Expression of Mouse Proopiomelanocortin in an Insulinoma Cell Line  
REQUIREMENTS FOR β-ENDORPHIN PROCESSING*

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Proopiomelanocortin (POMC) is a neuroendocrine precursor protein which is processed at paired basic amino acids in a tissue-specific manner. To study this phenomenon, a vaccinia virus recombinant, which directs the synthesis of mouse POMC (VV:mPOMC) was constructed and used to infect epithelial (BSC-40) and endocrine (Rin m5F) cell lines. Bonafide mPOMC was produced in both cell types and β-endorphin immunoreactivity was secreted in a nonregulated manner from BSC-40 cells and in a regulated manner from Rin m5F cells. Although the precursor was not cleaved to smaller β-MSH or β-endorphin immunoreactive peptides in BSC-40 cell extracts, Rin m5F cells produced primarily authentic γ-lipotropin and des-acytetyl β-endorphin1-27. Furthermore, production of these peptides was restricted to the regulated secretory pathway in Rin m5F cells. Site-directed mutagenesis was then used to change the inefficiently recognized Lys-Lys potential cleavage site near the carboxyl terminus of β-endorphin to Lys-Arg. Expression of the mutant precursor in Rin m5F cells resulted in the synthesis of both des-acytetyl β-endorphin1-27 and β-endorphin1-24.

Most neuroendocrine peptides are derived from larger precursor proteins or prohormones. Peptides are excised from the prohormone by endoproteolysis, usually at pairs of basic amino acids (e.g., Lys-Arg, Lys-Lys, Arg-Arg) (1). In many instances, further modification of the cleavage products, such as carboxyl-and/or amino shortening (2), amidation (3), and N-acetylation (4), must also occur for bioactive peptides to be generated.

A number of prohormones contain the sequence of more than one bioactive peptide. A well-studied example of this type of prohormone (polyprotein) is proopiomelanocortin (POMC). POMC is expressed in the anterior and neurointermediate lobes of the pituitary as well as in several regions of the brain and other tissues and is processed in a tissue-specific manner to produce a variety of peptide hormones (5). In the pituitary, these are primarily ACTH, β-LPH, and amino-terminal fragment in the anterior lobe, and smaller amino-terminal fragments, α-MSH, CLIP, γ-LPH, and several forms of β-endorphin in the neurointermediate lobe. The mechanisms responsible for tissue-specific processing of the prohormone are unknown, but several factors may be involved, including: (i) selective expression of distinct processing enzymes, (ii) differential compartmentalization of either the proteasomes or the precursor, and (iii) differential modification of the prohormone to regulate cleavage site accessibility.

For peptide hormone in which secretion can be controlled by external stimuli (regulated release), the proteolytic maturation steps that occur once the precursor has been committed to the regulated secretory pathway (6, 7). In contrast, prohormone that fails to enter the regulated secretory pathway is not cleaved and is secreted in bulk-flow carrier vesicles via the constitutive pathway (8). It is not clearly understood which structural features of prohormones may serve as determinants for targeting the precursor to the correct cellular compartment for cleavage (9) or for recognition by processing endopeptidases (10). However, several naturally occurring mutants of proinsulin (11) and proalbumin (12, 13) have demonstrated that the integrity of the paired basic amino acid target sequence is critical for proteolysis. Additionally, sequences (14) or posttranslational modifications (15) are critical for cleavage site recognition or targeting to the cellular compartment containing the processing endopeptidase(s).

In this communication, we describe the use of a vaccinia virus expression system to study the proteolytic maturation of mouse POMC (mPOMC) in two heterologous cell lines. In addition, the importance of the amino acid composition of a potential cleavage site was determined by expression of a mutant mPOMC generated by site-directed mutagenesis.

MATERIALS AND METHODS

Cell Culture and Vaccinia Virus—AtT-20 (17) (derived from an anterior pituitary corticotroph tumor) and Rin m5F (18) (derived from a pancreatic β cell tumor) cells were grown in DMEM containing 4.5 and 2.0 g/liter glucose, respectively (GIBCO), 10% fetal calf serum (FCS, Hyclone), and 28 μg/ml gentamicin at 37°C in 5% CO2. BSC-40 cells (19) were maintained at 40°C in 5% CO2 in either MEM containing 5% FCS and 28 μg/ml gentamicin, or, for mPOMC purification, phenol red-free MCDB 202 (20) containing 4% FCS, 50 μg/ml ovalbumin, and 28 μg/ml gentamicin.

