Expression and Processing of Mouse Proopiomelanocortin in Bovine Adrenal Chromaffin Cells

A MODEL SYSTEM TO STUDY TISSUE-SPECIFIC PROHORMONE PROCESSING*

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A common mechanism in the synthesis of peptide hormones and neurotransmitters is excision from larger precursor proteins. In most cases, maturation to bioactive forms occurs in a series of steps within the regulated secretory pathway of endocrine and neural cells (1). An early step in the process is endoproteolytic cleavage of the prohormone, typically at pairs of basic amino acids (e.g. LysArg-). Further modifications, such as removal of flanking basic amino acids by carboxy- and/or aminopeptidases, NH₂-terminal acetylation, and COOH-terminal amidation, may then occur (3).

Many prohormones are processed in a tissue specific manner. For example, proopiomelanocortin (POMC) is cleaved in the anterior lobe of the pituitary to ACTH, β-LPH, γ-LPH, and α-endorphin, but is processed in the intermediate lobe to α-melanocyte-stimulating hormone, corticotropin-like intermediate lobe peptide, and several carboxyl-shortened forms of β-endorphin (4) (refer to Fig. 1). Although the biochemical basis for tissue-specific processing is unclear, possible mechanisms include: (i) selective expression of distinct processing enzymes specific for cleaving different sites in the prohormone; (ii) modulation of the accessibility of potential cleavage sites by differential post-translational modification of the precursor (e.g. glycosylation, phosphorylation); and (iii) regulation of the microenvironment in the processing compartmen(s): differences in pH, or concentration of ions, co-factors, or protease modulators could potentially influence interactions of processing enzymes with their prohormone substrates.

Identification of the prohormone endoproteases from cell types with different processing capabilities is essential to an understanding of the tissue specificity of the processing reactions. Indeed, diverse enzyme activities, many of which can cleave substrates containing paired basic amino acids in vitro, have been isolated from both endocrine and neuroendocrine sources (5–10). However, none of these candidate prohormone endoproteases has been authenticated. A major obstacle has been distinguishing a bona fide maturation activity from the many other trypsin-like enzymes present in tissue homogenates. One approach to overcoming this difficulty is to first characterize the cleavage site specificity of prohormone processing endoproteases in vitro and use this information as a guide in identifying putative prohormone processing enzymes in vitro. A comparison of the processing specificities of difer-

The abbreviations used are: POMC, proopiomelanocortin; ACTH, adrenocorticotropin; LPH, lipotropin; PP, proprolactin; VV, vaccinia virus; m.o.i., multiplicity of infection; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RIA, radioimmunoassay; IR, immunoreactive; HEPES, 4-(2-hydroxyethyl)-1-piperazinene sulfonic acid; BSA, bovine serum albumin; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] glycine; TEMED, N,N,N',N'-tetramethylethlenediamine.

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ent cell types also provides a foundation for distinguishing between potential mechanisms of tissue-specific processing.

Previously, we examined the cleavage site specificity of the prohormone endoproteases(s) in an insulinoma cell line, Rin m5F (11, 12). Using a recombinant vaccinia virus (VV) expression vector, this cell line was shown to efficiently process mouse POMC (mPOMC) at many of the sites specifically cleaved in the intermediate pituitary, including the Lys-Lys-Arg-Arg sequence within ACTH and the Lys-Arg site within β-LPH. Unlike the intermediate pituitary, however, the Lys-Lys site within β-endorphin was inefficiently cleaved. By comparing the efficiency of cleavage as the four permutations of paired lysines and/or arginines, introduced at two of the cleavage positions in mPOMC, the sequence of the paired basic amino acids was shown to be critical for directing the degree of processing in insulinoma cells. However, the efficiency of cleavage for specific pairs of basic amino acid sequences was modulated by position within the prohormone. Furthermore, analysis of mutant POMC processing suggested that "paired basic amino acids" may not constitute the precise definition of a cleavage site in these cells. Mutation of an efficiently cleaved Lys-Arg- site to Met-Arg resulted in partial processing whereas substitution with His-Arg prevented endoproteolysis.

Expression of POMC in a cell type exhibiting a processing specificity distinct from Rin m5F cells (ideally mimicking anterior pituitary) would provide a manipulable model system for studies on the mechanisms of tissue-specific processing. We selected chromaffin cells of the adrenal medulla as such a model for several reasons. First, these cells process a variety of neuropeptide precursors in a tissue specific manner (see Ref. 13 for review). For example, proenkephalin is processed at select pairs of basic amino acids resulting in the synthesis of large enkephalin-containing peptides in chromaffin cells. In contrast, the same peptide precursor is completely processed in the brain to enkephalin pentapeptides by processing at all paired basic amino acid cleavage sites (14, 15). Furthermore, chromogranin A, the major soluble protein of chromaffin vesicles, is partially processed in the adrenal medulla at selected sites, whereas insulin-producing cells efficiently process this precursor to lower molecular weight forms (16, 17). Second, large (×10⁶ cells) pure populations of chromaffin cells can be isolated from bovine adrenal glands and maintained for weeks in culture (18). Finally, as a primary culture, this tissue may more accurately reflect processing and secretion of peptide hormones in endocrine tissues in situ than tumor-derived cell lines.

