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Review

Cathepsin L and Arg/Lys aminopeptidase: a distinct prohormone processing pathway for the biosynthesis of peptide neurotransmitters and hormones

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Abstract

Peptide neurotransmitters and hormones are synthesized as protein precursors that require proteolytic processing to generate smaller, biologically active peptides that are secreted to mediate neurotransmission and hormone actions. Neuropeptides within their precursors are typically flanked by pairs of basic residues, as well as by monobasic residues. In this review, evidence for secretory vesicle cathepsin L and Arg/Lys aminopeptidase as a distinct proteolytic pathway for processing the prohormone proenkephalin is presented. Cleavage of prohormone processing sites by secretory vesicle cathepsin L occurs at the NH_2-terminal side of dibasic residues, as well as between the dibasic residues, resulting in peptide intermediates with Arg or Lys extensions at their NH_2-termini. A subsequent Arg/Lys aminopeptidase step is then required to remove NH_2-terminal basic residues to generate the final enkephalin neuropeptide. The cathepsin L and Arg/Lys aminopeptidase prohormone processing pathway is distinct from the proteolytic pathway mediated by the subtilisin-like prohormone convertases 1/3 and 2 (PC1/3 and PC2) with carboxypeptidase E/H. Differences in specific cleavage sites at paired basic residue sites distinguish these two pathways. These two proteolytic pathways demonstrate the increasing complexity of regulatory mechanisms for the production of peptide neurotransmitters and hormones.

Keywords: cathepsin L; enkephalin; neuropeptides; prohormone processing; secretory vesicles.

Introduction: prohormone processing proteases for the biosynthesis of active peptide neurotransmitters and hormones

Peptides in the nervous system are essential for activity-dependent neurotransmission of information among neurons, and peripheral peptides are required for endocrine regulation of physiological functions. Moreover, the nervous and endocrine systems communicate with one another via these peptide neurotransmitters and hormones, collectively known as neuropeptides. Knowledge of the biosynthetic mechanisms for the production of neuropeptides is critical for understanding cell-cell communication in neurotransmission and peptide hormone actions.

Cellular production of neuropeptides requires proteolytic processing of their respective precursor proteins. This results in a multitude of distinct peptides with diverse physiological actions, such as enkephalin and opioid peptide regulation of analgesia (Law et al., 2000; Snyder and Pasternak, 2003), ACTH induction of steroid synthesis (Frohman, 1995), galanin involvement in cognition (Steiner et al., 2001), neuropeptide Y participation in regulating feeding behavior (Gehlert, 1999; Wieland et al., 2000), and numerous other neuroendocrine functions (Table 1). The primary structures for prohormones such as proenkephalin, proopiomelanocortin, and others indicate that neuropeptides within the precursors are usually flanked at their NH_2- and COOH-termini by pairs of basic residues, and sometimes by monobasic residues (Hook et al., 1994; Steiner, 1998; Seidah and Prat, 2002; Figure 1). These multi-basic and monobasic sites provide cleavage sites for proteolytic processing. Proteolytic processing of proenkephalin results in multiple copies of enkephalin that induce analgesia. The related POMC precursor (proopiomelanocortin) undergoes proteolytic processing to generate distinct peptide hormones consisting of ACTH, α-MSH, and β-endorphin. Proinsulin undergoes proteolytic processing to generate the A and B chains linked by disulfide bonds. Clearly, proteolysis represents key steps for the biosynthesis of essential peptide neurotransmitters and hormones.

Proteolytic cleavage of prohormones may occur at one of three positions at paired basic processing sites. These cleavages may consist of processing at the COOH- and NH_2-termini of the dibasic residues, or between the dibasic residues (Figure 2). Resultant peptide intermediates

Table 1 Neuropeptides in the nervous and endocrine systems.

