The Prohormone Convertases PC1 and PC2 Mediate Distinct Endoproteolytic Cleavages in a Strict Temporal Order during Proopiomelanocortin Biosynthetic Processing*

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The biosynthesis of peptide hormones and neuropeptides begins with endoproteolytic cleavages of high molecular weight precursors, followed by exoproteolytic removal of basic amino acids from the ends of the peptides and later posttranslational modifications such as α-amidation and formation of pyroglutamic acid residues (1–8). PC1 (also called PC3) and PC2 are the leading candidates to mediate the endoproteolytic processing of POMC in secretory granules of pituitary corticotropes and melanotropes (see Fig. 1) (9–20).

Two subtilisin-like endoproteases called PC1 and PC2 are distributed in a tissue-specific manner in the pituitary and in the brain. AtT-20 cells and corticotropes of the anterior pituitary express primarily PC1 and perform a limited number of cleavages of the proopiomelanocortin (POMC) precursor during biosynthesis. Melanotropes of the intermediate pituitary express both PC1 and PC2 and perform a more extensive set of cleavages during the biosynthetic processing of POMC. To investigate the role of PC2 in the biosynthetic processing of POMC, AtT-20 mouse corticotropes were stably transfected with a full length PC2 cDNA. The AtT-20 cells expressing PC2 acquired the ability to perform all the additional cleavages seen in the intermediate pituitary, but did not acquire the ability to α-N-acetylate the product peptides. The kinetics of the earliest steps in biosynthetic processing were unaltered by the expression of PC2, and the changes due to PC2 expression were seen only in the middle and late steps in biosynthetic processing. Thus, both the identity of the final product peptides and the kinetics of the processing steps in the AtT-20 cells expressing PC2 fit the patterns expected for melanotropes of the intermediate pituitary.

POMC processing is cell type-specific in the pituitary, and the distribution of PC1 and PC2 mRNAs might explain the differences in processing: corticotropes express primarily PC1 mRNA, while melanotropes express high levels of PC2 mRNA along with PC1 mRNA. AtT-20 cells are a mouse corticotrope cell line which expresses almost exclusively PC1 and has been extensively documented as a model for anterior pituitary corticotropes (2, 21).

As has been found for most prohormones, all of the cleavages of the POMC precursor occur at pairs of basic amino acids (see Fig. 1) (2, 22–26). The initial steps of POMC processing (steps 1, 2, and 3 in Fig. 1) occur with similar kinetics in both lobes of the pituitary. Cleavage of βLPH to make β-endorphin (step 4) occurs in both lobes of the pituitary, but melanotropes of the intermediate pituitary carry out much more extensive cleavage at this site. Two of the endoproteolytic cleavages that take place very late in the biosynthetic pathway occur only in intermediate pituitary melanotropes and in the brain and do not occur in anterior pituitary corticotropes: step 6 forms Lysoγ-MSH and step 7 forms β-endorphin (1–27). Another biosynthetic step which is restricted to melanotropes and to neurons in the nucleus of the solitary tract is the α-N-acetylation of ACTH and β-endorphin (2, 24).

Previous studies using the vaccinia virus system to express PC1 and PC2 have shown that both candidate endoproteases can mediate a number of cleavages of the POMC precursor, but did not fully explain the differences in cleavages seen between corticotropes and melanotropes in vivo (27–29). In particular, since the cleavages to produce the melanotrope-specific products, Lysoγ-MSH and β-endorphin (1–27), were undetectable in cells expressing both PC1 and PC2, there remained the important question of whether additional prohormone convertases needed to be identified to understand POMC processing in the pituitary and brain.

