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Kex2-like endoproteases PC2 and PC3 accurately cleave a model prohormone in mammalian cells: Evidence for a common core of neuroendocrine processing enzymes

(precursor processing/secretory pathway/proopiomelanocortin/paired basic residues)

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ABSTRACT **Two mammalian gene products, PC2 and PC3, have been proposed as candidate neuroendocrine-precursor processing enzymes based on the structural similarity of their catalytic domains to that of the yeast precursor-processing endoprotease Kex2. In this report we demonstrate that these two proteases can cleave proopiomelanocortin (POMC) in the secretory pathway of mammalian cells. Similarly to pituitary corticotrophs, PC3 expressed in processing-deficient BSC-40 cells cleaved native mouse POMC at the -Lys-Arg- sites flanking corticotropin. The -Lys-Arg- within β-lipotropin was less efficiently cleaved to release β-endorphin. Expression of PC2 together with PC3 resulted in efficient conversion of β-lipotropin, as occurs in pituitary melanotrophs. Furthermore, coexpression of PC2 together with mouse POMC in bovine adrenomedullary chromaffin cells resulted in conversion of β-lipotropin to γ-lipotropin and β-endorphin in the regulated secretory pathway. Finally, the processing selectivities of PC3 and PC2 expressed together in BSC-40 cells were determined by using a series of mutant mouse POMCs containing all possible pairs of basic residues at certain sites. The observed pattern of cleavage site selectivities mimicked that of the endogenous endoproteases of the insulinoma and bovine adrenomedullary chromaffin cells, suggesting that PC2 and PC3 may represent important core endoproteases in the catalysis of prohormone processing in many neuroendocrine cell types.**

A common step required for the maturation of many biologically active proteins and peptides is endoproteolysis of larger precursor proteins, usually at pairs of basic amino acids (especially -Lys-Arg- and -Arg-Arg-) (1). This mechanism was initially inferred from the sequences of several endocrine and neuroendocrine precursor proteins, including proinsulin (2, 3) and the corticotropin (ACTH)/β-endorphin precursor proopiomelanocortin (POMC) (4). Subsequent studies have revealed a broad spectrum of precursor proteins, both endocrine and nonendocrine, that are endoproteolytically processed at pairs of basic amino acids (5–7). However, differences in the processing of neuroendocrine and nonneuroendocrine precursor proteins do exist. Most endocrine precursors are cleaved exclusively in regulated secretory pathways, whereas many other precursors (e.g., growth factor precursors) are cleaved in constitutive pathways (8, 9).

Many prohormones are processed in a tissue-specific manner. For example, mouse POMC (mPOMC) is cleaved in the anterior lobe (AL) of the pituitary to ACTH, β-lipotropin (β-LPH) and β-endorphin-(1–31) but is processed in the intermediate lobe (IL) to α-melanocyte-stimulating hormone (α-MSH), corticotropin-like intermediate lobe peptide (CLIP), and several carboxyl-shortened forms of β-endorphin (ref. 10; see Fig. 1). To address the biochemical basis for this tissue-specificity we identified two manipulable heterologous cell types that mimic the processing of mPOMC in the pituitary. Primary cultures of bovine adrenomedullary chromaffin (BAM) cells express and process transfected mPOMC to a set of peptides identical to those in AL corticotrophs (26), whereas expression of mPOMC in a rat insulinoma, Rin m5F, resulted in a set of cleavages reminiscent of IL melanotrophs (ref. 9; see Fig. 1). However, when processing a series of mutant mPOMCs containing altered cleavage sites, both the insulinoma and BAM cells exhibited the same selectivity in mutant cleavage-site use (11, 26), suggesting that they share a common core of endoproteases.

Several lines of evidence suggest that the mammalian-precursor-processing endoproteases are functionally similar to the product of the yeast *KEX2* gene, Kex2, required for excision of the peptide mating pheromone (α-factor) from its precursor (12). Kex2 is a Ca^{2+}-dependent serine protease that specifically cleaves on the carboxyl side of pairs of basic residues (-Lys-Arg- and -Arg-Arg-) (13). Coexpression of Kex2 with mPOMC in BSC-40 cells (a line incapable of processing this peptide precursor) resulted in efficient proteolysis at pairs of basic amino acids, producing authentic pituitary peptides, including γ-LPH and β-endorphin-(1–31) (14).