Neuropeptide	Regulatory function
Enkephalin	Analgesia
β-Endorphin	Analgesia
Dynorphin	Analgesia
ACTH	Steroid production
α-MSH	Skin pigmentation
Insulin	Glucose metabolism
Glucagon	Glucose metabolism
Galanin	Cognition
NPY	Blood pressure (peripheral) and obesity (CNS)
Somatostatin	Growth regulation
Vasopressin	Water balance

Peptide neurotransmitters and hormones are collectively termed neuropeptides. Examples of several neuropeptides and their regulatory functions are listed. Abbreviations are adrenocorticotropin hormone (ACTH), α-melanocyte stimulating hormone (α-MSH), and neuropeptide Y (NPY).

require removal of basic residues from COOH- and/or NH_2-termini by carboxypeptidase and aminopeptidase enzymes, respectively.

Endoproteolytic processing at the COOH-terminal side of paired basic residues is accomplished by the subtilisin-like prohormone convertases (Figure 2), consisting of PC1/3 and PC2 as well as related PC enzymes (Hook et al., 1994; Steiner, 1998; Seidah and Prat, 2002). The carboxypeptidase E/H removes basic residue extensions from the COOH-termini of peptide intermediates (Fricker, 1988; Hook et al., 1998). The subtilisin-like PC1 and PC2 endopeptidases, in combination with carboxypeptidase E/H, represent a well-established proteolytic processing pathway for the biosynthesis of neuropeptides.

Significantly, recent studies demonstrate a newly identified prohormone processing pathway mediated by secretory vesicle cathepsin L (Yasothornsrikul et al., 2003) and Arg/Lys aminopeptidase (Hook and Eiden, 1984; Yasothornsrikul et al., 1998) for the production of enkephalin neuropeptides (Figure 2). Cathepsin L cleaves dibasic residue sites at their NH_2-termini and between the two basic residues (Yasothornsrikul et al., 1998). The peptide intermediates generated by cathepsin L subsequently require processing by Arg/Lys aminopeptidase to remove NH_2-terminal basic residues for production of the final neuropeptide.

The novel biological role of cathepsin L for prohormone processing in regulated secretory vesicles is the focus of this review. The biological role of cathepsin L in the production of active peptides contrasts with its previously known function as a lysosomal protease. These results demonstrate cathepsin L and Arg/Lys aminopeptidase as a distinct, alternative pathway that complements the PC enzyme and carboxypeptidase E/H pathway for prohormone processing.

Chromaffin granules represent a model neurosecretory vesicle system for elucidating proenkephalin (PE) and prohormone processing enzymes

Chromaffin granules of adrenal medulla in the sympathetic nervous system contain many neuropeptides

