Biosynthesis and Posttranslational Processing of Site-directed Endoproteolytic Cleavage Mutants of Pro-neuropeptide Y in Mouse Pituitary Cells

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Although pairs of basic amino acids are common endoproteolytic sites in prohormones, the enzymes responsible for these cleavages have not yet been characterized. To investigate the specificity of these endoproteases, cDNAs encoding pro-neuropeptide Y (pro-NPY) containing all four pairs of basic amino acids were expressed in AtT-20 cells. Pro-NPY was selected as a model substrate because it undergoes a single cleavage at the sequence -Lys-Arg- during posts translational processing. AtT-20 cells, a mouse anterior pituitary corticotrope line, were selected because they synthesize pro-adrenocorticotropic hormone (pro-ACTH)/endorphin and cleave a well characterized subset of the eight pairs of basic amino acids in the precursor. Altered cDNAs encoding pro-NPY with -Arg-Arg-, -Arg-Lys-, or -Lys-Lys- at the cleavage site were used to generate stable cell lines. The production of NPY and the carboxyl-terminal peptide was studied using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gel filtration, reversed-phase high performance liquid chromatography, ion-exchange high performance liquid chromatography, tryptic peptide mapping, and microsequencing. Direct amino acid labeling confirmed the identity of the pair of basic amino acids at the cleavage site. Even when the four pairs of basic amino acids were presented in the same structural context, the rate, extent, and type of cleavage was substrate-specific. Pro-NPY(-Arg-Arg-) was cleaved at a rate similar to that observed for the wild-type pro-NPY(-Lys-Arg-). In contrast, pro-NPY(-Arg-Lys-) was cleaved at a much lower rate, and pro-NPY(-Lys-Lys-) was cleaved very poorly. Following endoproteolytic cleavage, the pair of basic amino acids present did not alter the production of mature NPY with a COOH-terminal Tyr-NH₂. While two of the three mutant pro-NPY molecules were processed to wild-type carboxy-terminal peptide, the carboxy-terminal peptide derived from pro-NPY(-Arg-Lys-) contained an amino-terminal lysine residue, indicating that biosynthetic endoproteolysis occurred in the middle or at the amino terminus of the pair of basic amino acid residues at the cleavage site. Expression of wild-type or mutant pro-NPY inhibited cleavages within the endogenous pro-ACTH/endorphin; poorly cleaved pro-NPY mutants (Lys in the second position of the cleavage site) were the most potent inhibitors of pro-ACTH/endorphin cleavage.

The maturation of most bioactive peptides involves multiple posttranslational modifications, including endoproteolytic cleavage. Endoproteolysis occurs at selected pairs of basic amino acids and occasionally at single arginine residues (Steiner et al., 1974; Eipper et al., 1987; Schwartz, 1987; Fisher and Scheller, 1988; Mains et al., 1990). The specificity of cleavage is not understood; precursor molecules can contain pairs of basic amino acids that are never cleaved, and tissue-specific utilization of cleavage sites is often observed (Eipper et al., 1987; Mains et al., 1990). For example, the rodent pro-ACTH¹/endorphin precursor contains four -Lys-Arg- pairs; in corticotropes (anterior pituitary) three of these -Lys-Arg- pairs are cleaved in a strict temporal order within a 2-4-h period, while the fourth -Lys-Arg- pair is not cleaved (Eipper et al., 1987; Mains et al., 1990). Melanotropes (intermediate pituitary) express the same pro-ACTH/precursor but rapidly cleave all four of the -Lys-Arg- pairs. Corticotropes cleave only one of the two -Arg-Arg- pairs in rodent pro-ACTH/endorphin while melanotropes cleave both pairs. Additionally, pro ACTH/endorphin is cleaved at an -Arg- and Lys- pair in melanotropes, but not in corticotropes.