Vaccinia virus strain WR was used in these studies. VV:mPOMC
was constructed as described (21). Partially purified virus stocks for use in experiments were made as described (19). Infections were performed as follows: cells were rinsed in PBS-M (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8 mM Na2HPO4, and 1 mM MgCl2) and inoculated with virus diluted in sufficient PBS-M containing 0.01% BSA to cover cells. Cells were incubated at 37°C for 1 h. The inoculum was then replaced with fresh culture medium.

Antibodies and Radioimmunoassay (RIA)—All synthetic peptide standards were obtained from either Peninsula Laboratories or Bachem Biosciences. Synthetic POMC was obtained from Novo Biobols. Monoidinated porcine insulin for RIA was obtained from Du Pont-New England Nuclear. N-Acetylated (Ac) camel β-endorphin, rat and monkey β-MSH were iodinated using NaI211 (Du Pont-New England Nuclear), by the chloramine-T method (22). Guinea pig anti-rat insulin (Linco Research Inc.) was raised against rat insulin but is 30% cross-reactive with the prohormone. β-Endorphin RIAs were performed using a rabbit antiserum (FF) raised against human β-endorphin. The antibody is directed against the midportion of the molecule and cross-reacts equally with Ac and des Ac β-endorphins 1–31, 1–27, and 1–26. It also cross-reacts, although including P-LPH and POMC. The β-MSH RIAs were performed using a rabbit antiserum (Molly) raised against monkey β-MSH. Antiserum MOLY cross-reacts with mouse γ-MSH, β-MSH, and POMC. RIAs were performed using 50 μl of POMC-containing samples incubated overnight at 4°C with a goat antiserum and iodinated peptide in 200 μl of 50 mM H2PO4, 100 mM NaCl, 10 mM Na2EDTA, 50 mM NaN03, 0.1% BSA, 0.1% Triton X-100, pH 7.4 (RIA buffer I). Primary antibodies were precipitated with an excess of either goat anti-guinea pig IgG (Linco Research Inc.) or goat anti-rabbit IgG (Amel Products) in 0.5 ml of 12% (NH4)2SO4, 10 mM Na2HPO4, pH 7.5, in the presence of 0.2% nonimmune rabbit (or guinea pig) serum (NRS) at 4°C for 30 min.

HPLC Analysis of Peptides—For HPLC analysis, 50 μg of POMC were lyophilized and resolved by SDS-PAGE as described above. Gels were fixed and stained in Coomassie Brilliant Blue R250 and the band was excised to each tube and samples were again lyophilized to dryness before being assayed.

HPLC Analysis of Peptides in Cell Extracts—Cells were incubated with recombinant vaccinia at a m.o.i. of 1 and incubated in culture medium 1 h. This medium was removed and cells were rinsed once in a large volume of serum-free medium containing 0.07% BSA. Serum-free medium (1 ml for 35-mm and 2 ml for 60-mm plates) containing 0.07% BSA with or without secretagogues (100 nmophol dibutyryl cyclic AMP (PMA (Sigma) or 50 μM forskolin (Behring Diagnostics)) was added to each plate, and cultures were incubated at 37°C for 1 h. Medium was collected and assayed directly by RIA, or trfluoroacetic acid was added to 0.1% final concentration and samples were clarified by centrifugation for injection directly on the C18 column. Peptides were resolved in the trifluoroacetic acid/ACN gradient described above. 25-μl aliquots of a 1-min fraction were assayed directly by RIA. Larger volumes were aliquoted into RIA tubes and lyophilized to dryness before being assayed.

Site-directed Mutagenesis and Construction of VV:K233R-mPOMC—mPOMC containing the mPOMC cDNA (26) was partially digested with Stu1 endonuclease and protruding ends made blunt with Klenow fragment and T4 DNA polymerase. The plasmid was alkylated (25) with dimethylsulfoxide and 10 μM EcoRI and cloned into M13mp18 cut with EcoRI and amplified in E. coli. The cDNA insert was now lacking all but 20 base pairs of its 5'-untranslated sequences and was flanked by regenerated BamHI and EcoRI.
restriction sites. Single primer oligonucleotide-directed site-specific mutagenesis was performed as described (27) using a 15-mer oligonucleotide (5′-CACAAGAGGGGCGAG-3′). The mutagenized cDNA was excised with BamHI and EcoRI and cloned into the vaccinia vector VV:K235R-mPOMC.