In order to avoid any potential problems that might arise from the disruption of cellular functions associated with vaccinia infection (30) and to create cell lines which could be used to investigate PC1 and PC2 function in depth, we wished to create stable AtT-20 lines with altered levels of PC1 and PC2 expression. AtT-20 cells have a high level of PC1 mRNA and protein and extremely low levels of PC2 mRNA (7, 13, 14, 21). We used AtT-20 cells whose PC1 levels had been reduced by expression of antisense RNA to PC1 to demonstrate the crucial role of PC1 in the early steps in POMC processing (13). Our initial work, which utilized an expression vector driven by the mouse metallothionein-1 promoter, did not succeed at getting adequate PC2 expression in AtT-20 cells to decide whether PC2 had a role in peptide processing. We have found that an expression vector using the human cytomegalovirus promoter (31) produces levels of expression.
of various rat peptidylglycine α-amidating monoxygenase (EC 1.14.17.3) constructs in AtT-20 cells. Therefore, we used this expression vector to make stable AtT-20 lines expressing elevated levels of PC2, so that we could investigate possible changes in POMC peptide processing in a cell line whose ability to make secretory granules, and store products for later secretion had not been compromised.

In this work, biosynthetic labeling demonstrates that the earliest cleavages of POMC seen in corticotropes (Fig. 1) are unaltered by overexpression of PC2, while later cleavages normally only found in melanotropes are induced by expression of PC2. Identification of the product peptides showed that all of the endoproteolytic cleavages unique to melanotropes are accurately produced by AtT-20 cells expressing PC2, while α-N-acetylation is not induced by expression of PC2.

Even the kinetic patterns of the cleavages seen in melanotropes are matched upon expression of PC2 in AtT-20 cells, with some cleavages happening rapidly and others taking many hours to occur. Finally, studies of secretion showed that the new peptides produced by AtT-20 cells expressing PC2 are stored in a compartment from which secretion can be stimulated by secretagogues.

**MATERIALS AND METHODS**

Establishment of Stable Cell Lines Expressing Rat PC2—Starting with the pBluescript plasmid encoding the full sized rat PC2 (13), the cDNA was cut 3′ to the stop codon at nucleotide 2558 with BsaXI, the ends of the DNA were made blunt by treatment with S1 nuclease, and then XbaI was used to obtain the full insert. The 2-kb fragment was inserted into an expression vector which utilizes the cytomegalovirus promoter (pCIS.CXXNH, kindly provided by Dr. Cornelia Gorman, Genentech) (31) prepared by digestion with XbaI and HpaI, creating the expression plasmid pCIS.sPC2; the orientation was verified by restriction mapping. The plasmid was cotransfected along with the pMt.neo-1 plasmid into AtT-20 cells using the lipofection method, enabling drug selection with G418 (32). Drug-resistant cell lines were screened by Northern blot analyses using a probe for PC2 mRNA (13). Poly(A)⁺ RNA was prepared using the PolyAtract kit (Promega) following the manufacturer’s instructions.

Biosynthetic Labeling and Analyses of POMC Peptides—Wild-type AtT-20 and cell lines expressing the full length rat PC2 mRNA in the sense orientation were labeled with [3H]tryptophan (47 Ci/mmol; [3H]tyrosine (28 Ci/mmol; [3H]methionine (557 Ci/mmol; [3H]tryptophan (28 Ci/mmol; 10 μCi), or [35S]methionine as the wild-type cells, and several times more PC2 mRNA than the intermediate pituitary. The expression of high levels of PC2 mRNA was to determine whether the cleavages in POMC that are unique to melanotropes and do not occur in corticotropes (Fig. 1) could be introduced by overexpression of PC2 in this corticotrope cell line. Using the pCIS.sPC2 construct, two independent cell lines expressing high levels of PC2 mRNA were established by Northern analysis; both exhibited similar alterations in POMC processing, storage, and secretion and have been stable for over 5 months. Additional drug-resistant lines from the same transfections, which did not express significant amounts of PC2 mRNA, did not show changes in POMC metabolism. When poly(A)⁺ RNA was prepared in order to allow analysis of larger amounts of mRNA than tested previously (13), a faint PC2 mRNA signal was detected in wild-type AtT-20 cells; the best pCIS.sPC2 cell line expressed 50~100 times as much PC2 mRNA as the wild-type cells, and several times more PC2 mRNA than the intermediate pituitary. The expression of endogenous PC1 mRNA was not altered by the expression of transfected PC2 mRNA (data not shown).