Among the known propeptide precursors a preference for endoproteolytic cleavage at -Lys-Arg- sites has been observed, although all four possible permutations are utilized (Rholam et al., 1990; Schwartz, 1990). When one of the basic amino acids has been replaced by a nonbasic residue in naturally occurring mutants, many precursors are not cleaved (Judah and Quinn, 1978; Brennan and Carrell, 1978; Takahashi et al., 1987; Abdo et al., 1981; Robbins et al., 1981; Shibasaki et al., 1985; Yoshimasa et al., 1988; Diuguid et al., 1986). The relative ability of the processing enzymes of any cell type to cleave at each combination of paired basic amino acids, when presented in a constant tertiary structure, has not previously been compared. It is possible that unutilized cleavage sites are unavailable due to protein binding, chemical modification, or three-dimensional folding; it has been proposed that paired basic amino acids predicted to occur in α-helical domains are less likely to undergo cleavage than those occurring in β-turns (Geisow and Smyth, 1980; Rholam et al., 1986). Another

¹The abbreviations used are: pro-ACTH, pro-adrenocorticotropic hormone; pro-NPY, pro-neuropeptide Y; CSFM, complete serum-free medium; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate.
Pro-NPY Cleavage Mutants

Possible explanation for the specificity observed may be that the enzyme activity is specific for processing unused cleavage sites may be limiting or absent in a tissue-specific pattern.

Human pro-neuropeptide Y (pro-NPY) is a simple eukaryotic substrate which can be genetically altered to probe the ability of the processing enzyme(s) in AtT-20 cells to distinguish among the four possible pairs of Lys and Arg. After removal of a 28-amino acid signal sequence, the 69-amino acid wild-type NPY cDNA was inserted into a single nuclease basic site (Lys-Arg-). In addition, pMT.neo-1 also contains the gene for neomycin phosphotransferase, which confers resistance to the antibiotic G418 (geneticin). The resulting expression plasmids are: pMT.NPY-R14-Arg14, pMT.NPY-R20-Arg20, and pMT.NPY-R20-Lys20. These plasmids are identical to pMT.neo-1 (-Lys-, Arg-)(Dickerson et al., 1987) except for the mutated endopeptidase cleavage site.

Expression Plasmid Constructions—NPY cDNAs containing point mutations in the region coding for the endopeptidase cleavage site were isolated on HindIII-XbaI restriction fragments, and cloned separately with either 100 μM [3H]lysine (49 Ci/mmol, Amersham Corp.) or 100 μM [3H]lysine (49 Ci/mmol, Amersham Corp.), in a similar manner to that described above. Cell extracts were immunoprecipitated with carboxyl-terminal peptide-directed antibody (Dickerson et al., 1987), subjected to gel filtration of preincubated with 100 μM [3H]lysine (49 Ci/mmol, Amersham Corp.) or 100 μM [3H]lysine (49 Ci/mmol, Amersham Corp.), in a similar manner to that described above. Cell extracts were immunoprecipitated with carboxyl-terminal peptide-directed antibody (Dickerson et al., 1987), subjected to gel filtration of preincubated with 100 μM [3H]lysine (49 Ci/mmol, Amersham Corp.) or 100 μM [3H]lysine (49 Ci/mmol, Amersham Corp.), in a similar manner to that described above. 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chromatography as above. Samples were pooled and dried under vacuum, resuspended in 35 mM ammonium formate, pH 2.5, 30% acetonitrile, and analyzed by ion-exchange HPLC on a Bio-Sil TSK 530 CM cation-exchange column (300 × 4 mm, Bio-Rad). Samples were eluted with a 70-min linear gradient from 35 mM ammonium formate, pH 2.5, 30% acetonitrile to 323 mM ammonium formate, pH 2.5, 30% acetonitrile, at a flow rate of 1 ml/min (Emeson and Eipper, 1986).