**SDS-PAGE of β-Endorphin Peptides**—C, column eluates were lyophilized to dryness and resuspended in 10 mM H3PO4 (pH 6.8 with Tris), 2.5% SDS, 5% β-mercaptoethanol, and 0.02% bromophenol blue, heated 3 min in a boiling water bath, and resolved on a polyacrylamide slab gel by a modification of the method of Swank andunkresz (30). The separating gel contained 17% acrylamide/1% bisacrylamide, 0.1% SDS, 0.1 M H3PO4 (pH 6.8 with Tris), 0.07% ammonium persulfate (w/v), and 0.7% TEMED. The stacking gel contained one-half the concentrations of all components except for the ammonium persulfate and TEMED, which were unchanged. Electrobe buffer was 0.1 M H3PO4 (pH 6.8 with Tris), 0.1% SDS, and electrophoresis was carried out at 10 V/cm. Lanes with molecular weight standards (tryptic fragments of myoglobin [Sigma] and synthetic β-endorphin) were stained in Coomassie Brilliant Blue R-250. Lanes with samples were cut into 2.5-mm thick slices and incubated 3.5 h at 55 °C. Migration of molecular weight standards is indicated. Lane 1, AtT-20; lane 2, VV:mPOMC-infected BSC-40; lane 3, VV:WT-infected BSC-40; lane 4, VV:mPOMC-infected Rin m5F; lane 5, VV:WT-infected Rin m5F. B, amino-terminal sequence of prohormone secreted from VV:mPOMC-infected BSC-40 cells. Row 1 represents the nucleotide sequence of the cloned cDNA starting at the codon for the initiator methionine. Row 2 represents the deduced primary translation product. J indicates the site of signal peptide cleavage in AtT-20 cells (33). Row 3 denotes the results of automated sequence analysis of prohormone purified from the medium of VV:mPOMC-infected BSC-40 cells. * means no amino acid derivative was detected. These positions presumably correspond to cysteine residues as this amino acid is not detectable by the sequencing methods used.

**RESULTS**

**Expression of Mouse POMC in Heterologous Cells**—A recombinant vaccinia virus which directs the synthesis of mouse proopiomelanocortin (mPOMC) was constructed by previously described marker rescue techniques (21). To verify that this vaccinia recombinant (VV:mPOMC) directs the synthesis of the prohormone, BSC-40 (a monkey kidney epithelial line) and Rin m5F (a rat insulinoma line) cells were either mock infected, or infected with wild type vaccinia (VV:WT) or VV:mPOMC at a m.o.i. of 1. Cultures were harvested 16 h after injection and β-endorphin immunoreactivity (IR) quantitated by RIA. Only the VV:mPOMC-infected cells produced significant levels of β-endorphin IR (1.2 and 0.5 pmol of β-endorphin IR/10⁶ cells in BSC-40 and Rin m5F cells, and 4.5 and 1.2 pmol/10⁶ cells in the culture medium of BSC-40 and Rin m5F cells, respectively). The authenticity of the prohormone synthesized in these cells was confirmed by SDS-PAGE of mPOMC which had been metabolically labeled with [³⁵S]methionine and immunoprecipitated with an affinity-purified anti-ACTH antibody (Fig. A4). Both VV:mPOMC-infected BSC-40 and Rin m5F cells, but not the corresponding VV:WT-infected cultures, produced the same two size isoforms of the prohormone (32 and 36 kDa) as are found in the anterior pituitary corticotropic line, AtT-20. This correlates well with earlier studies that demonstrated two major size isoforms of mPOMC in vivo, due primarily to differences in glycosylation (31, 32). Correct removal of the 26 amino acid signal peptide (33) ("pre" sequence) was demonstrated by amino-terminal sequencing of 200 pmol of purified prohormone isolated from media of VV:mPOMC-infected BSC-40 cells (see "Materials and Methods"). The first nine amino acids of this precursor correspond to residues 27–35 of the preprohormone as deduced from the cDNA sequence (26) (Fig. 1B).