Changes in the POMC Products in Cells Expressing PC2—Fig. 2 shows the results of analyzing cell extracts from wild-type (wt) and PC2 (sPC2) cell lines incubated in medium containing [3H]Tyr for 8 h. Since AtT-20 cells secrete quite rapidly, newly synthesized products are recovered from both cells and medium after 8 h. The expression of PC2 resulted in the appearance of a major peak of α-MSH-sized material and a major loss in the ACTH (1-39) peaks (glycosylated and non-glycosylated) (Fig. 2, top). The expression of PC2 also

![Fig. 1. The mouse POMC precursor and cleavage sites.](image-url)
possibility that it would not be possible to stimulate secretion above the high basal rate. To investigate this question, wild-type and then chased in nonradioactive medium for four consecutive 1-h periods. The higher rate of secretion by wild-type AtT-20 cells. Only the rate of secretion increased rates of secretion were seen with two independent lines expressing PC2.

Expression of PC2 also led to changes in the newly synthesized POMC peptides seen in the culture medium. Fig. 3 demonstrates that the basal rate of secretion of αMSH-sized peptides, ACTH, and glycosylated ACTH, and β-endorphin-sized peptides by PC2 cells was consistently more rapid than secretion by wild-type AtT-20 cells. Only the rate of secretion of POMC was not altered by the expression of PC2. Similarly increased rates of secretion were seen with two independent lines expressing PC2.

One of the important characteristics of neuroendocrine cells is their ability to store peptides for extended times and then release the peptides when stimulated. The higher rate of basal secretion in PC2 cells than in wild-type cells raised the possibility that it would not be possible to stimulate secretion above the high basal rate. To investigate this question, wild-type and PC2 cells were incubated with [35S]Met for 30 min and then chased in nonradioactive medium for four consecutive 1-h periods. During the third period, cells were stimulated using 10 nM phorbol ester (as in Ref. 32). Samples of medium were immunoprecipitated with the N-ACTH antiserum, β-endorphin antiserum, and γMSH antiserum, and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4). Medium from the wild-type cells showed the expected pattern with all antisera; the secretion of labeled POMC and ABI decreased (since the cells were being chased) and primarily the two forms of ACTH(1-39) were secreted in response to the secretagogue. The secretion of POMC and ABI from the PC2 cells showed a similar pattern through the experiment (Fig. 4, bottom). However, unlike the wild-type cells, PC2 cells did not show a major stimulation of secretion of the two forms of ACTH(1-39), and instead showed a major stimulation of the secretion of αMSH-sized material (Fig. 4, bottom). Results obtained with the β-endorphin antiserum showed that PC2 cells secreted primarily β-endorphin-sized peptides in response to the secretagogue, while the wild-type cells secreted a major peak the size of glycosylated γMSH in response to secretagogue (not shown).

Identification of the POMC Products Whose Production Was Increased by Expression of PC2—To identify the peptide product comigrating with ACTH(1-39)NH2 and αMSH in cells expressing PC2, the ACTH(1-39)NH2-sized material was isolated from cells incubated in medium containing [35S]Met for 30 min and then chased in nonradioactive medium for four consecutive 1-h periods. The higher rate of basal secretion in PC2 cells than in wild-type cells raised the possibility that it would not be possible to stimulate secretion above the high basal rate. To investigate this question, wild-type and PC2 cells were incubated with [35S]Met for 30 min and then chased in nonradioactive medium for four consecutive 1-h periods. During the third period, cells were stimulated using 10 nM phorbol ester (as in Ref. 32). Samples of medium were immunoprecipitated with the N-ACTH antiserum, β-endorphin antiserum, and γMSH antiserum, and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4). Medium from the wild-type cells showed the expected pattern with all antisera; the secretion of labeled POMC and ABI decreased (since the cells were being chased) and primarily the two forms of ACTH(1-39) were secreted in response to the secretagogue. The secretion of POMC and ABI from the PC2 cells showed a similar pattern through the experiment (Fig. 4, bottom). However, unlike the wild-type cells, PC2 cells did not show a major stimulation of secretion of the two forms of ACTH(1-39), and instead showed a major stimulation of the secretion of αMSH-sized material (Fig. 4, bottom). Results obtained with the β-endorphin antiserum showed that PC2 cells secreted primarily β-endorphin-sized peptides in response to the secretagogue, while the wild-type cells secreted a major peak the size of glycosylated γMSH in response to secretagogue (not shown).