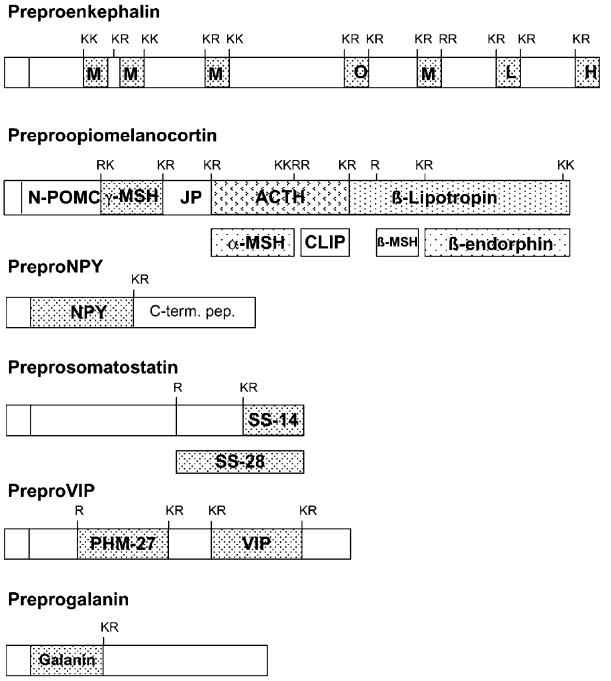

Figure 1 Structural features of prohormone precursors for proteolytic processing.
Prohormone precursor protein structures indicate that active peptide neurotransmitters and hormones are flanked by multibasic residues that represent sites of proteolytic processing to generate active neuropeptides. The precursor proteins are shown for preproenkephalin, preproopiomelanocortin, prepro-NPY (NPY, neuropeptide Y), preprosomatostatin, prepro-VIP (VIP, vasoactive intestinal polypeptide), and preprogalanin. The NH_2-terminal signal sequence is known to be cleaved by signal peptidases at the RER (rough endoplasmic reticulum) and the resultant prohormone undergoes trafficking to Golgi apparatus and packaged into secretory vesicles where prohormone processing occurs.

including enkephalin (Schultzberg et al., 1978), NPY (Higuchi et al., 1988; Carmichael et al., 1990), VIP (Holzwarth, 1984), galanin (Rokaeus and Brownstein, 1986), somatostatin (Brakch et al., 1995), and others. The abundance of these granules in chromaffin cells is demonstrated by electron microscopy (Figure 3). Chromaffin granules have been instrumental for the identification of processing enzymes for proenkephalin and other pro-neuropeptides present in chromaffin granules. In each case, the proteases identified in chromaffin granules represent processing enzymes for neuropeptide production in brain and endocrine tissues, illustrated by gene inactivation and gene knockout studies. For example, identification of carboxypeptidase E/H (CPE/H) activity in chromaffin granules led to isolation of the gene, whose inactivation results in a block at the CPE/H step for processing enkephalin-related and other neuropeptide intermediates (Naggert et al., 1995; Fricker and Leiter, 1999; Che et al., 2001). Moreover, studies of the PC1/3 and PC2 proteases in chromaffin granules (Azaryan et al., 1995a; Hill et al., 1995) are consistent with gene knockout studies that demonstrate selective roles for PC2 and PC1/3 in the biosynthesis of multiple neuropeptides (Furuta et al., 1997, 1998; Berman et al., 2000; Vishnuvardhan et al., 2000; Allen et al., 2001; Villeneuve et al., 2002; Zhu et al., 2002a,b; Miller et al., 2003a,b). Thus, chromaffin

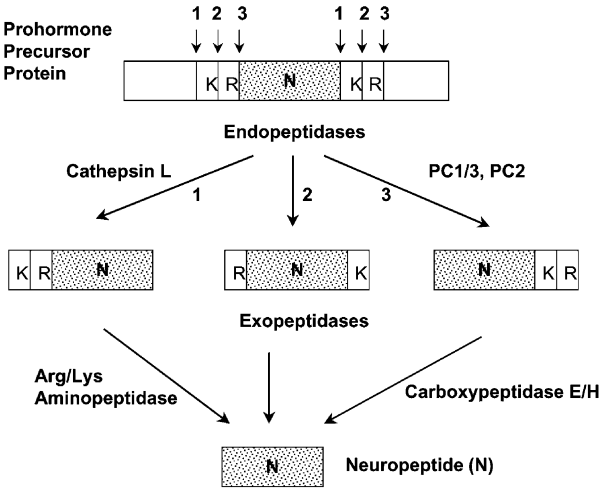

Figure 2 Proteases for prohormone processing.
Prohormone precursors typically contain active peptides flanked by paired basic residues. The dibasic processing sites undergo proteolytic cleavage at one of three sites (numbered 1, 2, and 3) which consist of cleavage at the NH_2- or COOH-terminal sides of the dibasic residues, or between the dibasic residues. Peptide intermediates generated by cleavage at the NH_2-terminal side of the dibasic site will then require Arg/Lys aminopeptidase to remove basic residues at the NH_2-termini. Cleavage of prohormones between the dibasic site results in intermediates that then require the exopeptidases Arg/Lys aminopeptidase and carboxypeptidase E/H to remove basic residues at NH_2- and COOH-termini. Finally, cleavage at the COOH-terminal side of paired basic residues results in intermediates that then require only carboxypeptidase E/H to generate the final neuropeptide.