RESULTS

Cell Lines—Mutations were introduced into human pro-NPY cDNA that encoded substitutions of the basic amino acids at the single paired basic endoproteolytic cleavage site of pro-NPY. Expression vectors containing each form of pro-NPY cDNA were then transfected into AtT-20/D-16v cells, multiple subclones were isolated, and the subclone exhibiting the highest level of expression was chosen for extensive characterization. During the screening to select the optimal clone from each set of transfections, only the level of pro-NPY expression was observed to vary among the subclones; the pattern of pro-NPY processing for a particular mutation did not vary among subclones.

Three new cell lines were derived: Mt.NPY/R38K39 cells, expressing pro-NPY containing -Arg38-Arg39; Mt.NPY/R38K39 cells, expressing pro-NPY containing -Arg38-Lys39; and Mt.NPY/K38K39 cells, expressing pro-NPY containing -Lys38-Lys39. The levels of pro-NPY expression achieved by the three new cell lines fell into two classes. The Mt.NPY/R38R39 cells were similar to the wild-type Mt.NPY/K38R39 cells and synthesized pro-NPY-related peptides at levels between 50 and 100%, the level of expression of endogenous pro-ACTH/endorphin. In contrast, the Mt.NPY/R38K39 and Mt.NPY/K38K39 cell lines expressed pro-NPY at levels approximately 3-10% the level of proACTH/endorphin. This level of expression was the maximal achieved for these last two mutants after eight separate transfections using calcium phosphate or lipofection, with over 75 drug-resistant lines screened for each of the R38K39 and K38R39 mutants. The low level of peptide production in Mt.NPY/R38K39 and Mt.NPY/K38R39 cell lines was matched by a proportionally decreased level of pro-NPY mRNA compared to Mt.NPY/K38R39 cells, as determined by Northern analysis (Dickerson et al., 1987). Although low when compared to the levels achieved in Mt.NPY/K38R39 cells and Mt.NPY/R38R39 cells, this level of expression is comparable to levels reported for similar expression studies in AtT-20 cells (between 0.1 and 20% the level of pro-ACTH/endorphin) (Moore et al., 1983; Comb et al., 1985; Thomas et al., 1986; Lapps et al., 1986).

The lower level of expression observed in Mt.NPY/R38K39 and Mt.NPY/K38R39 cells may be due to toxicity of the pro-NPY molecules containing a lysine residue in position 39 (RK and KK). These pro-NPY molecules were found to inhibit endogenous propeptide processing when expressed at levels much lower than required for inhibition of endogenous propeptide processing by pro-NPY containing an arginine residue in position 39 (KR and RR) (see below). Basal transcription from the metallothionein promoter may result in accumulation of toxic intermediates during normal cell growth, resulting in the death of potential high-level expressing RK and KK cell lines. We have attempted to avoid this potential cytotoxicity by performing transient expression studies, but AtT-20 cells are not efficiently transfected, either by calcium phosphate coprecipitation, DEAE-dextran, or by electroporation. Additionally, levels of transient expression have been prohibitively low for biosynthetic labeling when promoters from SV40, Rous sarcoma virus, metallothionein, and viva virus were utilized (data not shown). Since the secretion rates

(as percentage of peptide in the cells/h) do not differ among the four pro-NPY cell lines, it is unlikely that the apparent toxicity of the RK and KK mutations is caused by improper protein folding or misrouting, as seen in cases of more severe mutations of foreign proteins (Doms et al., 1988; Carroll et al., 1987). Cadmium treatment of wild-type AtT-20 cells does not affect pro-ACTH/endorphin synthesis or secretion (Dickerson et al., 1987).

To verify that the identity of the basic amino acids at the cleavage site in each of the mutant pro-NPY molecules was in fact what was expected from the site-directed mutagenesis, each cell line was incubated separately with either [3H]arginine or [3H]lysine, and labeled pro-NPY was analyzed by tryptic peptide mapping (data not shown). All three cell lines synthesizing mutated pro-NPY produced pro-NPY molecules that contained the predicted amino acid sequence in the cleavage site. Thus any difference in processing was due to the sequence at the cleavage site of each precursor, and not due to an anomalous cleavage sequence.