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three experiments using the \( \alpha \)-endorphin and \( \beta \)MSH antisera. The medium was analyzed as in Fig. 3, using the N-ACTH antiserum with samples of wild-type (top) and PC2 (bottom) medium. Basal secretion data from the second hour of chase are the same as in Fig. 2, and the stimulated (stim) samples are from the third hour. Similar results were seen with the slab gel technique in two other experiments examining stimulated secretion and in all studies in Fig. 6 (top) were collected and digested with chymotrypsin. Both peaks of \( \beta \)-endorphin-sized material yielded a \( [\text{3H}] \)Tyr-labeled peptide that comigrated with Tyr-Gly-Gly-Phe, the chymotryptic product from nonacetylated forms of \( \beta \)-endorphin, not with \( \alpha \)-N-acetyl-Tyr, the chymotryptic product from acetylated forms of \( \beta \)-endorphin (35). Thus the expression of PC2 led to cleavage of the Lys-Lys bond near the COOH-terminus of \( \beta \)-endorphin, a cleavage found in the intermediate pituitary but not in cortical cortotropes (Fig. 1). No evidence for \( \alpha \)-N-acetylation of ACTH or \( \beta \)-endorphin was detected in cells expressing PC2. The \( \gamma \)MSH-sized material synthesized by the PC2 cells was prepared by immunoprecipitation and gel filtration. Radioactive microsequencing of [\( ^{35} \text{S} \)]Met-labeled material showed the release of radiolabeled [\( ^{35} \text{S} \)]Met at the fourth cycle (not shown), consistent with the sequence KYVMGH... as expected for Lys-\( \gamma \)MSH, the \( \gamma \)MSH peptide found in the intermediate pituitary but not in the anterior pituitary (25). Thus AtT-20 cells expressing PC2 along with the endogenous PC1 carry out all of the endoproteolytic cleavages unique to melanotropes.

Changes in the Kinetics of POMC Biosynthetic Processing in Cells Expressing PC2—The above data establish that the

![Fig. 4. Stimulated secretion from wild-type AtT-20 cells and cells expressing PC2. Wild-type and PC2 AtT-20 cells were labeled and chased as in Fig. 3; during the third 1-h collection from the medium, 10 mM phorbol myristate acetate was included to stimulate secretion. The medium was analyzed as in Fig. 3, using the N-ACTH antiserum with samples of wild-type (top) and PC2 (bottom) medium. Basal secretion data from the second hour of chase are the same as in Fig. 2, and the stimulated (stim) samples are from the third hour. Similar results were seen with the slab gel technique in two other experiments examining stimulated secretion and in all three experiments using the \( \beta \)-endorphin and \( \gamma \)MSH antisera.](image4)

![Fig. 5. Identification of the \( \alpha \)MSH-sized peak as ACTH(1–13)NH\(_2\). Wild-type and PC2 cells were incubated with [\( ^{3} \text{H} \)]Tyr as in Fig. 2, and the \( \alpha \)MSH-sized material was prepared by immunoprecipitation and gel filtration. The intact labeled material was analyzed by RP-HPLC with 20 \( \mu \)g each of synthetic \( \alpha \)MSH and ACTH(1–13)NH\(_2\) added; the marker peptides were detected by absorbance at 220 nm. The chymotryptic digests of the same material were analyzed with 50 \( \mu \)g each of synthetic Ser-Tyr and \( \alpha \)-N-acetyl-Ser-Tyr (Ac-Ser-Tyr) added; marker peptides were detected by absorbance at 280 nm. The analyses of intact ACTH(1–13)NH\(_2\) were confirmed with an additional [\( ^{3} \text{H} \)]Tyr-labeled sample and with a [\( ^{35} \text{S} \)]Met-labeled sample.](image5)

Tyr, the product expected from \( \alpha \)MSH (Fig. 5, bottom) (36). These data show that AtT-20 cells expressing PC2 greatly increased the cleavage of ACTH(1–39) to create ACTH(1–13)NH\(_2\), but that no \( \alpha \)-N-acetylation of ACTH(1–13)NH\(_2\) was seen.