**Targeting of mPOMC to Regulated Secretory Pathway**—In AtT-20 cells, mPOMC-derived peptides are secreted via the regulated secretory pathway (34). Since localization of the precursor to this pathway appears to be required for correct processing to occur (7, 8), it was important to ascertain the targeting of the prohormone synthesized in cells infected with VV:mPOMC. The Rin m5F cell line provided a good model system for this study because it has a well-characterized regulated secretory pathway (35, 36).

The effect of vaccinia infection on regulated secretion was first determined by examining the secretagogue-stimulated release of insulin from mock-, VV:WT-, and VV:mPOMC-infected Rin m5F cells (Fig. 2, A and B). Although the virus suppressed the absolute levels of released endogenous hormone, as compared to mock-infected cultures (approximately 3-fold, 16-h postinfection), the extent of stimulation (6.5-fold) by the phorbol ester PMA was not affected, demonstrating that the regulated secretory pathway remains functional in vaccinia-infected cells. To determine whether mPOMC was correctly sorted into this pathway, secreted β-endorphin IR was quantitated in this experiment (Fig. 2C). As expected, only the VV:mPOMC-infected cells produced significant levels. Additionally, a 2.7-fold increase in secretion of β-endorphin IR over control levels was observed in the presence of PMA, demonstrating that virally produced mPOMC can be targeted into the correct secretory pathway in a heterologous environment. When similar experiments were performed in BSC-40 cells, however, secretion of β-endorphin IR could not be stimulated with either PMA or forskolin (secretagogues acting via the protein kinase C and CAMP-dependent pathways, respectively) (data not shown). This suggests that a regulated secretory pathway is lacking in this cell line; thus mPOMC must exit BSC-40 cells by way of the constitutive pathway only.

**Processing of mPOMC in Heterologous Cells**—The pro-
Processing of β-Endorphin in Insulinoma Cells

Fig. 2. Regulated secretion of mPOMC from Rin m5F cells. Parallel 35-mm plates of Rin m5F cultures were either mock infected, or infected with VV:WT or VV:mPOMC at a m.o.i. of 1. 16 h after infection, cells were rinsed and then incubated for 1 h at 37°C in 1 ml of serum-free medium containing 0.07% BSA and 100 nm PMA (solid bars). Controls (hatched bars) were treated identically except for the omission of PMA. Insulin and β-endorphin IR in each sample was quantitated by RIA. All experimental conditions were performed in triplicate and assayed in duplicate. The averages are shown. Error bars represent the standard error of the mean. A, insulin IR in mock infected cells. B, VV:WT- and VV:mPOMC-infected cells. C, β-endorphin IR in mock-, VV:WT-, and VV:mPOMC-infected Rin m5F cultures.

Fig. 3. Processing of the β-LPH domain of mPOMC in three cell types. A, processing of the β-LPH domain of mPOMC in anterior pituitary and AtT-20 corticotrophs (Ant.) and intermediate lobe melanotrophs (Fig. 3A).

The peptide which eluted at 48 min was identified as β-endorphin1-31, by coelution with synthetic camel β-endorphin1-31 from the C4 column. The β-MSH IR peak at 41 min was determined to be γ-LPH (the only β-MSH sequence in mPOMC is located at the carboxy terminus of γ-LPH). Since synthetic standards were not available for this peptide, it was identified by its apparent molecular weight of 4.6 kDa on an SDS-polyacrylamide gel (as compared to 4.4 kDa, the calculated molecular weight of mouse γ-LPH) (data not shown). The peptide which eluted at 55 min had both β-endorphin and β-MSH IR and ran as a single species of molecular mass 8.2 kDa on SDS-PAGE, identifying it as β-LPH (calculated molecular mass of 8.1 kDa). The intact precursor eluted at 69–71 min. SDS-PAGE analysis of these fractions demon-
strated that both of the size isoforms exhibited in Fig. 1 were present in this peak (data not shown). The β-MSH IR peptide eluting at 20 min was not identified in the present study.