In this report, we demonstrate efficient synthesis and proteolytic maturation of mPOMC in primary cultures of bovine adrenal chromaffin cells using a vaccinia virus expression vector. Processing of mPOMC in these cells was very similar to processing in anterior pituitary, contrasting with processing in both the intermediate lobe and Rin m5F cells. The precursor was cleaved primarily to ACTH and β-LPH, with only partial conversion of β-LPH to γ-LPH and β-endorphin. However, despite the differential processing at the tetradecapeptide sequence in ACTH and the γ-LPH/β-endorphin junction, the adrenal chromaffin cells and the insulinoma cells displayed very similar processing efficiency of both the ACTH/β-LPH junction and the β-endorphin cleavage site, for the native sequences as well as all mutant sequences. Thus a strong similarity in at least some of the insulinoma and adrenal chromaffin cell prohormone endoproteases is indicated. Together, these two cell types represent a manipulable model system to determine the factors which modulate the tissue specificity of prohormone processing.

**MATERIALS AND METHODS**

*Vaccinia Virus—VV strain WR was used in these studies. Viral recombinants directing expression of native and mutant mPOMC were constructed and virus stocks were maintained as described (12).*

*Chromaffin Cell Cultures—Bovine adrenal glands were obtained from the local slaughterhouse. All reagents were cell culture grade from either Sigma or Gibco unless otherwise specified. Chromaffin cells were isolated essentially as described (19). Briefly, eight glands were perfused retrogradely 3 times with 5 ml of W3 (145 mM NaCl, 5.4 mM KCl, 1.0 mM NaH₂PO₄, 11.2 mM glucose, 15 mM HEPES, 100 units/ml of penicillin, 100 units/ml of streptomycin, and 25 μg/ml of gentamicin, pH 7.4), incubating 10 min at 37 °C between perfusions. The glands were then perfused 3 times with 4 ml of W3 containing 2 mg/ml type I collagenase (Worthington) and 50 μg/ml of DNase I (Cooper BioMedical), incubating 15 min at 37 °C between perfusions. After the last perfusion, medullae were dissected out, minced, and incubated with 150 ml of W3 containing 1 mg/ml of collagenase and 25 μg/ml of DNase I for 80 min at 37 °C in a spinner flask. The cell suspension was filtered through nylon mesh and the cells were collected by centrifugation at 100 × g for 15 min.*

*For most experiments, the chromaffin cells were then purified on a Percoll gradient as described (19). Cultures enriched in enkephalin containing chromaffin cells were prepared essentially as described (20). Briefly, cell pellets from the filtrate of the glands were resuspended in 4 ml of W3 and layered on top of step gradients of bovine serum albumin (BSA) in two 16-ml tubes (1 ml of 30%, 2 ml of 23%, 3 ml of 19%, 3 ml of 15%, and 3 ml of 10% BSA/tube). The BSA solutions were made in W3 from a 35% stock solution (Path-o-cyte, Miles Diagnostics). Gradients were centrifuged at 16,000 × g (max) in a swinging bucket rotor for 30 min at 4 °C, and the cells at the interface between the 23 and 30% layers were collected.*

*Chromaffin cells from both Percoll and BSA gradients were washed twice in W3 containing 1.8 mM CaCl₂ and 0.8 mM MgSO₄ and were collected by centrifugation at 100 × g for 15 min. Cells were then resuspended in culture medium (50% Dulbecco's modified Eagle's medium and 50% Ham's F-12 containing 10% fetal calf serum (HyClone), 5 mM HEPES, pH 7.0, 100 units/ml penicillin, 100 units/ml streptomycin, and 25 μg/ml gentamicin) and plated on untreated plastic tissue culture dishes (Nunc) at an approximate density of 4 × 10⁶ cells/150-mm dish. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ (standard culture conditions) for 5–9 h to allow attachment of fibroblasts. Dishes were then gently swirled, and the medium containing unattached chromaffin cells was collected. Chromaffin cells were resuspended in fresh culture medium and replated on diastase tissue culture dishes (Nunc) which had been coated with 5 μg of rat tail collagen/cm² (type I, Sigma). Cell densities were approximately: 2 × 10⁵/35-mm plate for secretion experiments, 2 × 10⁶/100-mm plate for performing peptide analysis, and 4 × 10⁶/35-mm plate for immunofluorescence. These plates were maintained undisturbed for approximately 36 h under standard culture conditions to allow complete attachment of the chromaffin cells prior to infection.*
Chromaffin cells were inoculated with either wild type vaccinia virus or vaccinia virus recombinants expressing native or mutant mPOMC at a multiplicity of infection (m.o.i. of 1 as described (11). After 2 h, the inoculum was replaced with fresh culture medium and the cells were maintained at 37 °C for 24 h before harvesting or performing secretion experiments.

Purity of cultures and relative number of infected cells were assessed by immunofluorescence. Cells were fixed directly on culture dishes with formalin prior to addition of primary antibody. The number of chromaffin cells in a culture was determined by staining with 56 mM K+ (15 min) in the absence and presence of 2.2 mM CaCl2.

RESULTS

Expression of mPOMC in Primary Cultures of Adrenal Chromaffin Cells—Efficient expression and processing of mPOMC in a wide variety of established cell lines was previously achieved with a VV vector (11, 12). The wide host range and transient nature of this vector suggested that the same VV constructs could also direct expression in primary adrenal chromaffin cultures. Since all cells in a culture should be infected by the recombinant virus, including fibroblasts or other contaminating cell types, pure populations of chromaffin cells were essential. Chromaffin cells isolated from bovine adrenal glands were first purified on a Percoll gradient followed by differential plating (see “Materials and Methods”). After 60 h in culture >90-95% of the cells were chromaffin cells, as determined by staining with a dopamine β-hydroxylase antibody. The few cells which were not immunopositive for dopamine β-hydroxylase predominantly had fibroblast-like morphologies.