Pulse-Chase Biosynthetic Labeling—Initial steady-state labelings indicated that pro-NPY containing either -Arg-Lys- or -Lys-Lys- in the cleavage site was not processed efficiently in AtT-20 cells. To increase the sensitivity of detection of cleavage, a pulse-chase protocol was used to analyze pro-NPY processing in the four cell lines. After a 2-h incubation of AtT-20 cells expressing wild-type pro-NPY (Fig. 1, top panel, RR), much of the pro-NPY was already processed to NPY-sized material. After 6 h of chase, essentially all of the pro-NPY-sized material was converted to NPY-sized material. The mutant pro-NPY and NPY were secreted during the chase period, and could be isolated from the medium (data not shown). This accounts for the decrease in total pro-NPY-related radioactivity observed in the cell extract during the chase incubation compared to the pulse incubation (Dickerson et al., 1987). The second panel in Fig. 1 shows the results from the Mt.NPY/R38R39 cell line (RR); the time course of processing was similar to that seen in the cells expressing wild-type pro-NPY. Essentially all of the pro-NPY-sized material was converted to NPY after the 6 h of chase. The third panel in Fig. 1 shows the results from the Mt.NPY/R38K39 cell line (RK). In contrast to the Mt.NPY/K38R39 and Mt.NPY/R38R39 cells, at the end of the 2-h labeling period, most of the pro-NPY-related material was still present as pro-NPY-sized material. Furthermore, after the 6-h chase a significant amount of the pro-NPY-sized material was not converted to NPY. The bottom panel shows the results from the Mt.NPY/K38K39 cells (KK). The Mt.NPY/K38K39 cells show a dramatic decrease in conversion of pro-NPY to NPY. After a 6-h chase, less than 10% of the pro-NPY-related material was present as NPY-sized material.

The processing efficiency for each pro-NPY substrate averaged over eight different experiments is summarized in Fig. 2. Even when presented in the same structural context in pro-NPY, the -Lys-Lys- sequence was cleaved very poorly by the processing enzymes in the AtT-20 cells (Fig. 2). The -Arg-Lys- sequence was cleaved significantly more slowly than -Arg-Arg- or the wild-type -Lys-Arg- sequences (Fig. 1), although the extent of cleavage of the -Arg-Lys- mutant eventually exceeded 50% (Fig. 2).

The enzymes involved in processing pro-ACTH/endorphin are assumed to be responsible for processing pro-NPY. Our previous studies demonstrated that when levels of pro-NPY expression were greater than 90% that of pro-ACTH/endorphin, a major reduction in pro-ACTH/endorphin and pro-NPY processing was observed (Dickerson et al., 1987), indicating saturation of a common processing step. When wild-
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Fig. 1. Analysis of pulse-chase experiments. Two identical wells of Mt.NPY/K3'R3' cells (KR), Mt.NPY/R3'R3' cells (RR), Mt.NPY/R38K39 cells (RK), or Mt.NPY/K38K39 cells (KK) were preincubated for 16 h with 10 μM CdCl2 and ascorbate, and labeled for 2 h with [3H]Tyr. One well of each pair was then extracted for peptides (pulse), and the second well of each pair was incubated for a further 6 h in unlabeled complete medium and then extracted for peptides (chase). Extracts were immunoprecipitated with affinity-purified NPY antibody and separated by SDS-PAGE.