Recently, three mammalian DNA sequences, *fur*, PC2, and PC3 (called PC1 in ref. 15), have been reported that share significant structural similarity with the catalytic domain of Kex2 (15–20). RNA-hybridization studies demonstrate that furin is expressed in a wide variety of tissues and cell lines, including BSC-40 cells (21). In contrast, expression of PC3 and PC2 is restricted to neuroendocrine tissues and is not found in several cell lines shown incapable of processing prohormones, including BSC-40 cells (15, 18–20). PC3 is expressed at relatively high levels in anterior pituitary, AtT-20 cells (a mouse corticotroph tumor cell line), and bovine adrenal medulla, whereas lower mRNA levels are present in pancreatic beta cells and related cell lines (15, 19, 20). In contrast PC2 is expressed at high levels in pancreatic beta cells and in the IL of the pituitary (18, 20).

We recently demonstrated that the furin cDNA encodes a Golgi-localized endoprotease that can potentiate the endog-

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Abbreviations: POMC, proopiomelanocortin; mPOMC, mouse POMC; AL, anterior lobe; IL, intermediate lobe; BAM, bovine adrenomedullary chromaffin; β-LPH and γ-LPH, β- and γ-lipotropin, respectively; IrM, immunoreactive material; pfu, plaque-forming unit(s); VV, vaccinia virus; ACTH, corticotropin; α-MSH, α-melanocyte-stimulating hormone; hPC2, human PC2 protein; mPC3, mouse PC3 protein; VV:WT, VV:wild type.

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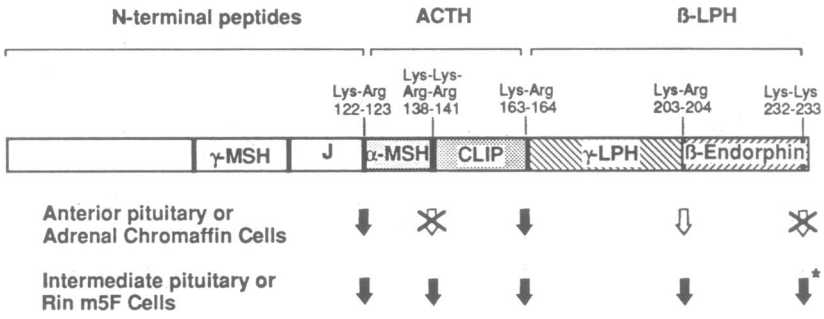

FIG. 1. Cell-type-specific processing of mPOMC. Shown is the cleavage-site use, in the ACTH and β-LPH domains, of POMC in pituitary AL corticotrophs and IL melanotrophs. Also depicted is the cleavage-site utilization of two heterologous cell types that mimic pituitary processing; transfected bovine adrenomedullary cells (BAM) and Rin m5F cells, a rat insulinoma (9, 11, 26). ⬇, Efficiently cleaved sites; ⇩, partially cleaved sites; ⊗, sites not cleaved. J, joining peptide; *, not processed in Rin m5F cells.

enous processing of pro-β-nerve growth factor in BSC-40 cells (21). Similar results were reported for the enhanced processing of the von Willebrand factor precursor (22, 23). In this report, we show that coexpression of mPOMC and PC3 in mammalian cells results in the production of peptides similar to those of AL corticotrophs, whereas coexpression with PC3 and PC2 together results in the IL pattern of cleavages. We also demonstrate that PC2 can cleave mPOMC in the regulated secretory pathway of BAM cells. Furthermore, we show that PC3 and PC2 can mimic the endoproteolytic selectivity for basic pairs previously found with mutant mPOMC substrates in insulinoma cells and chromaffin cells.

MATERIALS AND METHODS

Vaccinia Virus. Vaccinia virus (VV) strain WR was used in these studies. Full-length mouse PC3 (mPC3) and human PC2 (hPC2) inserts were isolated from plasmids pPC2 and pPC3 and ligated into the multiple cloning site of the vector pZVneo. The resulting constructs, pZVneo:hPC2 and pZVneo:mPC3, were used to introduce the hPC2 and mPC3 DNA sequences into the VV genome by homologous recombination (24). Viral recombinants directing expression of native and mutant mPOMC have been described (9, 11).