Approximately 36 h after replating, these primary cultures were either mock infected or infected with either wild type vaccinia (VV:WT), or a VV recombinant which directs the expression of mPOMC (VV:mPOMC). The majority (>80%) of cells in both VV:WT and VV:mPOMC stained immunopositive for VV whereas no positive staining was detected in mock-infected cultures. Replicate cultures were harvested 24 h after infection, and expression was assayed by quantifying γ-LPH immunoreactivity (IR) by radioimmunoassay (RIA). Synthesis of mPOMC was essentially undetectable in either mock or VV:WT-infected cells, while VV:mPOMC-infected cells accumulated 0.8 pmol/106 cells γ-LPH IR in each cell extract and culture medium (1.6 pmol/106 cells total).

Regulated Secretion—For measuring stimulated secretion, all conditions were performed in triplicate. Culture medium was removed from infected cells, and the plates were gently washed once in control collection medium followed by a 15-min incubation at 37 °C in the same medium. For K+ secretion experiments, KRH (125 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 2.2 mM CaCl2, 5.6 mM glucose, 1 mM ascorbic acid, 0.07% BSA, and 25 mM HEPES, pH 7.4) was used as control medium (22, 23), and for stimulation with Ba++, calcium-free BSS (125 mM NaCl, 4.75 mM KCl, 1.4 mM MgCl2, 10 mM glucose, 0.07% BSA, and 25 mM HEPES, pH 7.35) was used (24). The above media were then replaced with 1 ml of collection medium ± secretagogues. Cells were incubated at 37 °C for 15 min for K+ experiments, and either 30 or 45 min for Ba++ experiments. For stimulating secretion with K+, 56 mM KCl and the NaCl was reduced to 74 mM to maintain isosmotic conditions. For stimulation with Ba++, 3 mM BaCl2 was added to the BSS. Following the collection period, media were briefly centrifuged before assaying directly by radioimmunoassay (RIA).

Peptide Analysis—Extracts of infected cells were prepared and RIAs were performed as described (11). Antiseras used in RIAs were directed against specific domains of mPOMC: Henrietta (anti-ACTH), Molly (anti-γ-LPH), FF (anti-α-endorphin), and anti-ACTH 34-39 (anti-corticotropin-likse intermediate lobe).

Peptides in cell extracts and media samples were resolved on a C, reversed phase HPLC column as described (11). Briefly, the media was removed from the cells, clarified by low speed centrifugation (1000 X g for 5 min), and immediately frozen at -70 °C until further use. The cells were washed once with fresh warm medium and then harvested in 0.5 ml of 1 M acetic acid (pH 1.9 with HCl), 1 mM phenylmethylsulfonyl fluoride. Cell extracts were probe sonicated 10 s at 40 watts, clarified by microcentrifuge, lyophilized to dryness, and stored at -70 °C until further use. Molecular weights were estimated by migration on a Tricine SDS-polyacrylamide gel essentially as described (25). Briefly, C4 column fractions were lyophilized to dryness and reconstituted in 10 mM Tris-phosphate, pH 6.8, 2.5% SDS, 5% β-mercaptoethanol, and 0.02% bromphenol blue. Samples were heated for 5 min in a boiling water bath and resolved by electrophoresis. The separating gel contained 12.5% acrylamide, 0.83% bisacrylamide, 0.1% SDS, 13% glycerol, 0.98 M Tris-HCl, pH 8.45, 0.05% ammonium persulfate (w/v), and 0.05% TEMED. The stacking gel contained 4% acrylamide, 0.15% bisacrylamide, 0.1% SDS, 0.72 M Tris-HCl, pH 8.45, 0.05% ammonium persulfate, and 0.05% TEMED. The upper reservoir buffer contained 0.1% SDS, 0.1 M Tris, and 0.1 M Tricine, pH 8.25. The lower reservoir buffer was 0.2 M Tris-HCl, pH 8.9. Molecular weight standards consisted of low molecular weight proteins (Bio-Rad low MW kit), triptic fragments of myoglobin (Sigma MW-SDS-17 kit), and β-endorphin. Lanes with standards were stained with Coomasie Brilliant Blue R-250. Lanes with samples were cut into 2.5-mm slices and incubated overnight in 0.8 ml of RIA buffer I (11) at room temperature on a rotating platform. Aliquots of the eluates were analyzed by RIA. Sequence analysis of processed peptides was performed by manual Edman degradation as previously described (12).

FIG. 2. Regulated secretion of mPOMC from adrenal chromaffin cell cultures. One and one-half days after plating, replicate 35-mm plates of chromaffin cell cultures were infected with VV:mPOMC at a m.o.i. of 1. Twenty-four hours after infection, cells were washed and secretion of γ-LPH IR was assayed as described under “Materials and Methods.” Secretion from control and secretagogue-treated cultures is indicated by hatchet and solid bars, respectively. All experimental conditions were performed in triplicate and each sample was assayed in duplicate. The averages are shown, and error bars represent the standard error of the mean. A, stimulation with 56 mM K+ (15 min) in the absence and presence of 2.2 mM CaCl2; B, stimulation with 5 mM Ba++ (30 min) in the absence and presence of 2.2 mM CaCl2.
observed (Fig. 2A). A 30-min incubation with 3 mM BaCl₂ elicited an 11.5-fold increase in secreted \( \gamma \)-LPH IR, corresponding to a release of approximately 50% of the intracellular mPOMC IR. As expected, the barium-stimulated release was partially inhibited by 2.2 mM CaCl₂ (Fig. 2B).