To investigate the forms of \( \beta \)-endorphin produced in cells expressing PC2, the \( \beta \)-endorphin-sized material prepared by cells incubated with [\( ^{3} \text{H} \)]Tyr was isolated and then analyzed by cation exchange HPLC with synthetic marker peptides included (23). As expected from our previous work (35), wild-type cells were found to produce only material comigrating with intact, unmodified \( \beta \)-endorphin(1–31) (Fig. 6, top). The \( \beta \)-endorphin-sized material prepared by PC2 cells was fractionated into peaks comigrating with \( \beta \)-endorphin(1–31) and \( \beta \)-endorphin(1–27); no peak of radioactivity comigrated with acetylated \( \beta \)-endorphin(1–27) or with acetylated \( \beta \)-endorphin(1–31). The two [\( ^{3} \text{H} \)]Tyr-labeled peaks from the PC2 cells in Fig. 6 (top) were collected and digested with chymotrypsin. Both peaks of \( \beta \)-endorphin-sized material yielded a [\( ^{3} \text{H} \)]Tyr-labeled peptide that comigrated with Tyr-Gly-Gly-Phe, the chymotryptic product from nonacetylated forms of \( \beta \)-endorphin, not with \( \alpha \)-N-acetyl-Tyr, the chymotryptic product from acetylated forms of \( \beta \)-endorphin (35). Thus the expression of PC2 led to cleavage of the Lys-Lys bond near the COOH-terminus of \( \beta \)-endorphin, a cleavage found in the intermediate pituitary but not in the anterior pituitary (25). Thus AtT-20 cells expressing PC2 along with the endogenous PC1 carry out all of the endoproteolytic cleavages unique to melanotropes.

![Fig. 6. Identification of \( \beta \)-endorphin(1–27) in PC2 AtT-20 cells. Wild-type and PC2 cells were incubated with [\( ^{3} \text{H} \)]Tyr as in Fig. 2, and the \( \beta \)-endorphin-sized material was prepared by immunoprecipitation and gel filtration. The intact labeled material was analyzed by cation exchange chromatography with 15 \( \mu \)g of each of the indicated marker peptides added; similar results were obtained with peptides labeled with [\( ^{35} \text{S} \)]Met. The material from the PC2 cells comigrating with \( \beta \)-endorphin(1–27) and \( \beta \)-endorphin(1–31) was dried, desalted with a Sep-Pak, digested with chymotrypsin, and analyzed with 50 \( \mu \)g of the indicated markers added. Marker peptides were detected by absorbance at 280 nm.](image6)
expression of PC2 leads to several changes in the biosynthesis and secretion of smaller products from POMC, and the steady-state labeling pattern suggests that the effects of PC2 are restricted to a subset of POMC-derived peptides, with no major changes in the steady-state intracellular levels of POMC, ABI, and joining peptide. To investigate the kinetics of biosynthetic processing, wild-type and PC2 cells were labeled with [3H]Tyr for 30 min and then further incubated in nonradioactive medium (chased) for various periods of time; an example of such an experiment is shown in Fig. 7. At the end of the pulse period and throughout the chase, the amounts of POMC precursor and ACTH biosynthetic intermediate were unaltered by the expression of PC2. Although glycosylated and nonglycosylated ACTH(1-39) accumulated in the wild-type cells, they did not accumulate in the PC2 cells. Instead, the PC2 cells accumulated a peak of ACTH(1-13)NH₂. The rate of conversion of βLPH to β-endorphin was also increased substantially by the expression of PC2. As noted above, the expression of PC2 was correlated with increased basal secretion of both ACTH(1-39) and of ACTH(1-13)NH₂, leading to higher levels of these peptides in the medium of PC2 cells compared to wild-type cells.