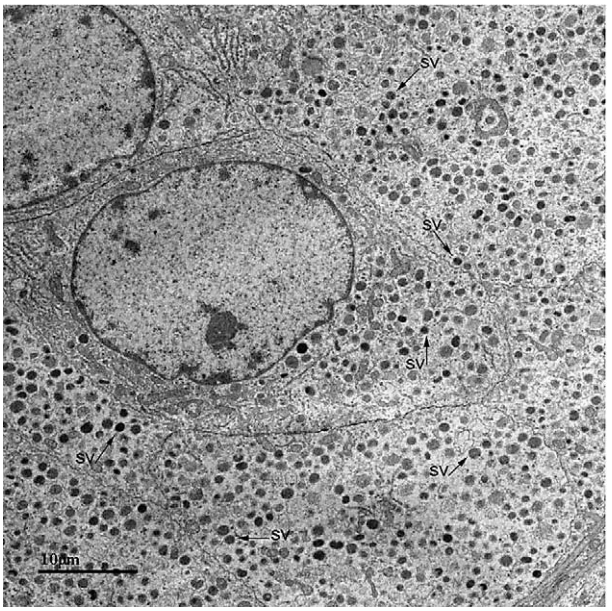

Figure 3 Dense core secretory vesicles, chromaffin granules, in neuroendocrine chromaffin cells.
Neuroendocrine chromaffin cells of adrenal medulla (bovine) contain numerous dense core secretory vesicles (SV, indicated by arrows) that are visualized by electron microscopy. These secretory vesicles contain numerous neuropeptides including enkephalin, NPY, VIP, galanin, somatostatin, and others that are co-secreted with catecholamine neurotransmitters upon neural stimulation of the adrenal medulla.

granules have been instrumental for elucidating prohormone processing proteases.

The cysteine protease PTP ('prohormone thiol protease') represents the major proenkephalin-cleaving activity compared to other proteases in chromaffin granules

The major proenkephalin (PE) processing protease in chromaffin granules was initially identified as the cysteine protease complex termed 'prohormone thiol protease' (PTP; Krieger and Hook, 1991; Yasothornsrikul et al., 1999). With full-length recombinant enkephalin precursor as substrate, purification of the PE cleaving activity led to isolation of four proteases consisting of the cysteine protease PTP (Krieger and Hook, 1991; Schiller et al., 1995; Yasothornsrikul et al., 1999), the subtilisin-like PC1/3 and PC2 proteases (Azaryan et al., 1995a), and a 70 kDa aspartyl protease that resembles the pituitary 'POMC converting enzyme' (PCE; Azaryan et al., 1995b). PTP represented the major proenkephalin cleaving activity, with lower levels of PE cleaving activity observed by native PC1/3 and PC2 purified from chromaffin granules. PTP converts proenkephalin into appropriate intermediates present *in vivo*, and generates active (Met) enkephalin (Krieger and Hook, 1991; Krieger et al., 1992). Cellular studies showed that a potent inhibitor of PTP, E64d, reduced (Met)enkephalin in chromaffin cells (Tezapsidis et al., 1995). These results demonstrate the cysteine protease activity of PTP for proenkephalin processing.

Activity-based proteomic profiling of cysteine proteases and mass spectrometry demonstrates cathepsin L as a prohormone processing enzyme

The high molecular mass nature of native PTP activity of 180–200 kDa (Schiller et al., 1995) suggested that PTP contains several protein subunits, since proteases generally possess lower molecular masses than that of native PTP. It was then important to identify the catalytic subunit of PTP responsible for proenkephalin cleaving activity. A biotinylated form of E-64, known as DCG-04, allowed activity-based affinity labeling of the active enzyme subunit. Specific affinity labeling of PTP with ^{125}I-DCG-04 identified the 27 kDa band as the active enzyme. Direct labeling with ^{125}I-DCG-04 resulted in detection of 27 kDa and 31 kDa bands. However, in the presence of CA074, an inhibitor of cathepsin B which does not affect PE cleaving activity, only the 27 kDa band was affinity labeled (Yasothornsrikul et al., 2003). Thus, the 27 kDa band was responsible for PTP activity.

