation at 37°C, the medium was centrifuged and the supernatant collected as described above (section 2.3) and treated with cold TCA (10%) for 1 h at 4°C to precipitate labeled proteins. The precipitate

was washed with ether several times and air dried. The TCA-precipitated proteins were immunoprecipitated with DP4 or DP3 antiserum as described in [7] except that protein A-Sepharose beads (Pharmacia, Piscataway, NJ) were used to precipitate the antigen-antibody complex. The anti-ACTH immunoprecipitated proteins were electrophoresed on 13.5% polyacrylamide-SDS (SDS-PAGE) 10 cm tube gels using the Laemmli procedure [13]. For the preparation of [^{3}H]POMC as substrate for in vitro enzymatic cleavage, the anti-ACTH immunoprecipitated proteins were electrophoretically separated on an acid gel (without urea) [14]. Cytochrome *c* was run in a parallel gel and the electrophoresis terminated when cytochrome *c* migrated 6.0 cm from the origin. The gel was sliced (1.5 mm slices) and each gel slice eluted with 1 ml of 0.1 N HCl/0.05 mg/ml BSA. Aliquots (5 μl) of the eluant from each slice were counted for radioactivity and the fractions containing the POMC peak (R_f = 0.15 relative to cytochrome *c*) were pooled and lyophilized for use as substrate.

2.7. *Incubation of expressed [^{3}H]POMC with pro-opiomelanocortin-converting enzyme*

[^{3}H]Arg-POMC (2–3 × 10^4 cpm) expressed, radiolabeled and purified as described above (section 2.6) was incubated with purified bovine intermediate lobe pro-opiomelanocortin-converting enzyme (10–30 ng) [7] in 0.1 M sodium citrate buffer (pH 4.0) for 5 h at 37°C in a total volume of 150 μl. At the end of this period, aliquots of the incubate were immunoprecipitated with DP4 or DP3 antisera. The immunoprecipitates were then analyzed by SDS-PAGE [13].

3. RESULTS

3.1. *Analysis of POMC from transfected CV-1 cells*

Fig.1 shows the Western blots of medium from CV-1 cells which were transfected under various conditions. In both panels, only lane 1, medium from cells infected with the recombinant virus vTF7-3 and transfected with the plasmid containing the POMC cDNA sequence in the correct orientation (pKA2) showed a POMC band immunostained with both ACTH and β-endorphin antisera (fig.1, arrow). This band was not present in cells transfected with a plasmid containing the POMC cDNA sequence in the wrong orientation (pKA5, lane 2), in cells infected with wild type vaccinia virus and transfected with either pKA2 or pKA5 (lanes 3 and 4, respectively), or in untransfected cells (lane 5). Analysis of the size of