Using the vaccinia expression system, processing of mPOMC in BSC-40 and Rin m5F cells was next examined. Cells were harvested 16 h after infection with VV:mPOMC, extracts resolved by reversed phase HPLC with a C4 column, and fractions assayed for both β-endorphin and β-MSH IR.

In agreement with our earlier findings with human proenkephalin (39), BSC-40 cells were found to be incapable of processing mPOMC to smaller β-endorphin- and β-MSH-containing peptides (Fig. 3C) (21). In contrast, Rin m5F cells processed the precursor almost completely to peptides which coelute with authentic γ-LPH and des-Ac β-endorphin

Cation exchange chromatography using AtT-20 γ-LPH and synthetic P-endorphin-27 as standards (see "Materials and Methods") was used on the mPOMC cDNA to substitute an arginine for the lysine codon corresponding to position 29 in P-endorphin (LYS28 in mouse prePOMC), thereby changing the oxytocic secretory pathway, we analyzed the mPOMC-derived products secreted in 1 h by these cells in the presence and absence of secretagogues (Fig. 4). In the absence of secretagogues, primarily intact precursor was found in the medium (Fig. 4A).

In the presence of 100 nM PMA and 50 μM forskolin, processed forms (β-endorphin, and γ-LPH) were the principle secreted products (Fig. 4B). Importantly, a similar amount of unprocessed mPOMC was secreted from both control and stimulated cells. This demonstrated very efficient processing in the regulated secretory pathway of the Lys-Arg cleavage sites in the β-LPH domain of mPOMC. Limited, if any, proteolytic maturation of the prohormone could be detected in the constitutive secretory pathway.

Cleavage Site Requirements—Because Rin m5F cells, like AtT-20 and anterior pituitary corticotrophs, synthesize primarily the nonacetylated, noncarboxyl shortened form of β-endorphin (i.e. β-endorphin, by cleaving Lys28-Pro29 in the β-LPH domain of mPOMC), this cell line appears to be incapable of either N-acetylation or efficiently cleaving the Lys28-Pro29 target site near the carboxyl terminus of the precursor (Lys28-Lys29 in β-endorphin, see Fig. 3A). There are several possible explanations for inefficient proteolysis of the Lys-Lys site, including: (i) proximity of the site to the carboxyl terminus (only two amino acids away) or other structure of the precursor may interfere with enzyme binding, or (ii) the composition of the paired basic amino acid target site itself may be critical for recognition by the processing endopeptidase. To address this question, site-directed mutagenesis was used on the mPOMC cDNA to substitute an arginine codon for the lysine codon corresponding to position 29 in β-endorphin (Lys29 in mouse proPOMC), thereby changing the Lys-Lys cleavage site to Lys-Arg (see "Materials and Methods"). This mutant cDNA was cloned into the vaccinia expression system, and the resultant virus (VV:K233R-mPOMC) used to infect BSC-40 and Rin m5F cultures.

As expected, only unprocessed K233R-mPOMC was found in BSC-40 cell extracts, coeluting with the native prohormone from the C4 column (data not shown). However, the peptide profile from extracts of VV:K233R-mPOMC-infected Rin m5F cells (Fig. 5A) was distinct from that of the native precursor. The γ-LPH molecules synthesized from both the native and mutant precursors were identical, demonstrating consistent efficient cleavage at the Lys-Arg sites flanking this sequence in the prohormone. In contrast to the processing of native mPOMC, however, the β-endorphin IR peak from the C4 column shifted from 48 min (β-endorphin, for VV:mPOMC (Fig. 3D) to 51 min (the elution time of des-Ac β-endorphin, for VV:K233R-mPOMC). In addition, the 55 min peak (β-LPH) had more β-endorphin than β-MSH IR. This is in contrast to the β-LPH peak obtained from either AtT-20 cells or VV:mPOMC-infected Rin m5F cells, which had more β-MSH IR (compare Figs. 5A and 3D).

Several factors could account for the additional β-endorphin IR found in the K233R-mPOMC peak eluting at 55 min: (i) the β-endorphin antibody may have an increased affinity for the mutant β-LPH, or (ii) there may be a second β-endorphin IR species eluting at the same position. To distinguish between these possibilities, an aliquot of the material eluting at 55 min was resolved by SDS-PAGE, and eluates of
C4 column. An aliquot (about 1 ng) of the 55-min peptides were further analyzed by cation exchange chromatography. AtT-20 $\gamma$-LPH migrates at position 6.4 kDa; and 8-MSH 17.2 kDa; c, 14.6 kDa; d, 8.2 kDa; e, 6.4 kDa; and f, $\beta$-endorphin-14. When analyzed by this method, AtT-20 $\beta$-LPH migrates at position d.