Cell Culture and Regulated Secretion. BSC-40 cells, an African green monkey kidney epithelial-like line, was maintained as described (14). BSC-40 cells do not express detectable levels of PC2 or PC3 mRNA (15). Primary cultures of BAM cells were established and maintained as described (26). Stimulated secretion was performed as described (26). All conditions were performed in triplicate.

Peptide Analysis. Samples were prepared, and RIAs were done, as described (9, 11). Antisera used in RIAs were directed against specific domains in mPOMC: FF, directed against the midportion of β-endorphin and cross-reacting with larger β-endorphin-containing peptides (such as β-LPH and POMC); Molly, directed against the midportion of γ-LPH but also cross-reacting with β-LPH and POMC; and Henrietta, directed against the midportion of ACTH and also cross-reacting with larger ACTH-containing peptides including POMC but not with processed forms of ACTH (i.e., α-MSH or CLIP). Peptides were resolved on a C_4-reversed-phase HPLC column (Vydac 214TP54) with an acetonitrile (Baker) gradient containing 0.1% trifluoroacetic acid (Pierce HPLC grade), as described (9, 11, 14). Peptides were resolved by cation-exchange HPLC (Nest WCX 1850-02), as described (9, 11). Molecular weights were estimated by migration on a Tricine/SDS/polyacrylamide gel, as described (9, 11). Sequence analysis of processed peptides was done by manual Edman degradation, as described (9, 11).

RESULTS

Cells coinfected with VV:mPOMC and VV:wild type (VV:WT) (Fig. 2*A*) secreted predominantly intact mPOMC, as evidenced by the prominent peak containing ACTH, γ-lipotropin (γ-LPH), and β-endorphin immunoreactive material (IrM). In contrast, coinfection with VV:mPOMC and VV:KEX2 resulted in greatly reduced amounts of intact mPOMC and the appearance of several prominent peaks of IrM that coeluted with authentic 13-kDa and 4.5-kDa ACTH, γ-LPH, and β-endorphin-(1–31) (Fig. 2*B*).

Analysis of the products secreted from cells coinfected with VV:mPOMC and VV:mPC3 (Fig. 2*C*) revealed that mPOMC was partially converted to peptides that coeluted with 13-kDa and 4.5-kDa ACTH, γ-LPH, and β-endorphin-(1–31). Unlike Kex2, processing of mPOMC by mPC3 yielded a major peak of γ-LPH and β-endorphin IrM coeluting with authentic β-LPH. The less abundant doublet of γ-LPH IrM eluting 2–6 min earlier than β-LPH was not identified.

In contrast, coinfection with VV:hPC2 produced a prominent peak of β-endorphin IrM coeluting with β-endorphin-(1–31) but no peaks of ACTH or γ-LPH immunoreactivity coeluting with ACTH or γ-LPH standards (Fig. 2*D*). Rather, ACTH and γ-LPH IrM coeluted later as a doublet (48 and 51 min). A third peak (55 min) containing β-endorphin IrM, in addition to ACTH and γ-LPH IrM, was also detected.

The peptide profile of cells that had been triple-infected with VV:mPOMC, VV:mPC3, and VV:hPC2 (Fig. 2*E*) was distinct from that seen in the medium from cells coexpressing mPOMC and either PC3 or PC2 individually. Peaks of immunoreactivity that coeluted with 13-kDa and 4.5-kDa ACTH, γ-LPH, and β-endorphin-(1–31) were detected. Note that the prominent peak coeluting with β-LPH in cells expressing only PC3 (Fig. 2*C*, 53 min) and the major doublet containing ACTH and γ-LPH IrM in cells expressing only PC2 (Fig. 2*D*, 48 and 51 min) were also greatly reduced. As a control, medium from cells coinfected with VV:mPOMC and VV:WT was transferred onto cells infected with either VV:KEX2, VV:mPC3, or VV:hPC2 for 4 hr. mPOMC in this medium remained intact, showing that the observed cleavages had not occurred after secretion.

The identities of the peptides that coeluted with 13-kDa and 4.5-kDa ACTH, γ-LPH, β-endorphin-(1–31), and β-LPH were confirmed by using a combination of biochemical and immunologic techniques coupled with N-terminal sequencing. By similar techniques each peak of the peptide doublet containing ACTH and γ-LPH IrM produced by cells coexpressing mPOMC and PC2 (Fig. 2*D*, 48 and 51 min) was identified as an ACTH/γ-LPH uncleaved intermediate containing glycosylated (48 min, 17 kDa) or nonglycosylated (51 min, 9.5 kDa) ACTH domains.