**Processing of mPOMC in Adrenal Chromaffin Cells**—Processing of native mPOMC by adrenal chromaffin cells was characterized by identifying peptide products of the ACTH and \( \beta \)-LPH domains of the precursor (refer to Fig. 1). Extracts of VV:mPOMC-infected cells were resolved by reversed phase HPLC, and mPOMC-derived peptides were identified by retention time coupled with domain-specific RIA (Fig. 3). When assayed for ACTH, \( \gamma \)-LPH, and \( \beta \)-endorphin IR, peaks of immunoreactivity co-eluting with \( \beta \)-LPH, \( \beta \)-endorphin, \( \gamma \)-LPH, and two prominent ACTH isoforms were found.

The principal \( \gamma \)-LPH IR product co-eluted with the major peak of \( \beta \)-endorphin IR at the position of \( \beta \)-LPH standard (48 min). Furthermore, both immunoreactivities co-migrated on an SDS-polyacrylamide gel as a single band (data not shown). The apparent molecular mass of this peptide (8-9 kDa) correlated well with the calculated molecular mass of authentic \( \beta \)-LPH (8.2 kDa). A second peak of \( \gamma \)-LPH IR co-eluted with authentic \( \gamma \)-LPH (35 min) and a \( \beta \)-endorphin peak of equivalent amount co-eluted with authentic \( \beta \)-endorphin, indicating that about 40% of the \( \beta \)-LPH produced by these cells was further processed to \( \gamma \)-LPH and \( \beta \)-endorphin.

Prominent peaks of ACTH IR, eluting at 29 and 33 min, were observed in extracts of VV:mPOMC-infected cultures. By SDS-polyacrylamide gel electrophoresis (SDS-PAGE) these peptides had apparent molecular masses of approximately 14 and 4.5 kDa, respectively (data not shown). In the anterior pituitary, \( \sim \)50% of the ACTH is glycosylated to a 13-kDa isoform, with the 4.5-kDa peptide corresponding to unglycosylated ACTH (28). Chromaffin cells most likely produce a similar heterogeneity in the post-translational modifications of the ACTH domain of mPOMC. To confirm that the chromaffin cell enzymes cleaved POMC at the \( \text{Lys} \text{Arg} \) preceding the \( \alpha \)-LPH domain of mPOMC. This was determined by the profile of \( \gamma \)-LPH IR, slightly more \( \beta \)-LPH was produced than the fully processed \( \gamma \)-LPH, and the precursor protein accounted for approximately one-third of the total immunoreactivity (data not shown). Because no differences in mPOMC processing were observed in enriched enkephalin-containing cells compared to unprocessed cells, we concluded both cell subtypes processed mPOMC identically. All further experiments were therefore performed with unprocessed chromaffin cell populations.

**Stimulated Secretion of Processed Peptides**—Anterior pituitary corticotrophs secrete both \( \beta \)-LPH and its processed forms (\( \beta \)-endorphin, and \( \gamma \)-LPH) in response to secreta-
gogues, demonstrating that all three peptides are final products of processing. Accordingly, experiments were performed to determine whether the β-LPH and any of the intact prohormone present in chromaffin cell extracts (Fig. 3) were also final products of maturation, or were instead present as processing intermediates in a secretory compartment, preceding formation of mature (secretion-competent) storage vesicles.

Secretion from VV:mPOMC-infected chromaffin cells was stimulated for 45 min with 3 mM BaCl₂ in the absence of CaCl₂ as described above. Media from 100-mm plates of control and stimulated cultures were resolved on the C₄ column and fractions were assayed for ACTH, γ-LPH, and β-endorphin IR (Fig. 4). Very low levels of mPOMC IR were detected in control medium. The predominant form was intact prohormone, although P-LPH was also detected (Fig. 4A). However, the medium of cultures incubated with BaCl₂ contained predominantly processed peptides, including ACTH, β-LPH, γ-LPH, and β-endorphin. The medium was collected, adjusted to 0.1% trifluoroacetic acid and the hormone present in chromaffin cell extracts (Fig. 4). Although P-LPH was also detected (Fig. 4A), further processing of the P-LPH was observed in the presence of barium (Fig. 4B). Furthermore, the pattern of peptide products released was very similar to that found in cell extracts (compare Figs. 3 and 4B) except that prohormone accounted for only a minor portion of the secreted immunoreactivity. Thus β-LPH was apparently a final product of maturation whereas most of the intracellular prohormone was sequestered in a nonsecretable compartment(s), presumably rough endoplasmic reticulum and/or Golgi. These results indicate that processing of mPOMC occurred within the regulated secretory pathway of chromaffin cells and, after transmitting this pathway, >90% of the precursor had been proteolytically processed to a set of peptides identical to those found in anterior lobe corticotrophs.

Expression of Mutant mPOMC in Chromaffin Cell Cultures—The processing of mPOMC by chromaffin cell cultures was strikingly distinct from the processing observed in Rin m5F cells (summarized in Fig. 5). The insulinoma cells cleaved mPOMC not only at the -LysArg- sites flanking ACTH but also at the -LysLys-Arg- site within ACTH. Additionally, β-LPH was processed completely to β-endorphin, and γ-LPH. Note, however, that both cell types inefficiently cleaved the -LysArg- site within β-endorphin and efficiently processed the -LysLys-Arg- site at the ACTH/β-LPH junction (see Fig. 5).