Type pro-NPY expression was induced to lower levels, pro-NPY was processed almost completely to NPY and there was no detectable effect on processing of endogenous pro-ACTH/endorphin (Dickerson et al., 1987). High levels of pro-NPY expression in Mt.NPY/R38R39 cells were also necessary before inhibition of endogenous pro-ACTH/endorphin processing occurred. In contrast, inhibition of pro-ACTH/endorphin processing was observed in Mt.NPY/R38K39 cells and Mt.NPY/K38K39 cells, even though production of mutant pro-NPY in these cell lines was never greater than 3–10% of pro-ACTH/endorphin synthesis. Fig. 3 shows the β-endorphin immunoprecipitations from aliquots of cell extracts from wild-type AtT-20 cells and Mt.NPY/K38K39 cells. At the end of the 2-h pulse there was a 3-fold molar increase in the amount of pro-ACTH/endorphin in Mt.NPY/R38K39 cells and Mt.NPY/K38K39 cells, compared to nontransfected AtT-20 cells; a significant decrease in conversion of β-lipotropin to β-endorphin was also observed at the end of the 6 h chase period. Inhibition of endogenous pro-ACTH/endorphin processing was never observed in Mt.NPY/

Fig. 2. Extent of conversion of pro-NPY to NPY. All four cell lines were analyzed as described in Fig. 1 in eight separate experiments; some incubations utilized [3H]Leu instead of [3H]Tyr. Total pro-NPY and NPY production was determined by summing the relative molar amounts of pro-NPY and NPY in the medium and cell extract following the chase period. Percent conversion was calculated by dividing the amount of NPY-sized material by the total amount of NPY-sized material plus pro-NPY-sized material, after correcting for the number of tyrosine or leucine residues in each molecule.

Fig. 3. Analysis of pro-ACTH/endorphin processing. Aliquots from the same cell extracts used for immunoprecipitation in Fig. 1 were immunoprecipitated with affinity-purified β-endorphin antibody and separated by SDS-PAGE. PAE, pro ACTH/endorphin; βLPH, β-lipotropin hormone; β-endo, β-endorphin.

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K38R39 or Mt.NPY/R38R39 cells at such low levels of pro-NPY expression.

Characterization of Pro-NPY-derived Products—It was of particular interest to determine in detail the pattern of post-translational processing of the mutant pro-NPY molecules since the exact site of the initial endoproteolytic cleavage event has not been established. In tissue that endogenously produces NPY, the carboxyl terminus of NPY ends in the sequence -Tyr36-NHz (Tatemoto et al., 1982; Tatemoto et al., 1985; Marek and Mains, 1989). NPY derived from the wild-type pro-NPY molecule in stably transfected AtT-20 cells was properly ω-amidated (Dickerson et al., 1987). [3H]Tyrosine labeled NPY derived from AtT-20 cells expressing wild-type and the two mutant forms of pro-NPY had identical tryptic peptide patterns (Fig. 4). Since the amount of radioactivity in the four Tyr-containing tryptic fragments occurred in the expected 1:2:1:1 ratio (Tyr-NH*:NPY(20–25):NPY(1–19):NPY(26–33)), the NPY molecules contained an ω-amidated Tyr36 residue and an intact Tyr1 residue (Dickerson et al., 1987). When intact NPY from Mt.NPY/K38K39 cells was subjected to RP-HPLC, a single peak of radioactivity was identified that comigrated with synthetic human NPY (data not shown). Thus the amino acid sequence at the cleavage site had no effect on the extent of ω-amidation following proteolytic cleavage in all four cell lines investigated.

The carboxyl-terminal peptide (pro-NPY(40–69)) is the other major product of pro-NPY processing. The amino-terminal residue of the carboxyl-terminal peptide derived from wild-type pro-NPY expressed in AtT-20 cells (Mt.NPY/K38K39 cells) was Ser19, as expected from the deduced amino acid sequence (Minth et al., 1984; Dickerson et al., 1987). To determine the amino-terminal residue of the carboxyl-terminal peptide produced from the mutant pro-NPY molecules, [3H]leucine-labeled carboxyl-terminal peptide from Mt.NPY/R38R39 and Mt.NPY/R38K39 cell lines was microsequenced (Fig. 5). The top panel in Fig. 5 shows data for carboxyl-terminal peptide derived from wild-type pro-NPY and is included as a standard (Dickerson et al., 1987). The middle