In addition to authentic γ-LPH, a minor earlier eluting peak of γ-LPH IrM was found in the medium of cells coexpressing mPOMC and either PC3 alone or PC3 and PC2 together (Fig. 2 *C* and *E*, 38 min). This peptide coeluted with the processing intermediate, lysyl-arginyl carboxyl-extended γ-LPH, on both the C_4 reversed-phase column and a cation-exchange HPLC column. Identification of β-endorphin-(1–31) and lysyl-arginyl carboxyl-extended γ-LPH demonstrates that, like Kex2, mPC3 can cleave on the carboxyl-terminal side of the paired basic sequence (residues 203 and 204).

Although Kex2, PC3, and PC2 all share high sequence similarity, each displays a specific cleavage-site selectivity.

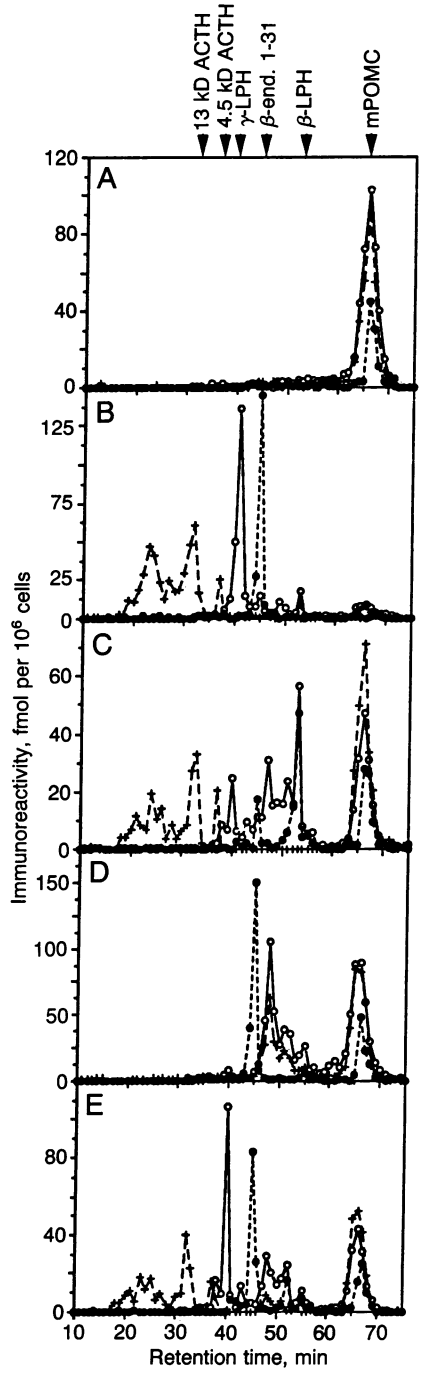

FIG. 2. mPOMC processing by Kex2, mPC3, and hPC2. Replicate 150-mm plates of BSC-40 cells (≈ 2×10^7 cells) were coinfected with VV recombinants expressing VV:mPOMC [2 plaque-forming units (pfu) per cell] and VV:WT (*A*), VV:KEX2 (*B*), VV:mPC3 (*C*), VV:hPC2 (each at 5 pfu per cell, 7 pfu per cell total) (*D*), or VV:mPC3 and VV:hPC2 together (each at 3 pfu per cell, 8 pfu per cell total) (*E*). After 16 hr, the medium was removed and replaced with serum-free minimal essential medium/0.07% bovine serum albumin. After an additional 2 hr, this medium was recovered, adjusted to 0.2% trifluoroacetic acid and applied to the C_4 reversed-phase column. Retained material was eluted with a gradient of acetonitrile, and 1-min fractions were collected and assayed for ACTH (+), β-endorphin (●), or γ-LPH (○) IrM. Elution positions of mPOMC-derived peptides from the mouse corticotroph cell line, AtT-20, are indicated.