Two-D gels showed that the 27 kDa band labeled with DCG-04 was resolved into 3 spots of 27–29 kDa (Figure 4). The sequences of tryptic peptides derived from these spots (determined by mass spectrometry) corresponded to bovine cathepsin L (Yasothornsrikul et al., 2003). These results indicated cathepsin L as the catalytic subunit of the PTP protease complex.

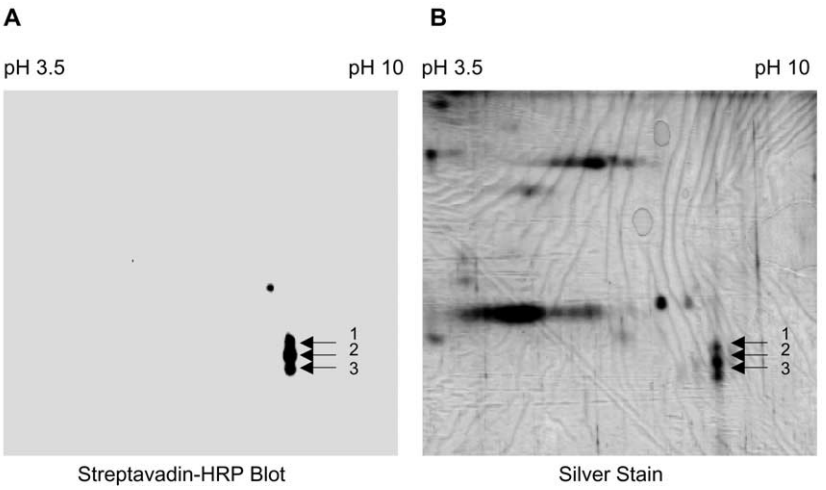

Figure 4 Activity-based profiling of cysteine proteases for proenkephalin processing.
Activity-based proteomic profiling of cysteine proteases of the 'prohormone thiol protease' (PTP) involved in proenkephalin processing was achieved by affinity-labeling with DCG-04 (panel A). The corresponding protein spots (panel B) that were affinity labeled were subjected to peptide sequencing by mass spectrometry of tryptic peptide digests.

Distinct form of secretory vesicle cathepsin L compared to lysosomal cathepsin L

The secretory vesicle form of cathepsin L differs biochemically from lysosomal cathepsin L (Yasothornsrikul et al., 2003). Secretory vesicle cathepsin L is a component of a protease complex of 180–200 kDa, whereas lysosomal cathepsin L is a single polypeptide. In addition, the DCG-04 labeled secretory vesicle cathepsin L is composed of three spots on a 2-D gel (Figure 4), whereas DCG-04 labeled lysosomal cathepsin L is composed of a single spot on a 2-D gel (Yasothornsrikul et al., 2003). These different forms may relate to the biological function of secretory vesicle cathepsin L in enkephalin production, compared to the degradative functions of lysosomal cathepsin L.

Cleavage specificity of cathepsin L for paired basic residue prohormone processing sites

Cathepsin L possesses cleavage specificity for dibasic and monobasic 'prohormone' processing sites (Figure 5), demonstrated by cathepsin L processing of the enkephalin-containing peptides BAM-22P and peptide F (Yasothornsrikul et al., 2003). Cathepsin L cleaves at the dibasic residue sites of BAM-22P F (↓Arg↓-Arg) and peptide F (↓Lys↓-Lys and ↓Lys-Arg). Cathepsin L also cleaved at ↓Arg sites, another cleavage site for pro-neuropeptide processing. The mass spectrometry profiles of cathepsin L cleavage of enkephalin-containing peptides demonstrate the production of biologically active (Met)enkephalin. In addition, cathepsin L converted full-length [^{35}S]-enkephalin precursor into identical product bands as those generated by native PTP. These cleavage studies demonstrate the specificity of cathepsin L for prohormone processing sites.