In order to compare "signatures" of Rin m5F and chromaffin cell processing enzymes we chose to study the specificity of the chromaffin cell endoprotease(s) using the same series of mPOMC cleavage site mutants previously expressed in Rin m5F cells (12). Processing of all four permutations of lysine and arginine (LysArg-, ArgArg-, ArgLys-, and LysLys-) was determined to identify any sequence specificity of the processing enzyme(s). To control for positional effects, these four sequences were introduced at two positions in the precursor: the efficiently cleaved -LysArg- site at the ACTH/β-LPH junction, and at the inefficiently cleaved -LysLys-Arg- site within β-endorphin. To test the presumed requirements for paired basic amino acids, HisArg- and MetArg- sequences were substituted for the efficiently cleaved -LysLys-Arg- site at the ACTH/β-LPH junction. The structures of these eight constructs are summarized in Fig. 5. The eight VV constructs containing the altered mPOMC cDNAs (VV:mutant-mPOMC) directed very similar levels of prohormone expression in adrenal chromaffin cell cultures as did VV:mPOMC (~0.8 pmol/10⁶ cells accumulated in each cell and culture medium during a 24-h infection).

Processing of the ACTH/β-LPH Cleavage Site Mutants—Replicate plates of adrenal chromaffin cell cultures were infected with VV:mutant-mPOMC as described above. Processing of the mutant prohormones was assessed by resolving the processing of native and mutant mPOMC in adrenal chromaffin cells. Approximately 36 h after plating, two parallel plates of chromaffin cell cultures were infected with VV:mPOMC at a m.o.i. of 1. Twenty-four hours after infection, cells were washed and incubated 45 min in CaCl₂-free BSS in either the absence (A) or presence (B) of 3 mM BaCl₂ as described under "Materials and Methods." This medium was collected, adjusted to 0.1% trifluoroacetic acid, and resolved on the C₄ column as described in the legend to Fig. 3. Aliquots of 1-min fractions were assayed for β-endorphin (●), γ-LPH (○), and ACTH (●) IR.

Fig. 4. mPOMC-derived peptides secreted from adrenal chromaffin cells. Approximately 36 h after plating, two parallel plates of chromaffin cell cultures were infected with VV:mPOMC at a m.o.i. of 1. Twenty-four hours after infection, cells were washed and incubated 45 min in CaCl₂-free BSS in either the absence (A) or presence (B) of 3 mM BaCl₂ as described under "Materials and Methods." This medium was collected, adjusted to 0.1% trifluoroacetic acid, and resolved on the C₄ column as described in the legend to Fig. 3. Aliquots of 1-min fractions were assayed for β-endorphin (●), γ-LPH (○), and ACTH (●) IR.

Fig. 5. Structure of mPOMC cleavage site mutants and their processing in insulinoma cells. The domains of the mPOMC precursor protein, separated by pairs of basic amino acids (vertical bars), are indicated in this schematic drawing, as are the amino acid sequences of the cleavage sites in the native prohormone (K, lysine; R, arginine; H, histidine; M, methionine). Sites cleaved in adrenal chromaffin cell cultures are indicated. The five ACTH/β-LPH and three COOH-terminal β-endorphin cleavage site mutants, the sequence of their altered cleavage sites, and the extent of their processing in Rin m5F cells are described in the boxes (—, inefficiently cleaved; +, partially cleaved; ++, efficiently cleaved). MSH, melanoctye-stimulating hormone; CLIP, corticotropin-like intermediate lobe peptide.
extracts of these cells on the reversed phase column and identifying mPOMC-derived peptides by retention time coupled with domain-specific RIA (Fig. 6). The peptide profiles of the five ACTH/β-LPH mutants fell into three classes: K163R-mPOMC (-LysArg- changed to -ArgArg-), the first class of mutant, was processed identically to the native prohormone (Fig. 6A). Authentic β-LPH, γ-LPH, and β-endorphin₁₃₁ were synthesized, as well as the two major forms of ACTH, indicating efficient cleavage of the -ArgArg- at this position in the precursor.

The second class of mutants consisted of KR163RK-mPOMC (-ArgLys-), R164K-mPOMC (-LysLys-), and K163H-mPOMC (-HisArg-) (Fig. 6B). All produced normal levels of β-endorphin₁₃₁ but almost no γ-LPH, β-LPH, or ACTH. Instead, two novel peaks containing both ACTH and γ-LPH IR (eluting at 44 and 47 min) and two novel peaks containing ACTH, γ-LPH, and β-endorphin IR (eluting at 51 and 53 min) were observed. These results suggested: 1) that the mutant -LysLys-, -ArgLys-, and -HisArg- sites were not cleaved by the chromaffin cell endoprotease(s); and 2) that the partial cleavage of the -LysArg- site within β-LPH was not affected by the mutation. If this interpretation were correct, the two earlier eluting novel peaks should correspond to a fusion of ACTH and γ-LPH sequences, while the two later eluting peaks should be ACTH/β-LPH fusions. The production of two forms of each peptide is expected, due to heterogeneity within the ACTH domain. To corroborate these assignments, the four novel peptides arising from KR163RK (-ArgLys-) peptides, taken as representative of this class of mutants, were resolved by SDS-PAGE. Eluates of 2.5-mm gel slices were assayed for ACTH, γ-LPH, and β-endorphin IR. Each peak ran essentially as a single species on the gel, and the immunoreactive profiles completely overlapped (data not shown). The first two peaks with HPLC retention times of 44 and 47 min had apparent molecular masses of 16.5 and 8.5 kDa, respectively, consistent with the predicted sizes of glycosylated and nonglycosylated ACTH/γ-LPH fusions (the calculated molecular mass of the nonglycosylated fusion is 8.9 kDa). The later eluting peaks with HPLC retention times of 52 and 54 min had apparent molecular masses of approximately 20 and 15 kDa, respectively, consistent with being glycosylated and nonglycosylated ACTH/β-LPH fusions (the calculated molecular mass of the nonglycosylated form of this fusion is 13 kDa).