Kex2 efficiently cleaves mPOMC at the -Lys-Arg- doublets flanking ACTH as well as at the -Lys-Arg- doublet at the γ-LPH/β-endorphin junction, producing ACTH, γ-LPH, and β-endorphin-(1–31) (refer to Fig. 1). PC3 also cleaves the two -Lys-Arg- doublets flanking ACTH but does not efficiently process the -Lys-Arg- at the γ-LPH/β-endorphin junction. As a result, ACTH and β-LPH are the major peptides released from the prohormone by PC3. PC2, on the other hand, cleaves mPOMC at the -Lys-Arg- flanking the amino terminus of ACTH and the -Lys-Arg- at the γ-LPH/β-endorphin junction but not at the -Lys-Arg- flanking the ACTH carboxyl terminus. Thus coexpression of PC2 and PC3 with mPOMC results in the efficient conversion of β-LPH to γ-LPH and β-endorphin-(1–31).

An important criterion for a *bona fide* prohormone endoprotease is that is must be able to function in the regulated secretory pathway of endocrine cells. We have demonstrated elsewhere that BAM cells efficiently cleave heterologous mPOMC at the -Lys-Arg- sites flanking ACTH and that processing is specific to the regulated secretory pathway (26). Therefore, BAM cells cannot be used to detect regulated pathway-specific cleavage of mPOMC by heterologous PC3. However, BAM cells cleave less than half the β-LPH produced at the γ-LPH/β-endorphin junction (refer to Fig. 1), so the activity of PC2 in the regulated secretory pathway of these cells could be assessed.

Replicate plates of BAM cell cultures were coinfected with VV:mPOMC and either VV:WT, VV:KEX2, or VV:hPC2. To simplify analysis, any mPOMC products present in the constitutive secretory pathway of either BAM cells or contaminating fibroblasts were chased from the cells before collecting secreted products; this was accomplished by treating the cultures for 2 hr with cycloheximide (16 hr after infection). A 30-min incubation with $BaCl_2$ elicited at least a 26-fold increase in secreted γ-LPH IrM over control samples, corresponding to a release of ≈40% of the intracellular mPOMC IrM. The γ-LPH:β-LPH ratio in medium samples from Ba^{2+}-treated cells coinfected with VV:mPOMC and either VV:WT or VV:KEX2 was very similar, demonstrating that Kex2 was unable to process the γ-LPH/β-endorphin junction in the regulated secretory pathway (Table 1). In contrast, the γ-LPH:β-LPH ratio in cells coexpressing mPOMC and hPC2 increased, showing that PC2 was capable of prohormone maturation in the regulated secretory pathway. None of these peptides were secreted in the absence of Ba^{2+}.

To determine whether PC3 and PC2 may represent the core endoproteases postulated in our earlier studies, the selectivity for cleavage of basic residue pairs of each enzyme was obtained by using the series of mPOMC mutants described (9, 11). As in insulinoma and BAM cells, all four permutations of lysine and arginine (-Lys-Arg-, -Arg-Arg-, -Arg-Lys-, and -Lys-Lys-) was determined to identify sequence preference

Table 1. Conversion of β-LPH to γ-LPH in the regulated pathway

	Ratio of γ-LPH to β-LPH in VV:mPOMC		
	+ VV:wild type	+ VV:KEX2	+ VV:hPC2
Experiment 1	0.51	0.52	1.14
Experiment 2	0.64	0.74	2.20

At 36 hr after plating, replicate plates of chromaffin cells were coinfected with VV:mPOMC (2 pfu per cell) and either VV:WT, VV:KEX2, or VV:hPC2 each at 5 pfu per cell (7 pfu per cell total). Sixteen hours after infection, cells were washed and incubated for 2 hr in medium containing cycloheximide at 50 μg/ml. The cells were again washed and incubated 45 min in Ca^{2+}-free balanced salt solution containing cycloheximide at 50 μg/ml with or without 3 mM $BaCl_2$. This medium was collected, adjusted to 0.1% trifluoroacetic acid, and resolved on the C_4 column, as described in Fig. 2. Aliquots of 1-min fractions were assayed for γ-LPH IrM, and the amount of γ-LPH IrM material coeluting with γ-LPH and β-LPH was measured. Extent of β-LPH conversion to γ-LPH is presented as a ratio of γ-LPH IrM in the γ-LPH peak to IrM in the β-LPH peak. No corresponding γ-LPH IrM peptides were detected in control samples (no Ba^{2+}).

of the processing enzymes. To control for positional effects, these four permutations were examined at two positions in the precursor: the -Lys-Arg- site at the ACTH/β-LPH junction and the -Lys-Lys- site within β-endorphin.