When the total radioactivity in each peak was calculated for each of the pulse-chase experiments and plotted as a function of the time of chase, the data clearly showed that the disappearance of POMC and the transient appearance and then disappearance of ABI were unaltered by the expression of PC2 (Fig. 8). The disappearance of POMC followed the same time course when determined using the endorphin antisemur; POMC disappearance followed a single exponential curve with a half-life of 30 min, as found previously (41). The accumulation of ACTH(1-39) (the sum of glycosylated and nonglycosylated forms) showed the expected pattern in the wild-type cells, while in the PC2 cells, ACTH(1-39) had the kinetics of a biosynthetic intermediate in the pathway to ACTH(1-13)NH₂. There is a significant amount of secretion of ACTH(1-39) and ACTH(1-13)NH₂ in the 2-h chase, especially from the PC2 cells. When the corresponding plots were made for POMC, βLPH, and β-endorphin from the endorphin immunoprecipitation and SDS-polyacrylamide gel electrophoresis analyses, the wild-type cells could be seen to accumulate primarily βLPH, while the PC2 cells accumulated β-endorphin (not shown). The equimolar production of N-ACTH-related and β-endorphin-related product peptides from the POMC precursor (42) was not altered by the expression of PC2; only the identity of the peptides and the rate of secretion were changed by the expression of PC2. Thus the expression of PC2 had no effect on cleavages 1, 2, and 3 (Fig. 1), but greatly enhanced cleavages 4 and 5.

Previous studies demonstrated that melanotropes carry out cleavages 6 (to form Lyso-γ₃MSH) and 7 (to form β-endorphin(1-27)) much later in the biosynthetic pathway than the other cleavages (2, 22, 25, 42). In order to determine whether PC2 cells exhibited a similar time course as melanotropes, wild-type and PC2 cells were labeled for 30 min and chased for 2 h in nonradioactive medium as in Fig. 7. Although a significant amount of ACTH(1-13)NH₂ was present at this time, no clear peak of γ₃MSH or β-endorphin(1-27) was found in the PC2 cells after 2 h of chase (not shown). Therefore, longer labeling and chase times were used; when wild-type and PC2 cells were labeled for 2 h in [3H]Tyr (Fig. 9, top), again no clear peak of γ₃MSH was seen in the PC2 cells. After 2 h of chase (i.e. 4 h from the start of labeling), a peak at the position of γ₃MSH was seen in the PC2 cells. This peak continued to increase at longer chase times (Fig. 9, bottom). Similar kinetics were seen for the appearance of β-endorphin(1-27) (not shown). Thus, new cleavages due to the expression of PC2 (steps 6 and 7 in Fig. 1) only became apparent after 2-4 h from the start of labeling.

**DISCUSSION**

In order to investigate the role of the candidate endoprotease PC2 in propeptide biosynthetic processing, AtT-20 mouse pituitary corticotrope cells were stably transfected with a vector encoding rat PC2. Similar studies have defined the specificity of the endoprotease furin (43-47). Expression of PC2 led to the appearance of two endoproteolytic cleavages observed in intermediate pituitary melanotropes but not in anterior pituitary corticotropes (summarized in Fig. 10): cleavage 7 occurs at the Lys-Lys near the COOH-terminal end of β-endorphin(1-31) and produces β-endorphin(1-27); cleavage 6 occurs within the Arg-Lys preceding γ₃MSH to

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Figure 7. Kinetic analysis of peptide processing in wild-type and PC2 AtT-20 cells. Wild-type (filled symbols) and PC2 (empty symbols) AtT-20 cells were incubated in medium containing [3H]Tyr for 30 min and then chased for the indicated times in nonradioactive medium. Cell extracts (corresponding to 3.4 × 10⁶ cpm of trichloroacetic acid-precipitable material in the pulse extracts) were analyzed as in Fig. 2 using the N-ACTH antiserum (first four panels) or the β-endorphin antiserum (last panel). Chase medium, containing the peptides secreted under basal conditions, was also analyzed from the 2-h chase time with the N-ACTH antiserum (fifth panel). Similar pulse-chase data were obtained in two experiments with [3H]Tyr labeling and two other experiments with [35S]Met labeling.
Prohormone Convertases Mediate Endoproteolytic Cleavages