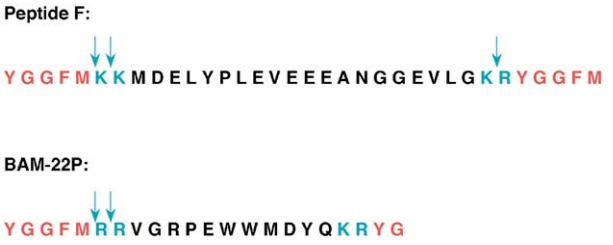

Figure 5 Cathepsin L cleaves prohormone processing sites to generate enkephalin.
The enkephalin-containing peptide substrates Peptide F and BAM-22P were incubated with cathepsin L and peptide products were identified by MALDI-TOF mass spectrometry. Sites that were cleaved by cathepsin L are indicated by the arrows.

Cellular localization of cathepsin L to secretory vesicles

Cathepsin L should be localized to secretory vesicles to be considered a prohormone processing enzyme. The secretory vesicle localization of cathepsin L in chromaffin cells was assessed by immunofluoresence confocal microscopy, which indicated co-localization of cathepsin L with secretory vesicle (Met)enkephalin (Yasothornsrikul et al., 2003). Both cathepsin L and (Met)enkephalin were visualized as discrete, punctate staining that is consistent with a secretory vesicle localization (Figure 6A). Moreover, cathepsin L is also co-localized with NPY in secretory vesicles (Figure 6B); NPY is another peptide hormone produced and stored within chromaffin granules (Carmichael et al., 1990). Further studies by high resolution immunoelectron microscopy also demonstrated the colocalization of cathepsin L with (Met)enkephalin within chromaffin granules (Figure 6C).

The presence of cathepsin L within secretory vesicles predicts that the enzyme should be co-secreted with enkephalin upon stimulated secretion from chromaffin cells. Cosecretion of [^{35}S]-cathepsin L and (Met)enkephalin occurred during stimulation of chromaffin cells by nicotine or by KCl depolarization (Yasothornsrikul et al., 2003). The combined microscopic and functional secretion of cathepsin L demonstrate its localization to secretory vesicles of the regulated secretory pathway.