PC3 efficiently processed the native -Lys-Arg- doublet between ACTH and β-LPH, so the three mutant mPOMC molecules containing altered cleavage sites at the ACTH/β-LPH junction were coexpressed in BSC-40 cells with PC3, as described above (Fig. 3). K163R-mPOMC (-Lys-Arg- → -Arg-Arg-) was processed by PC3 identically to the native prohormone (data not shown). Processing of either R164K-mPOMC (-Lys-Arg- → -Lys-Lys-) or KR163RK-mPOMC (-Lys-Arg- → -Arg-Lys-) by PC3 resulted in normal levels of β-endorphin-(1–31) but almost no γ-LPH, β-LPH, or ACTH (Fig. 3). Instead, two peaks containing ACTH and γ-LPH IrM (eluting at 48 and 51 min) and two peaks containing ACTH, γ-LPH, and β-endorphin IrM (eluting at 55 and 57 min) were seen. As described above, the 48- and 51-min peaks were identified as ACTH/γ-LPH intermediates. The 55- and 57-min peaks migrated as single bands on SDS gels containing ACTH, γ-LPH, and β-endorphin IrM with apparent molecular masses of 19- and 14.5-kDa, respectively. These molecular masses agree with the expected molecular masses of glycosylated and nonglycosylated ACTH/β-LPH intermediates (the calculated molecular mass of nonglycosylated ACTH/β-LPH intermediate is 13 kDa). Thus, like both the Rin m5F and chromaffin cell endoprotease(s), PC3 efficiently processes -Lys-Arg- and -Arg-Arg- but does not process -Lys-Lys- or -Arg-Lys- at the ACTH/β-LPH junction. Coexpression of PC2 with PC3 and these mutant mPOMCs did not affect their cleavage (data not shown).

Processing of the β-endorphin cleavage site mutants by PC2 was then examined in BSC-40 cells. Coinfection with K232R-mPOMC (-Lys-Lys- → -Arg-Lys-) resulted in the same peptide profile as with native precursor (Fig. 4*A*). Carboxyl-shortened forms of β-endorphin [i.e., β-endorphin-(1–26) and β-endorphin-(1–27)] were not seen, indicating the mutant -Arg-Lys- site was not cleaved. In contrast, coinfection with K233R-mPOMC (-Lys-Lys- → -Lys-Arg-) resulted in the appearance of a new peak of β-endorphin IrM eluting at the position of β-endorphin-(1–27) (49 min), in addition to the peak of β-endorphin-(1–31) (45 min, Fig. 4*B*). Identity of this 49-min peptide was confirmed by demonstrating coelution with synthetic β-endorphin-(1–27) on cation-exchange

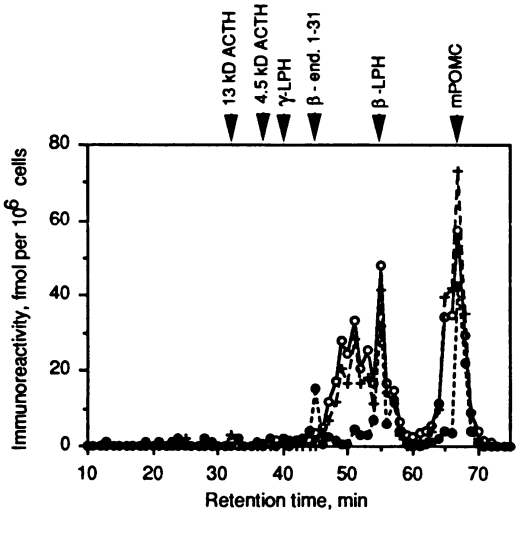

FIG. 3. Processing of R164K mPOMC (-Lys-Arg- → -Lys-Lys-) cleavage site mutant by PC3. BSC-40 cells were coinfected with VV:R164K-mPOMC (2 pfu per cell) and VV:mPC3 (5 pfu/cell, 7 pfu per cell total). Media samples were prepared and assayed as described in Fig. 2. Symbols denoting IrM are as for Fig. 2.