![Fig. 4. Analysis of NPY. Mt.NPY/K38R39 cells (KR), Mt.NPY/ R38R39 cells (RR), and Mt. NPY/R38K39 cells (RK) were incubated with [3H]Tyr and labeled NPY-sized material was prepared by immunoprecipitation and chromatography on Sephadex G-50. NPY-sized material was digested with trypsin and tryptic peptides were separated by RP-HPLC. Peak identities were established by amino acid analysis of synthetic human NPY tryptic peptides (Dickerson et al., 1987). Characterization of NPY from Mt.NPY/K38R39 cells (KR) was performed in a later set of experiments and the Tyr-NH2 peak eluted three fractions earlier. NPY(20–25) contains two tyrosyl residues, while the other tryptic fragments each contain a single tyrosyl residue.

![Fig. 5. Microsequencing of carboxyl-terminal peptides. [3H]Leu-labeled carboxyl-terminal peptide prepared from cell extracts of Mt.NPY/K38R39 cells (KR), Mt. NPY/R38R39 cells (RR), and Mt.NPY/R38K39 cells (RK). Immunoprecipitates were chromatographed on Sephadex G-50, and the carboxyl-terminal peptide pools were subjected to automated Edman degradation. The deduced amino acid sequence from the human cDNA is shown above the radioactivity released for each Edman cycle. The Mt.NPY/R38R39 sequence was repeated on two separately purified pools of carboxyl-terminal peptide, and the Mt.NPY/R38K39 sequence was repeated on three separate pools of carboxyl-terminal peptide.](http://www.jbc.org/)
panel shows data for carboxyl-terminal peptide derived from Mt.NPY/RK39R9 cells. Similar to wild-type cells, radioactivity was released in cycles 6, 10, and 11, indicating that Ser40 is indeed the amino terminal residue of the carboxyl-terminal peptide. Thus, altering the endoproteolytic cleavage site from -K38-R38- to -R38-K39- had no effect on the final carboxyl-terminal peptide produced from pro-NPY. The bottom panel of Fig. 4 shows the results of microsequencing carboxyl-terminal peptides derived from Mt.NPY/RK39R9 cells. Here radioactivity was detected in cycles 7, 11, and 12, indicating that the carboxyl-terminal peptide retains an additional residue (Lys39) before Ser40.

Since the production of Lys39-carboxyl-terminal peptide by Mt.NPY/RK39R9 cells was unexpected and has important implications for the steps in the processing pathway, this observation was confirmed by two independent means. Cells expressing wild-type pro-NPY and all three mutant pro-NPYs were incubated with [3H]lysine, and the cells were extracted and immunoprecipitated with a carboxyl-terminal peptide antibody. Lysine-labeled carboxyl-terminal peptide was in fact detected in Mt.NPY/RK39R9 cells but not in Mt.NPY/K38R39-, R38R9-, or -K39R9 cells. Since there are no lysine residues in the carboxyl-terminal peptide of wild-type pro-NPY, the radioactivity must be derived from the lysine found in the cleavage site of the mutant pro-NPY(RK39R9). Tryptic digestion of the [3H]lysine-labeled carboxyl-terminal peptide from Mt.NPY/RK39R9 cells liberated free [3H]lysine, as expected if the peptide contained a lysine residue at its amino terminal residue (data not shown). Second, the carboxyl-terminal peptide from Mt.NPY/RK39R9 cells exhibited a greater retention time than the wild-type carboxyl-terminal peptide during cation-exchange HPLC (Fig. 6), as expected for a molecule with an additional positively charged residue (Emeson and Eipper, 1986). Interestingly, the trifluoroacetic acid and hexafluoroisopropanol buffer systems (Bennett et al., 1981) were not able to separate the two forms of carboxyl-terminal peptide (wild-type and Lys39-extended) during RP-HPLC.