Gel slices were assayed for $\beta$-endorphin and $\beta$-MSH IR (Fig. 5B). Although there is a $\beta$-endorphin and $\beta$-MSH IR peptide, coeluted with synthetic des-Ac $\beta$-endorphin1-7 and $\beta$-endorphin1-26, respectively (data not shown), implying efficient proteolytic processing of the mutant Lys-Arg cleavage site.

**DISCUSSION**

In this study, a recombinant vaccinia virus was used to express mouse POMC in epithelial (BSC-40) and endocrine (Rin m5F) cell lines. Using this expression system, high levels of intracellular and secreted mPOMC (or mPOMC-derived peptides) were obtained only 16 h postinfection. In agreement with studies on human proenkephalin (39), BSC-40 cells were found to be incapable of storing mPOMC for stimulated secretion or of processing the precursor to smaller $\beta$-endorphin- or $\beta$-MSH-containing peptides (Fig. 3C). Moreover, the prohormone secreted by these cells was not degraded in the culture medium, greatly facilitating large scale purification for sequence analysis. In addition, because synthesis occurred in mammalian, rather than in bacterial cells, this mPOMC should contain similar posttranslational modifications of the precursor as occur in vivo, making it ideal for use as an authentic substrate for in vitro characterization of putative prohormone processing enzymes.

Other fibroblast and epithelial cell lines have also been found to be incapable of processing a variety of prohormones and have therefore been termed "maturation deficient" (40-42). It is interesting to note, that, while only intact POMC is found intracellularly in gene transfer experiments with COS-1 (42), BSC-40, or P388D1 cells, the protein secreted by these cell lines, with the exception of BSC-40, is degraded in the culture medium. These results emphasize a major advantage of the broad host range of a vaccinia expression system—a variety of different cell lines can be rapidly screened to identify those which express the heterologous protein in the desired manner.

In contrast to BSC-40 cells, the Rin m5F cell line was found to both efficiently target mPOMC into the regulated secretory pathway and process the prohormone to bona fide $\beta$-endorphin- and $\beta$-MSH-containing peptides (Fig. 3D); thus, Rin m5F cells are "maturation proficient." Targeting of mPOMC to the regulated secretory pathway was determined by the extent of stimulation of secretion of $\beta$-endorphin IR (2.7-fold) by second messenger analogues. This stimulation, however, was somewhat less than that obtained for the endogenous hormone insulin (5.5-fold) (Fig. 2). There are several possible explanations for the apparent difference. (i) The two prohormones may be packaged into distinct vesicle populations, as has been observed in anterior pituitary somatotrophs (44), with the insulin-containing granules being more susceptible to stimulation. (ii) Since basal secretion consists primarily of intact precursor whereas media from stimulated cells contains mainly mature peptides, the observed differences in stimulation may reflect differential affinities of the $\beta$-endorphin and insulin antisera for precursors and processed products. (iii) Sorting into the regulated secretory pathway may be less efficient for mPOMC than for insulin. Indeed, a greater stimulation of insulin secretion can be obtained in AtT-20 cells stably transfected with the gene for human insulin than for the endogenous mPOMC-derived peptides (44, 45). This has been attributed to a greater intrinsic "sorting index" of proinsulin than mPOMC. The relative "sorting indices" of mPOMC and proinsulin may thus carry over to Rin m5F cells, a system complementary to the AtT-20 studies.