Results of N-ACTH immunoprecipitation analyses from two identical experiments of the type described in Fig. 7 were averaged and plotted for both wild-type (filled symbols) and PC2 cells (empty symbols). For simplicity of presentation, error bars between the experiments are only shown for the POMC profile. The sum of glycosylated and nonglycosylated ACTH(1-39) is plotted simply as ACTH, since previous work (2) are summarized. Data from peptides released into the media are also indicated.

Wild-type and PC2 AtT-20 cells were labeled in medium containing [3H]Tyr for 2 h and then chased for the indicated times in nonradioactive medium. Cell extracts (corresponding to 6.3 × 10⁶ cpm of trichloroacetic acid-precipitable material in the pulse extracts) were analyzed as in Fig. 2 using the γMSH antiserum. Similar results were seen in two other experiments.

The secretion of all of the smaller peptides whose synthesis is proven useful in previous work (13,50). The function of PC1 and PC2 has been studied by transiently infecting cells using the vaccinia virus system (27,28). Using this system, PC1 and PC2 were shown to perform some of the cleavages normally seen in POMC processing, such as cleavage 2 (Fig. 1). There is agreement from the vaccinia expression data (27,28) and our antisense RNA data (13) that cleavages 1 and 2 can be mediated by PC1. While previous studies agreed that cleavages 2, 3, and 5 could be performed by PC2, they disagreed on whether cleavages 1 and 4 could be performed by PC2, nor the KK (Lys-Lys) cleavage to produce Lyso-γ₁-MSH (cleavage 6) nor the KK (Lys-Lys) cleavage to produce β-endorphin(1-27) (cleavage 7); cleavages at these sites occur in intermediate pituitary and brain (2) (Fig. 1). One of the major advantages of the vaccinia virus system is that much of the normal cellular protein synthesis is shut down by the vaccinia infection (27-30), and for many purposes the high level of production of protein achieved with the vaccinia expression system is very useful. However, for studies of cellular function, the suppression of cellular protein syn-

FIG. 8. Summary of the kinetics of peptide biosynthetic processing in wild-type and PC2 AtT-20 cells. Results of N-ACTH immunoprecipitation analyses from two identical experiments of the type described in Fig. 7 were averaged and plotted for both wild-type (filled symbols) and PC2 cells (empty symbols). For simplicity of presentation, error bars between the experiments are only shown for the POMC profile. The sum of glycosylated and nonglycosylated ACTH(1-39) is plotted simply as ACTH, since previous work (2) are summarized. Data from peptides released into the media are also indicated.

FIG. 9. Further kinetic analysis of peptide processing in wild-type and PC2 AtT-20 cells. Wild-type and PC2 AtT-20 cells were labeled in medium containing [3H]Tyr for 2 h and then chased for the indicated times in nonradioactive medium. Cell extracts (corresponding to 6.3 × 10⁶ cpm of trichloroacetic acid-precipitable material in the pulse extracts) were analyzed as in Fig. 2 using the γMSH antiserum. Similar results were seen in two other experiments.

FIG. 10. Summary of POMC processing due to prohormone convertases PC1 and PC2. Data from Figs. 2-9 and from our previous work (2,13) are summarized. These new peptides were only produced 2 or more h after the initial synthesis of POMC, as also found in the intermediate pituitary. The expression of PC2 also greatly accelerated the cleavage (step 4) of β-lipotropin to create β-endorphin, and the cleavage (step 5) of ACTH(1-39) to create ACTH(1-13)NH₂: cleavages 4 and 5 were detected within 1 h of the synthesis of POMC. The expression of PC2 did not, however, have any effect on the rate of initial cleavage of intact POMC, on the cleavage at the NH₂-terminal of ACTH, or the cleavage at the NH₂-terminal of joining peptide (cleavages 1 to 3); occurrence of these three early cleavages was blocked by the expression of antisense RNA to PC1 (13). The secretion of all of the smaller peptides whose synthesis was dependent on PC2 was stimulated substantially by the addition of secretagogue. In addition, cells expressing PC2 showed a significantly elevated basal rate of secretion of all the product peptides; the reasons for this altered rate of basal secretion are not known.