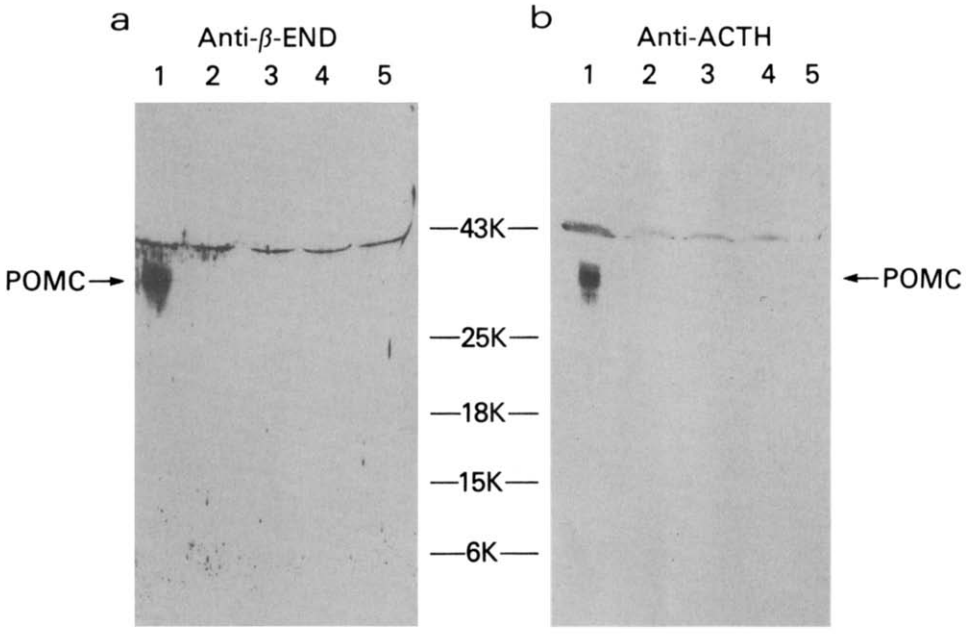

Fig.1. (a) SDS-PAGE and immunoblots (Western blots) of medium from CV-1 cells immunostained with β-endorphin (DP3) antiserum. (b) SDS-PAGE and immunoblots of medium from CV-1 cells immunostained with ACTH (DP4) antiserum. Lanes 1–5 in each panel represent medium from CV-1 cells infected with vTF7-3 recombinant vaccinia virus and transfected with pKA2 (lane 1) or pKA5 (lane 2); or infected with wild type vaccinia virus and transfected with pKA2 (lane 3) or pKA5 (lane 4); or untransfected CV-1 cells (lane 5). Molecular mass markers (K = 10^3) are indicated and the arrows show the POMC band immunostained with β-endorphin and ACTH antisera in lane 1 of panels a and b, respectively.

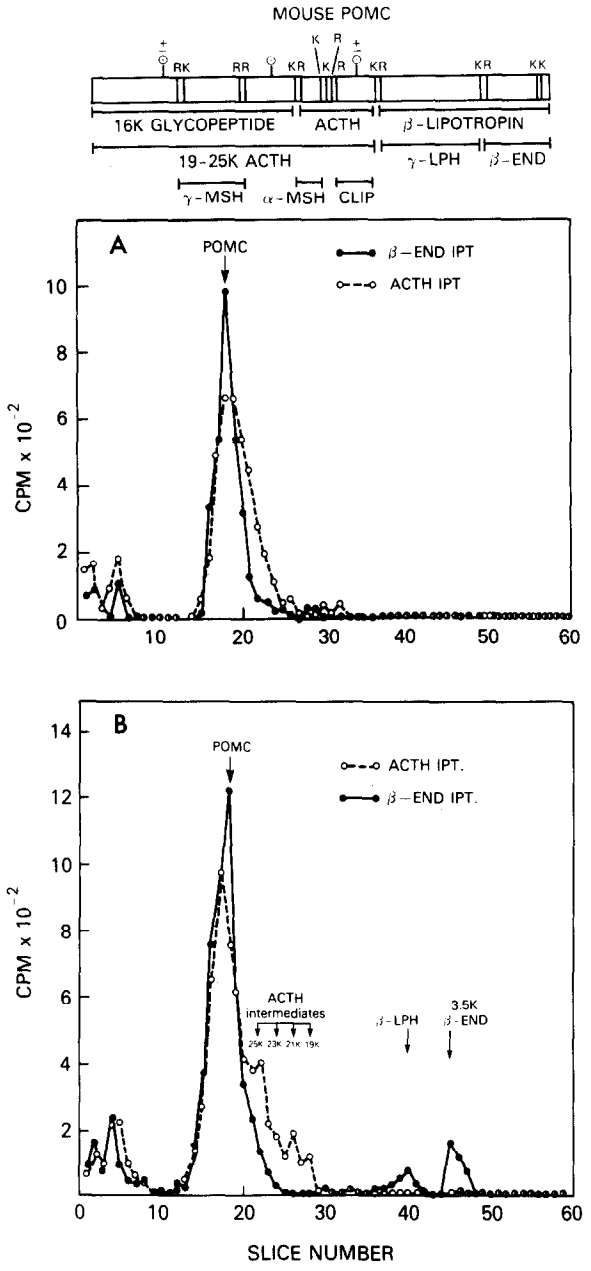

Fig.2. (A) SDS-PAGE of anti-ACTH (○---○) and anti-endorphin (●——●) immunoprecipitated [^{3}H]Arg-labeled POMC eluted from an acid gel (see section 2). A diagrammatic representation of mouse POMC showing the paired basic residues (K = Lys, R = Arg) and glycosylation sites (⊙) is shown above panel A. (B) SDS-PAGE of anti-ACTH (○---○) and anti-endorphin (●——●) immunoprecipitated [^{3}H]proteins generated after incubation of [^{3}H]Arg-labeled, purified POMC (see A) with bovine intermediate lobe POMC-converting enzyme for 5 h at 37°C in 0.1 M sodium citrate buffer, pH 4.0.