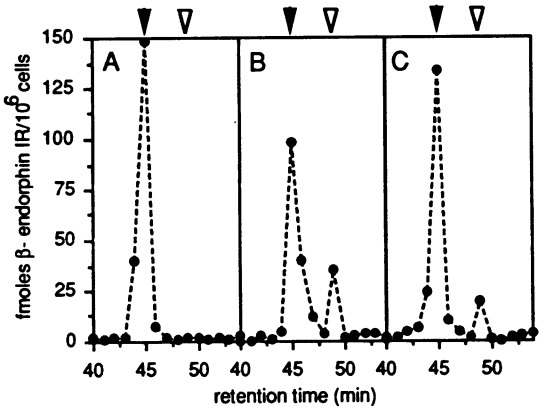

FIG. 4. Processing of carboxyl-terminal β-endorphin cleavage site mutants by PC2. (*A*) VV:K232R-mPOMC (-Lys-Lys- → -Arg-Lys-) coinfected with VV:hPC2. (*B*) VV:K233R-mPOMC (-Lys-Lys- → -Lys-Arg-) coinfected with VV:hPC2. (*C*) VV:KK232RR-mPOMC (-Lys-Lys- → -Arg-Arg-) coinfected with VV:hPC2. Only β-endorphin (end) IrM profile is depicted; ACTH and γ-LPH IrM profiles were unaffected by the mutations. See legends to Figs. 2 and 3 for experimental details. ▼, β-end 1–31; ▽, β-end 1–27.

HPLC (data not shown). Coinfection with KK233RR-mPOMC (-Lys-Lys- → -Arg-Arg-) resulted in a less efficient conversion of β-endorphin-(1–31) to β-endorphin-(1–27) (Fig. 4*C*). For both mutant precursors, the ACTH and γ-LPH IrM peptide profiles were identical to that of native mPOMC (data not shown). Only β-endorphin processing was affected. Again, as with the endogenous Rin m5F and BAM cell endoproteases, PC2 preferentially cleaved -Lys-Arg- and -Arg-Arg- sites at the β-endorphin carboxyl terminus but processed -Lys-Arg- more efficiently than -Arg-Arg-. Addition of PC3 in these experiments did not alter processing of these mutant cleavage sites.

DISCUSSION

In this report, we demonstrate that expression of the candidate mammalian prohormone endoproteases hPC2 and mPC3 in BSC-40 cells promotes the processing of a model prohormone, mPOMC, at paired basic amino acid sequences. The predominant peptides produced upon coexpression of mPOMC and mPC3 were ACTH and β-LPH, much like pituitary corticotrophs and VV:mPOMC-infected BAM cells (refer to Fig. 1). Efficient processing of the two -Lys-Arg- sites flanking the ACTH domain but inefficient processing of the -Lys-Arg- within β-LPH (γ-LPH/β-endorphin junction) is thus indicated. The prominent peptide products secreted from BSC-40 cells coexpressing mPOMC and PC2 included β-endorphin-(1–31) and an ACTH/γ-LPH intermediate, demonstrating efficient processing of -Lys-Arg- sites at the ACTH amino terminus and at the γ-LPH/β-endorphin junction but not at the -Lys-Arg- site at the ACTH carboxyl terminus (Fig. 2*D*). Together mPC3 and hPC2 cleaved the prohormone in a concerted manner; β-LPH was efficiently processed to γ-LPH and β-endorphin-(1–31) (Fig. 2*E*) much as occurs in pituitary IL melanotrophs (10) and VV:mPOMC-infected insulinoma cells (9, 11).

These results are consistent with a model for tissue-specific processing of POMC based on modulation of PC2 levels. PC3 alone appears to yield the processing phenotype of anterior pituitary (and BAM cells), whereas expression of hPC2, in addition, contributes to the IL (and insulinoma)-processing phenotype. Support for this model is found in the tissue distribution of the two proteases; PC3 is present in both the AL and IL of the pituitary, whereas PC2 is preferentially expressed in the IL (20).