The third pattern of processing was obtained with K163M-mPOMC (-LysArg- changed to -MetArg-) (Fig. 6C). Low but detectable levels of authentic γ-LPH, β-LPH, and ACTH were produced in cells expressing this construct. Again, normal levels of β-endorphin₁₃₁ were found. As in Rin m5F cells, the ACTH, γ-LPH, and β-endorphin IR peak from VV:K163M-mPOMC-infected chromaffin cells corresponding to intact precursor (eluting at 68 min) was retained several minutes longer on the C4 column than either native or any of the other mutant mPOMC. In addition, three novel peaks were apparent in VV:K163M-mPOMC-infected chromaffin cells, eluting as a partially resolved triplet. The earliest of these peaks (eluting at 57 min) contained only ACTH and γ-LPH IR, while the other two (eluting at 60 and 62 min) contained ACTH, γ-LPH, and β-endorphin IR. Rin m5F cells infected with this construct gave rise to a mPOMC-derived -MetArg- containing peptide that had a considerably longer retention time (15 min) than its -HisArg- containing counterpart. By analogy with the Rin m5F results, the novel triplet of peaks in the C₄ profile of VV:K163M-mPOMC-infected chromaffin cells may correspond to the earlier eluting quartet of novel peptides produced by the second class of mutants (-LysLys-, -ArgLys-, and -HisArg- cleavage sites, Fig. 6B). This hypothesis was corroborated by SDS-PAGE of column fractions containing each of the three peaks. The first peak migrated on the gel as predicted for the glycosylated ACTH/γ-LPH fusion. The second peak resolved by gel electrophoresis into two ACTH and γ-LPH IR peptides, but only one of them was also immunoreactive for β-endorphin. The apparent Mᵣ of these peptides was also consistent with an ACTH/γ-LPH fusion (nonglycosylated) and a glycosylated ACTH/β-LPH fusion (i.e. the two peptides present in the second HPLC peak originating from the -MetArg- construct correspond to the peptides in the two middle HPLC peaks originating from the -ArgLys- construct). The third peak from the HPLC triplet migrated on the gel as predicted for the nonglycosylated ACTH/β-LPH fusion (data not shown).

**Processing of the β-Endorphin Cleavage Site Mutants**—The processing patterns observed at the ACTH/β-LPH cleavage

![Fig. 6. Processing of the five ACTH/β-LPH cleavage site mutants in chromaffin cells.](image-url)
site suggested a clear preference of the chromaffin cell pro-
hormone endoprotease(s) for specific sequences of basic
amino acids; -LysArg- and -ArgArg- were more efficiently
cleaved than -ArgLys- and -LysLys- at that position. To
determine whether this hierarchy was position independent,
processing of the four permutations of lysine and arginine
was examined in the context of the β-endorphin cleavage site.
As demonstrated above (Fig. 3), VV:mmPOMC-infected chro-
maffin cells synthesized β-endorphin_{1-31}, rather than the pro-
toelytically processed forms (cleavage at -Lys^{28}-Lys^{29}) β-
endorphin_{1-27} or β-endorphin_{1-26}. Thus, the -LysLys- site near
the COOH terminus of the molecule was not processed by
these cultures (refer to Fig. 1).

The mutant prohormones containing the three other per-
mutations of lysine and arginine (refer to Fig. 5) were ex-
pressed in chromaffin cell cultures as described above. Ex-
tracts of these cells were resolved by reversed phase HPLC,
and column fractions were analyzed as before. Both the level
of mpOMC IR and processing of the ACTH domain of all
three constructs was identical to that of the native precursor
(data not shown). When assayed for γ-LPH and β-endorphin
IR, the peptide profiles of each mutant prohormone was
distinct, although extracts of all three cultures did contain
similar levels of authentic γ-LPH and intact prohormone
(Fig. 7).

K232R-mPOMC (-LysLys- changed to -ArgLys-) was ap-
parently processed in the same manner as the native precursor
(Fig. 7A). Carboxyl-shortened forms of β-endorphin and β-
LPH were not observed, indicating that the -ArgLys-
site was not cleaved.

K233R-mPOMC (-LysLys- changed to -LysArg-) produced
a more complex pattern of β-endorphin IR peptides (Fig. 7B).
The primary form of β-endorphin produced co-eluted with
authentic β-endorphin_{1-27} (46 min), the product expected if
the mutant cleavage site was processed. Less than 20% of the
β-endorphin eluted at the position expected for the unproc-
tessed mutant β-endorphin_{1-31} (42 min). Two major β-LPH
peaks (containing both γ-LPH and β-endorphin IR) were
observed, eluting at 48-49 and 51 min, respectively. The
earlier peak co-eluted with authentic β-LPH (containing an
intact COOH terminal cleavage site). The increased retention
time of the second β-LPH-related peptide suggests removal
of the four hydrophilic residues at the COOH terminus of the
β-endorphin domain by processing at the mutant cleavage
site.