It is important to note that cleavages 4 and 5 occur rapidly after cleavages 1, 2, and 3 in the intermediate pituitary, which has high endogenous levels of PC2 mRNA. It may be that the low level of cleavages 4 and 5 in wild-type cells and in some anterior pituitary corticotropes is due to a low level of PC2 expression (48,49); suppression of the low level of endogenous PC2 expression using antisense RNA in cultured cells may be a useful method to address this question, as antisense RNAs to peptidylglycine α-amidating monoxygenase and PC1 have proven useful in previous work (13,50).

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The function of PC1 and PC2 has been studied by transiently infecting cells using the vaccinia virus system (27,28). Using this system, PC1 and PC2 were shown to perform some of the cleavages normally seen in POMC processing, such as cleavage 2 (Fig. 1). There is agreement from the vaccinia expression data (27,28) and our antisense RNA data (13) that cleavages 1 and 2 can be mediated by PC1. While previous studies agreed that cleavages 2, 3, and 5 could be performed by PC2, they disagreed on whether cleavages 1 and 4 could be performed by PC2 (27,28). Unlike our results, data obtained using the vaccinia virus-infected cells indicated neither PC1 nor PC2 mediated the RK (Arg-Lys) cleavage to produce Lyso-γ₁-MSH (cleavage 6) nor the KK (Lys-Lys) cleavage to produce β-endorphin(1-27) (cleavage 7); cleavages at these sites occur in intermediate pituitary and brain (2) (Fig. 1). One of the major advantages of the vaccinia virus system is that much of the normal cellular protein synthesis is shut down by the vaccinia infection (27-30), and for many purposes the high level of production of protein achieved with the vaccinia expression system is very useful. However, for studies of cellular function, the suppression of cellular protein syn-
thesis raises the possibility that key cellular functions such as the creation of new, fully functional secretory granules will be disrupted by the vaccinia infection.

Another difference between our data and the data from the vaccinia virus-infected cells (7, 28) is that there was no α-N-acetylation of ACTH(1-39)NH₂ in the stably transfected AtT-20 cells, while the vaccinia virus-infected cells produced acetylated ACTH(1-39)NH₂. Consistent with the lack of acetylation of ACTH in this work was the concomitant lack of acetylation of β-endorphin; in the pituitary and in the brain, the α-N-acetylation of ACTH(1-39)NH₂ and β-endorphin always go together, and current data argue the same enzyme may acetylate both peptides (2, 24, 51). ACTH(1-39)NH₂ is an excellent substrate for most of the acetyltransferases in cells (51, 52), and any access of the cytosolic or nuclear acetyltransferases to ACTH(1-39)NH₂ in the infected cells could lead to the production of acetylated ACTH(1-39)NH₂.

Finally, cleavages 6 and 7 only occur in cells expressing high levels of PC2 and then only occur several hours after the initial synthesis of POMC. It is important to note that cleavage 6 produced the same product found in the rat intermediate pituitary (Lys⁵-γ,γ-MSH) (25). The small amount of cleavage of transfected mutant prionopeptide Y with an Arg-Lys pair at the cleavage site (similar to cleavage site 6 in POMC) of transfected mutant proneuropeptide Y with an Arg-Lys pair at the cleavage site (similar to cleavage site 6 in POMC) of transfected mutant proneuropeptide Y with an Arg-Lys pair at the cleavage site (similar to cleavage site 6 in POMC) could lead to the production of acetylated ACTH(l-13)NH₂.

Prohormone Convertases Mediate Endoproteolytic Cleavages

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