Substitution of the carboxyl-terminal peptide for tryptic mapping or microsequencing. The carboxyl-terminal peptide produced by Mt.NPY/K38R39 cells comigrated with carboxyl-terminal peptide produced by wild-type Mt.NPY/K38R39 cells during cation-exchange HPLC (Fig. 6), rather than with the Lys39-carboxyl-terminal peptide produced by the mutant Mt.NPY/RK39R9 cells. This indicated that the carboxyl-terminal peptide from Mt.NPY/K38R39 cells did not retain an amino-terminal lysine residue.

**DISCUSSION**

We have examined the sequence preference of the endoprotease(s) in the secretory pathway of AtT-20 cells, using closely related mutant forms of pro-NPY as test substrates. To attempt to explain the cleavage pattern observed in the endogenous pro-ACTH/endorphin in AtT-20 cells, one must deal with two major questions. First, why are certain -Lys-Arg- and -Arg-Arg- sites cleaved more readily than others? Second, why are -Arg-Lys- and -Lys-Lys- sites not cleaved in pro-ACTH/endorphin in AtT-20 cells? To evaluate the contribution of the paired basic sequence itself to this specificity, we have introduced all four combinations of paired basic cleavage sites into AtT-20 cells in the constant three-dimensional structure of pro-NPY, a molecule which we have previously demonstrated to be susceptible to cleavage in AtT-20 cells (Table I). In this paradigm, -Lys-Arg- and -Arg-Arg- cleaved with high efficiency. The -Arg-Lys- pair cleaved, although less efficiently; thus an endoprotease capable of cleaving at -Arg-Lys- sequences is present in AtT-20 cells, even though this cleavage event does not occur in the endogenous pro-ACTH/endorphin precursor. This lack of cleavage of pro-ACTH/endorphin may therefore be due to conformational differences, rather than lack of the appropriate enzyme; Seger and Bennett (1986) argue that the presence of an O-linked oligosaccharide near the -Arg-Lys- site in pro-ACTH/endorphin prevents the cleavage at -Arg-Lys-. The very inefficient cleavage of pro-NPY containing the sequence -Lys-Lys-in the cleavage site is consistent with the lack of biosynthetic secretory pathway endoproteases in AtT-20 cells capable of efficiently recognizing a -Lys-Lys-site. The fact that the enzyme(s) in AtT-20 cells exhibit broad enough specificity to cleave KR and RR sequences in foreign substrates, but fail to cleave these sequences in the KKKR context within ACTH itself, suggests that mechanisms to block endoproteolysis exist.

The number of endoproteases involved in the processing of prohormones is not known; our data could be explained by the presence of a single biosynthetic endoprotease in AtT-20 cells. No vertebrate endoproteolytic processing enzyme has been purified and cloned, but the cDNA for the yeast KEX2 endoprotease has been cloned and expressed after transfection (Fuller et al., 1989; Mizuno et al., 1989). Candidate biosynthetic endoproteases have been reported which cleave at the NH₂ terminus of the pair of basic amino acids (Guschanski et al., 1987), in the middle of the pair of basic amino acids (Mizuno et al., 1988; Loh, 1986; Maret and Fauchere, 1988; Shen et al., 1989) and at the COOH terminus of the pair (Docherty et al., 1984; Loh, 1986; Clamagirand et al., 1987; Brennan and Pech, 1988; Shen et al., 1989). Several candidate enzymes cleave at more than one site, depending on the substrate; even in yeast, in which KEX2 usually cleaves at the COOH terminus of the basic amino acid pair (Fuller et al., 1989; Mizuno et al., 1989), some transfected foreign pro-

![Fig. 6. Ion-exchange HPLC of carboxyl-terminal peptides.](http://www.jbc.org/)
peptides are cleaved in the middle of the pair of basic amino acids (Barr et al., 1988). Several candidate enzymes cleave at Lys Arg and Arg Arg but not at Lys Lys (Mizuno et al., 1985; Maret and Fauchere, 1988; Fuller et al., 1989; Mizuno et al., 1989), and insulin granules have an enzyme activity highly selective for -Lys-Arg over -Arg-Arg- sequences (Rhodes et al., 1989).