Partial proteolysis was observed with KK222RR-mPOMC
(-LysLys- changed to -ArgArg-) (Fig. 7C). The major forms
of β-endorphin and β-LPH eluted from the reversed phase
column at the expected positions for peptides containing
intact mutant cleavage sites. However, approximately 20% of
the β-endorphin IR peptides consistently co-eluted with the
carboxyl-shortened forms (47 and 51 min).

In summary, the pattern of mutant cleavage site utilization
in adrenal chromaffin cells was similar at the ACTH/β-LPH
junction and within β-endorphin_{1-31}. Neither -LysLys- nor
-ArgLys- sites in mPOMC served as efficient substrates,
whereas -LysArg- and -ArgArg- could both be cleaved. Ef-
ciency of -ArgArg- processing, however, was influenced by
position within the precursor. This sequence was cleaved more
completely between the ACTH and β-LPH domains of
mPOMC than near the COOH terminus of β-endorphin,
although -LysArg- was nonetheless efficiently processed at
both positions. Finally, a mutant -MetArg-, but not -His
Arg-, site could partially support proteolysis at the ACTH/β-
LPH junction.

**DISCUSSION**

In this report we demonstrated that primary cultures of
bovine adrenal chromaffin cells express high levels of
mPOMC (>1.6 pmol of mPOMC IR/10^6 cells in 24 h) when
infected with a number of recombinant VV vectors. The native
precursor was efficiently processed to peptides endogenous to
the anterior pituitary, including ACTH, β-LPH, γ-LPH, and
β-endorphin_{1-31} (Fig. 3). As in corticotrophs, processing oc-
curred within the regulated secretory pathway (Fig. 4) (26).
Analysis of peptides released from secretagogue-stimulated
cultures demonstrated that both intact ACTH and β-LPH
were final products of maturation, rather than simply proc-
tessing intermediates. However, the majority of the unproc-
tessed precursor was retained intracellularly, presumably
within the endoplasmic reticulum/Golgi. Thus, the selective
cleavage site utilization in transfected chromaffin cell cultures
was apparently the same as in anterior corticotrophs. The
-LysArg- sites flanking the ACTH domain were very efficiently processed, while the -LysArg- site within β-LPH was only partially cleaved and both the -LysLysArgArg- tetrabasic site within ACTH and the -LysArgArgArg- site in endorphin remained intact.

Chromaffin cells endogenously express and direct a variety of proteins containing multiple pairs of basic amino acids to the regulated secretory pathway. Of these chromogranins A, B, and C comprise about 87% of the total soluble proteins stored in bovine chromaffin vesicles, with lesser amounts of dopamine β-hydroxylase (4%), proenkephalin-derived peptides (1%), and neuropeptide Y (0.2%) (16, 31). The extent of processing of these major vesicle soluble proteins varies markedly; dopamine β-hydroxylase with six pairs of basic amino acids in its sequence (four are -ArgArg- and -LysArg-) (32) does not appear to be processed at all, whereas 50% of chromogranin A and C and much greater levels of chromogranin B- and proenkephalin-derived molecules are found as processed forms (31). However, a significant proportion of the chromogranins- and proenkephalin-derived peptides present in mature chromaffin vesicles corresponds to partially processed proteins (14, 31, 33, 34). Thus, like mPOMC processing reported here, cleavage site utilization of endogenous chromaffin cell peptide precursors is not obviously linked to the sequence of paired basic amino acids in the cleavage site. For example, peptide B, a prominent chromaffin vesicle proenkephalin-derived peptide, is excised from the precursor by selective cleavage of a -LysArg- doublet while an internal -LysArg- doublet within the peptide E sequence remains unprocessed. This is similar to the positional preference for the -LysArg- sequences observed during the processing of mPOMC by the cultured chromaffin cells. Thus, chromaffin cell processing activity shows positional and sequence hierarchy constraints as much in endogenously processed proproteins as in the heterologously expressed mPOMC.

The mPOMC processing in chromaffin cells processing contrasted with the cleavage site utilization previously reported in the rat insulinoma, Rin m5F, which more closely resembled that of neurointermediate lobe melanotrophs (12). In addition to the mPOMC cleavage sites processed in the adrenomedullary cells, both the -LysArg- β-LPH and the tetrabasic sequence in ACTH were efficiently processed by the insulin cell endoprotease(s). Consistent with the more extensive processing of POMC in Rin m5F cells, the endogenous chromogranin A is similarly more efficiently processed in insulin-secreting cells than chromaffin cells (16, 17). Together, the differential POMC processing recorded in these two heterologous cell types provides a manipulable system to study the factors responsible for the tissue specificity of prohormone processing.

To begin characterizing the cleavage site specificity and identify an enzymatic signature of the chromaffin cell prohormone endoprotease(s), processing at a series of altered cleavage sites in mPOMC was examined (Figs. 6 and 7). All four permutations of lysine and arginine (-LysArg-, -ArgArg-, -LysLys-, and -ArgLys-) were introduced at two positions in the precursor: the ACTH/β-LPH junction and near the COOH terminus of β-endorphin. In two additional constructs, -HisArg- and -MetArg- were substituted for the efficiently cleaved -LysArg- at the ACTH/β-LPH junction. Despite the differential mPOMC processing by chromaffin medullary cells and Rin m5F cells, the two cell types displayed the same sequence selectivity at all mutant sites: -LysArg- and -ArgArg- sequences were preferentially cleaved, whereas -ArgLys-, -LysLys-, and -HisArg- directed cleavages were very inefficient. In addition, the lack of cleavage at -HisArg- is consistent with the lack of processing of this POMC mutant in Rin m5F cells (12) as well as the processing of similar naturally occurring mutant sites; neither proalbumin Lille (-HisArg-) (35) or a mutant proinsulin (-LysHis-) (36) are cleaved in vivo. Since histidine should be charged in the acidic environment of maturing secretory granules where processing is thought to occur, a positive charge followed by an arginine is apparently insufficient for recognition by either the insulinoma or the chromaffin cell enzyme(s).