HPLC analysis of the media from Rin m5F cells demonstrated stimulated secretion of only processed forms of mPOMC (Fig. 4). Because the $C_4$ chromatogram of these secreted mPOMC-derived peptides reflects that of cell extracts, processing in the regulated secretory granules is implicated. Conversely, the major form of mPOMC secreted from control cells was unprocessed precursor, indicating that minimal maturation takes place in the constitutive secretory pathway. This apparent coupling of proteolytic processing and targeting to the regulated secretory pathway is analogous to that observed in mammalian systems.
to the sorting and secretion of mPOMC in AtT-20 cells (8). Analysis of mPOMC-derived peptides in Rin m5F cells revealed β-endorphin$_{1-31}$ and γ-LPH as principle products (Fig. 3D), demonstrating cleavage at Lys$^{232}$-Arg$^{241}$ and Lys$^{232}$-Arg$^{241}$ but not at Lys$^{232}$-Lys$^{233}$. These results are consistent with the observed processing of the endogenous proinsulin at Lys-Arg and Arg-Arg sites, as well as with results from expression of other heterologous prohormones in insulinoma cells (40, 41, 46). Although the precise site of cleavage within the target sites has yet to be determined for mPOMC in Rin m5F cells, correct removal of the extending Lys and Arg residues by an amine and/or carboxypeptidase must occur to produce both bona fide γ-LPH and β-endorphin$_{1-31}$. The specificity of the Lys-Arg and Arg-Arg specific endoproteases thought to be involved in proinsulin processing in insulin-producing tumors would suggest that cleavage occurs on the carboxyl side of the second basic amino acid (47). The presence of high levels of carboxypeptidase H activity (or enkephalin convertase, an enzyme which specifically removes carboxyl extended basic amino acids (48)) in Rin m5F cells is consistent with this mechanism.2

Processing of β-LPH to β-endorphin and γ-LPH is much more efficient in Rin m5F cells than in anterior pituitary corticotrophs or AtT-20 cells (compare Fig. 3, D and B). In addition, preliminary results indicate efficient cleavage within the ACTH domain of the molecule. Thus, the proteolytic processing of mPOMC in Rin m5F cells is more reminiscent of processing in intermediate pituitary melanotrophs. In contrast to the intermediate lobe, however, which primarily synthesizes forms of β-END$_{1-27}$ and β-END$_{1-26}$ by cleaving Lys$^{232}$-Lys$^{233}$ in mPOMC, the predominant form of β-END$_{1-26}$ IR produced by Rin m5F cells is β-endorphin$_{1-31}$ to determine whether the sequence of the target site rather than structural constraints of the precursor precluded cleavage near the carboxyl terminus of the β-END$_{1-26}$ domain, Lys-Lys was replaced with Lys-Arg through the use of site-directed mutagenesis. Expression of the mutant precursor (K233R-mPOMC) in Rin m5F cells resulted in efficient synthesis of β-endorphin$_{1-27}$, while very little β-endorphin$_{1-31}$ accumulated in the cells (Fig. 5). This demonstrates that sequence of the paired basic amino acids, rather than just position of the potential cleavage site within the precursor, can influence prohormone processing. In addition to β-endorphin$_{1-27}$, about 10% of the processed peptide in VV/K233R-mPOMC-infected Rin m5F cells was in the form of β-END$_{1-31}$. If cleavage indeed occurred on the carboxyl side of the Lys-Arg site, these results would indicate efficient removal of extending Arg and Lys residues, but inefficient removal of His (at position 27 in β-endorphin) by an endogenous carboxypeptidase H-like activity. This would correlate well with in vitro carboxypeptidase H activity studies, which demonstrate much less efficient (but detectable) removal of carboxyl terminal His versus Arg or Lys from synthetic substrates (49). In the intermediate pituitary and in several regions of the brain, however, the levels of the various forms of β-END$_{1-27}$ and β-END$_{1-31}$ are very similar (50). The difference in the extent of carboxyl shortening of β-endorphin in these tissues as compared to Rin m5F cells may possibly be explained by a much longer exposure time of the mPOMC endoproteolytic cleavage products to carboxypeptidase H activity in vivo than in our transient expression system.

In conclusion, we have expressed mouse POMC in heterologous cell lines using a recombinant vaccinia virus. Maturation deficient BSC-40 cells provided the means for synthesizing sufficient levels of intact precursor to permit isolation for biochemical studies, whereas the maturation proficient Rin m5F cells were employed to demonstrate that composition of the paired basic amino acid target sequence can be a critical factor governing cleavage site utilization. Using this as a model system, the requirements of potential cleavage sites for recognition by processing enzymes and the role of specific prohormone sequences for targeting to the regulated secretory pathway can be more precisely defined.

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