Processing of mPOMC (and other prohormones) normally occurs in the regulated secretory pathway of neuroendocrine cells. To determine whether either hPC2 or mPC3 could catalyze conversion in this pathway, BAM cells were developed as a model system. BAM cells express higher endogenous levels of PC3 than PC2 RNA and process mPOMC to a set of peptides identical to those produced by corticotrophs. Thus, the proposed model for tissue-specific processing would predict that increased PC2 expression would convert the processing phenotype of BAM cells (corticotroph-like) to an IL melanotroph-type phenotype. Indeed, coexpression of mPOMC and hPC2 in BAM cells resulted in the enhanced conversion of β-LPH to γ-LPH in the regulated secretory pathway (Table 1). Kex2, which can also cleave β-LPH in the BSC-40 cell constitutive pathway (Fig. 2*B*), had no effect on β-LPH processing in the regulated pathway, demonstrating that PC2 is, indeed, adapted for processing in the regulated pathway. However, quantitative conversion from a corticotroph-like to a melanotroph-like phenotype could not be effected in BAM cells, although foreign proteins are efficiently expressed when using VV vectors. Tissue-specific processing may, therefore, be a two-tiered event, involving both cleavage-site structure and accessibility acting in conjunction with differential expression of a core of processing enzymes.

Several cleavages seen in both IL melanotrophs and transfected Rin m5F cells were not efficiently catalyzed by PC2 and PC3 in transfected BSC-40 cells. These cleavages include the tetrabasic sequence in ACTH (to yield α-MSH and CLIP peptides) and the -Lys-Lys- near the carboxyl terminus of β-endorphin (this site is not cleaved in insulinoma cells). Because the ACTH antiserum used in these studies cross-reacts only with ACTH-related peptides containing the intact tetrabasic sequence, it would not efficiently detect cleavage at the tetrabasic site. One explanation for lack of efficient ACTH processing to αMSH is that this cleavage is executed by a distinct enzyme. However, recent studies *in vitro* show that a Ca^{2+}-dependent protease isolated from rat insulinoma secretory vesicles, the type II endoprotease (25), efficiently releases β-endorphin-(1–31) and an ACTH/γ-LPH intermediate peptide from the mPOMC precursor (peptides produced by PC2 *in vivo*) and also cleaves the ACTH tetrabasic sequence yielding α-MSH and CLIP. (C. Rhodes, B.A.T., G.T., and J. Hutton, unpublished work).

With the series of mutant mPOMC cleavage sites, striking similarities in the processing selectivities of BAM and Rin m5F cells for basic pairs were observed (Table 2), suggesting use of a common core of enzymes. Neither -Lys-Lys- nor -Arg-Lys- sites in mPOMC served as efficient substrates for either enzyme. -Arg-Arg- was cleaved as efficiently as -Lys-Arg- when placed at the ACTH/β-LPH junction but was cleaved less efficiently than -Lys-Arg- when placed near the carboxyl terminus of β-endorphin. However, both PC3 (ACTH/β-LPH cleavage) and PC2 (β-endorphin cleavage) were required to fully reconstruct the selectivity profile of the insulinoma and chromaffin-cell endoproteases. Thus, the processing selectivities of PC3 and PC2, along with their tissue distribution, are consistent with their proposed role as a core set of endoproteases responsible for prohormone maturation in a number of neuroendocrine cells.

Table 2. Processing selectivities of mPC3 and hPC2

Virus	Cleavage site	Rin m5F cells	BAM cells	PC3 (BSC-40)
	ACTH/β-LPH junction			
VV:mPOMC	(-Lys-Arg-, native)	++*	++*	++†
VV:K163R	(-Arg-Arg-)	++*	++*	++†
VV:R164K	(-Lys-Lys-)	–	–	–
VV:KR163RK	(-Arg-Lys-)	–	–	–
	Carboxyl terminus of β-endorphin			
VV:mPOMC	(-Lys-Lys-, native)	–	–	–
VV:K233R	(-Lys-Arg-)	++*	+‡	+§
VV:KK232RR	(-Arg-Arg-)	+†	+/–§	+/–¶
VV:K232R	(-Arg-Lys-)	–	–	–

Data were compiled from Figs. 3 and 4 and refs. 11 and 26. ++, Efficient cleavage; +, less-efficient cleavage; +/–, partial cleavage; –, inefficient cleavage. *90% processing; †50% processing; ‡75% processing; §25% processing; ¶10% processing.

Note Added in Proof: RIA analysis of the peptides secreted from transfected BAM cells (Table 1) using an amide-specific αMSH antiserum (Y. P. Loh, National Institutes of Health) revealed that only Ba^{2+}-treated cells coexpressing mPOMC and hPC2 produced authentic α-MSH peptide. These results demonstrate that, in addition to cleaving β-LPH, hPC2 can also cleave the ACTH tetrabasic sequence in the regulated secretory pathway.

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