this POMC band indicated a molecular mass ranging from 30 to 36 kDa. Similar results were obtained when CV-1 cells were transfected with pKA2 in the presence of [^{3}H]Arg. A radiolabeled POMC peak (33 kDa) which was immunoprecipitated with ACTH and β-endorphin antibodies was detected (data not shown). However, in both the Western blot (fig.1) and radiolabeled experiments, a large molecular mass band (≥43 kDa) was also present. In addition, very small amounts of [^{3}H]Arg-labeled, ACTH-related degradation products were detectable. Purification of the anti-ACTH immunoprecipitated [^{3}H]Arg-labeled prohormone by acid gel electrophoresis virtually eliminated all of the contaminating peaks, yielding a relatively pure preparation of POMC suitable for use as substrate for processing enzymes (see fig.2A).

Radioimmunoassay for ACTH using an antibody that recognizes intact POMC as well as processed products on an equimolar basis showed that this transient transfection system yielded 1–2 μg immunoreactive ACTH/10^6 cells. This is equivalent to a minimum yield of 220 pmol ACTH, or 220 pmol POMC per 10^6 cells (since there is 1 mol of ACTH per POMC molecule). Typically incorporation of [^{3}H]Arg into POMC was 5×10^5 cpm/10^6 cells. Thus, the specific activity of POMC produced was generally in the range of $0.1–0.2 \times 10^4$ cpm/pmol.

3.2. *Processing of [^{3}H]Arg-POMC by POMC-converting enzyme*

Fig.2B shows the SDS gel profile of [^{3}H]Arg-labeled products generated from expressed [^{3}H]Arg-POMC by incubation with POMC-converting enzyme (PCE). Several forms of ACTH intermediates, 19–25 kDa, as well as β-LPH and β-endorphin were observed. These forms represent products cleaved at paired basic residues of POMC (see fig.2) and their sizes correspond to those seen in the mouse intermediate lobe in situ [15]. Incubation of [^{3}H]Arg-POMC in the absence of enzyme did not yield any processed products (data not shown).

4. DISCUSSION

In this study, we have applied a previously described method using a recombinant vaccinia virus containing the sequence for a T7 RNA

polymerase and a plasmid carrying the T7 RNA polymerase promoter and terminator to produce microgram quantities of the prohormone, POMC/10^6 CV-1 cells. Moreover, we have demonstrated that [^{3}H]amino acid-labeled POMC produced in this manner is suitable for use as a substrate for processing enzymes. However, it has to be noted that the specific activity of radiolabeled POMC from CV-1 cells was at least 10-fold lower than that synthesized in mouse intermediate lobe after a 30–60 min pulse-labeling (unpublished data). Hence, more enzyme is required to cleave sufficient prohormone to yield detectable products.

Another consideration of the method is the post-translational modification state of POMC produced by CV-1 cells. The broad (30–36 kDa) POMC band seen on the gels suggests that heterogeneous glycosylated forms of POMC may be produced by the transfected CV-1 cells. Cell-free translated, non-glycosylated POMC has a molecular mass of 28.5 kDa [16]. Indeed, multiple glycosylated forms of POMC, 30–36 kDa, have been reported in mouse and rat pituitary [17–19]. Also, consistent with this interpretation was the observation that cleavage of expressed POMC by PCE yielded different forms of ACTH intermediates which were similar in size to those glycosylated forms found in the mouse pituitary [15,18]. Nevertheless, although the size of POMC expressed in CV-1 cells was similar to that found in mouse pituitary, it remains unknown if the carbohydrate structure is identical to its endogenous counterpart.

Vaccinia virus as an expression system has also been used by Thomas and co-workers [19,20] to produce pro-enkephalin and POMC. In their studies, a recombinant vaccinia virus containing the pro-enkephalin or POMC cDNA sequence was used to infect different cell lines including BSC-40, a green monkey kidney cell line. Their procedure yielded 22 pmol immunoreactive ACTH/10^6 BSC-40 cells [20] in the medium, which is one-tenth of that described in this study. Moreover, in our method, construction of a vaccinia recombinant virus containing POMC DNA was not required.

In conclusion, the demonstration that a vaccinia virus transient transfection system can be applied to a mammalian cell line to produce microgram quantities of intact, glycosylated POMC which can be cleaved by the bovine secretory vesicle prohormone-converting enzyme, paves the way for producing various genetically modified prohormone substrates for structure-function studies in the future. Such studies will further the understanding of the role prohormone conformation plays in determining the specificity of cleavage.

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