A comparison of the limited number of studies examining cleavage of prohormones with altered basic cleavage sites suggests that the hierarchy of cleavage-site utilization differs between cell lines and prohormones. The ACT-20 endopeptidase(s) might have an active site with an absolute requirement for two basic residues at the cleavage site, and a preference for cleaving after an arginine residue. This preference for cleaving on the carboxyl side of an arginine residue would result in cleavage between the pair of basic amino acids in substrates such as pro-NPY containing -Arg-Arg-Lys- at the cleavage site (Mt.NPY/R3'K3' cells). A preference for -Lys-Arg- sequences over -Arg-Lys- sequences was observed in Rin m3P cells by Thorne et al., 1989), using site-directed mutagenesis of the cDNA for pro-ACTH/endorphin to alter the cleavage site within β-endorphin. Stoller and Shields (1989) have recently made mutations in the prosomatostatin cDNA which result in substitutions at the single dibasic cleavage site, replacing the wild-type -Arg-Lys- sequence with -Arg-Pro-, -Lys-Arg-, or -Arg-Arg-. When the mutated prosomatostatins were expressed in GH3 cells, cleavage at -Arg-Lys- occurred with equal efficiency to cleavage at -Lys-Arg- and -Arg-Arg-. The different hierarchy of cleavage site preference, compared to these data with AtT-20 cells, may be due to structural differences between prosomatostatin and pro-NPY, or may be a function of different populations of processing enzymes in GH3 cells and AtT-20 cells. Stoller and Shields (1989) found that GH3 cells required a low pH step for processing of prosomatostatin, while chloroquine had no effect on processing or secretion of pro-ACTH/endorphin in AtT-20 cells and intermediate pituitary melanotropes (Mains and May, 1988).

After endoproteolytic cleavage at any of the four combinations of paired basic sites in pro-NPY, processing of the carboxyl terminus of NPY by carboxypeptidase H (Fricker, 1988) and PAM proceeded with great fidelity. In contrast, processing of the amino terminus of the carboxyl-terminal peptide depended upon the sequences present in the pro-NPY cleavage site. The Lys30 carboxy-terminal peptide produced by Mt.NPY/R3'K3' cells provided clear evidence that the initial cleavage event did not occur to the carboxyl-terminal side of the pair of basic residues in the cleavage site. Although cleavage at the single -Arg30-Lys30 sequence of pro-ACTH/endorphin does not occur to any significant extent in AtT-20 cells or corticotropes, it does occur in the melanotropes of the intermediate pituitary, resulting in the production of Lys30-γ-MSH; as in Mt.NPY/R3'K3' cells, the lysine residue derived from the -Arg-Lys-cleavage site is retained by the product (Seger and Bennett, 1986).

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TABLE I

**Pro-NPY Cleavage Mutants**

| Cell line         | Pro-NPY cleavage site | Pro-NPY processing | Inhibits endogenous processing | NPY Carboxyl-terminal peptide |
|-------------------|-----------------------|--------------------|-------------------------------|-------------------------------|
| Mt.NPY/K36R39     | -Lys-Glu-Lys-Arg-Ser-  | Control            | Yes                           | -Tyre-NH₂                     |
| Mt.NPY/R3'K3'     | -Arg-                 | Control            | Yes                           | -Tyre-NH₂                     |
| Mt.NPY/R3'K3'     | -Arg-Lys              | Slower             | Yes                           | -Tyre-NH₂                     |
| Mt.NPY/K36R39     | -Lys-1               | Much slower        | Yer                           | (-Tyre-NH₂)                   |

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