The partial processing of a -MetArg- site in K163M-mPOMC by the chromaffin cell enzymes is also in agreement with our previous studies in Rin m5F cells (12). It is not known whether the -MetArg- doublet is processed by a single basic directed enzyme or whether the long unbranched side group of methionine, a property shared only with lysine and arginine, provides a critical structural component for recognition by a paired basic endoprotease. The only apparent difference between the mutant cleavage site utilization in chromaffin cells and insulinoma cells was the extent of processing at the two “partially cleaved” sites. While the extent of cleavage of the mutant -MetArg- site in K163M-mPOMC (ACTH/β-LPH) and -MetArg- in KK232HR-mPOMC (β-endorphin1-27) was only 20–35% in the chromaffin cells, close to 50% processing was observed in Rin m5F cells (12).

For all the mutant precursors, the effect on processing was restricted to the altered cleavage site. Neighboring sites were apparently cleaved in the native prohormone. For example, the proportion of intact forms of β-LPH to processed γ-LPH and β-endorphin (somewhat greater than 1:1) was essentially the same for all constructs, independent of the extent of processing at either the ACTH/β-LPH junction or the COOH terminus of β-endorphin. However, processing at the native -LysArg- within β-LPH did appear to influence cleavage of the mutant -LysArg- in K233R-mPOMC. While greater than 80% of the β-endorphin produced in VV:K233R-mPOMC infected chromaffin cells had been carboxyl-shortened to β-endorphin1-27, only about 50% of the β-LPH had apparently been processed at the mutant site.

The simplest explanation for differential processing of native mPOMC by chromaffin and insulinoma cells would be the tissue-specific expression of endoproteases having distinct substrate specificities. However, the identical pattern of mutant cleavage site utilization in the two cell types suggests a similarity in the enzymes which performed these reactions. Consistent with this hypothesis is the recent identification of three DNA sequences; fur, PC2, and PC3 (or PC1), whose predicted translated products share significant structural homology with the yeast KEX2 precursor protein endoprotease and are co-expressed in pituitary, pancreas, and adrenal gland (37–40). The fur gene, which is expressed in a wide variety of tissues and cell lines, encodes a Golgi-localized KEX2-like endoprotease, furin, which can efficiently process pro-β-nerve growth factor and pro-von Willebrand factor in the constitutive secretory pathway of mammalian cells (40, 41). In contrast, expression of PC2 and PC3 is apparently restricted to endocrine and neural tissues including adrenal medulla, and insulinoma cells as well as the anterior and neurointermediate lobes of pituitary (38, 39). Whether furin, PC2, and/or PC3 can correctly process POMC in the regulated secretory pathway is currently being addressed.

If chromaffin and insulinoma cells do indeed have one or more prohormone endoproteases in common, either of two mechanisms could account for the differential processing of ACTH and β-LPH. First, Rin m5F cells may express additional enzymes not present in chromaffin cells (nor in anterior adrenal medulla). This could account for the different processing patterns of the mutant mPOMC constructs. Alternatively, the chromaffin and insulinoma cells may have different levels of expression of the common endoproteases. This would account for the differential processing of mutant mPOMC constructs in these two cell types. Interactions between chromaffin and insulinoma cells may account for the differential processing of these mutant mPOMC constructs. If these mechanisms are correct, it should be possible to define and characterize the endoproteases involved in mPOMC processing in the chromaffin and insulinoma cells.

2. J. Hayflick and G. Thomas, unpublished observations.
pituitary corticotrophs), which efficiently cleave the -LysLysArgArg- sequence in ACTH and the internal -LysArg- sequence in \( \beta-LPH \). A second possibility would be modulation of cleavage site accessibility. Instead of regulating enzyme expression, such a mechanism would rely on controlling pro-hormone/enzyme interaction, potentially through post-translational modifications, complex formation with accessory proteins, or other adjustments in the microenvironment of processing compartments. For example, O-glycosylation has been implicated in modulating the tissue-specific exclusion of \( \gamma \) melanocyte-stimulating hormone from the NH\(_2\) terminus of mPOMC (42). Perhaps processing of ACTH and \( \beta-LPH \) is more efficient in Rin m5F cells because these cleavage sites are presented in a more accessible conformation than in chromaffin cells.

Thus, a possibility which must be considered is that tissue-specific processing is a two-tiered mechanism. Regulation of factors which influence cleavage site accessibility and enzyme specificity and/or rates of catalysis may act in conjunction with differential expression of a small core of processing enzymes. Although the biochemical basis of tissue-specific processing remains conjectural, the development of insulinoma and adrenal chromaffin cell cultures as a readily manipulable model system provides the means of addressing many of these fundamental questions.

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Note Added in Proof—During review of this manuscript, we completed a study demonstrating PC2 and PC3 correctly process mPOMC to sets of peptides present in pituitary corticotrophs (or BAM cells (PC3)), and melanotrophs (PC3 and PC2) (43). Together, they mimicked the processing signature presented here supporting their role as a common core of neuroendocrine processing enzymes.

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