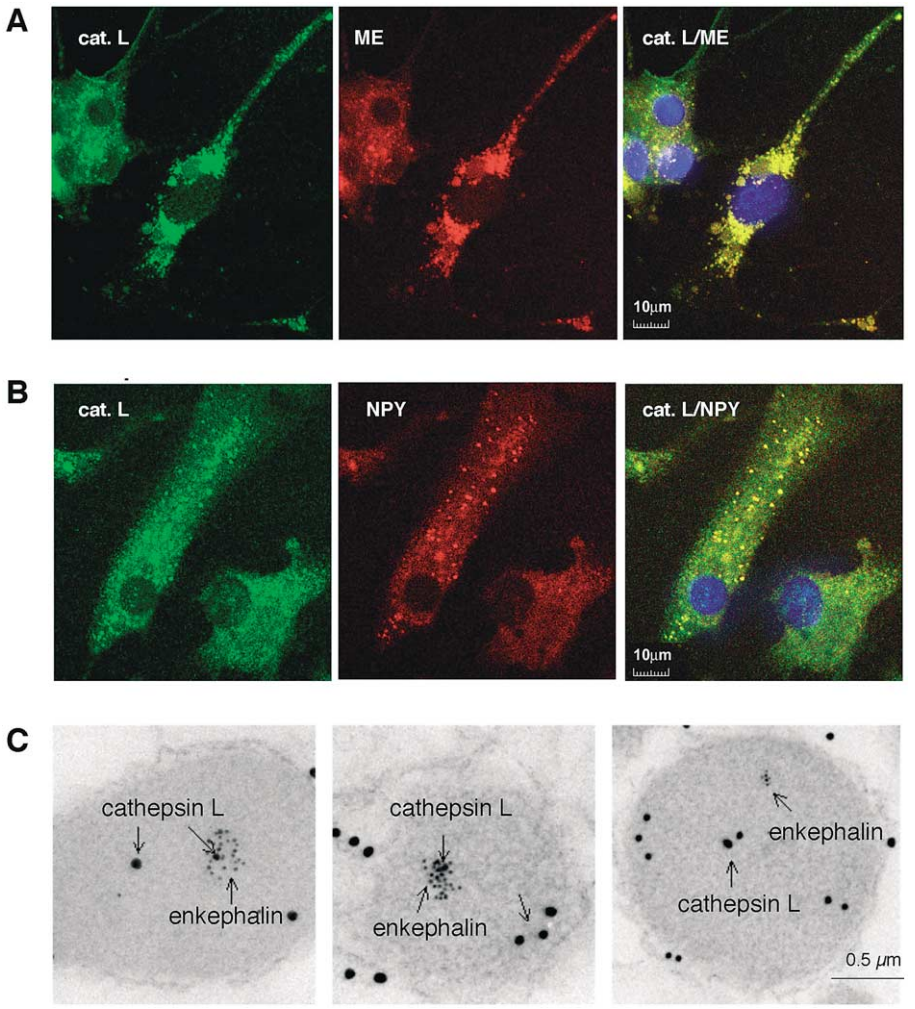

Figure 6 Secretory vesicle localization of cathepsin L with enkephalin.
(A) Colocalization of cathepsin L with enkephalin in chromaffin cells demonstrated by confocal immunofluorescence microscopy. Cathepsin L and (Met)enkephalin (green and red fluorescence, respectively) in chromaffin cells were visualized by immunofluorescence confocal microscopy. Excellent colocalization of cathepsin L and (Met)enkephalin was demonstrated by the merged images with colocalization indicated by yellow fluorescence. In chromaffin cells, the majority of cathepsin L is colocalized with (Met)enkephalin that is present within secretory vesicles. (B) Colocalization of cathepsin L with neuropeptide Y in chromaffin cells by confocal microscopy. Cathepsin L and neuropeptide Y (NPY) in chromaffin cells were visualized by immunofluorescence confocal microscopy (green and red fluorescence, respectively). Excellent colocalization of cathepsin L and NPY was demonstrated by the merged images with colocalization indicated by yellow fluorescence. In chromaffin cells, the majority of cathepsin L is colocalized with NPY-containing secretory vesicles. (C) Immunoelectron microscopy demonstrates cathepsin L in enkephalin-containing secretory vesicles. Immunoelectron microscopy of isolated chromaffin granules indicated colocalization of cathepsin L (15 nm gold particles) with enkephalin (6 nm gold particles).

Inactivation of the cathepsin L gene in knockout mice reduces enkephalin levels in brain

Evaluation of cathepsin L as a candidate proenkephalin (PE) processing enzyme for the production of enkephalin peptides was assessed in cathepsin L knockout mice. Results demonstrated a 50% reduction in levels of brain (Met)enkephalin (ME), compared to wild-type mice (Figure 7). The radioimmunoassay for ME does not recognize the proenkephalin precursor or extended forms of ME. These results clearly demonstrate a role for cathepsin L in the production of brain enkephalin. It will be of interest to analyze the effects of the absence of cathepsin L on the production of other neuropeptides. Ongoing evaluation of several other neuropeptides in cathepsin L deficient mice demonstrate a role for cathepsin L in the production of several neuropeptides (Hook et al., unpublished observations). Comparison of the role of cathepsin L with PC2 and PC1/3 in knockout mice in neuropeptide production will indicate how processing enzymes may selectively regulate the production of a variety of peptide neurotransmitters and hormones.

Arg/Lys aminopeptidase subsequent to cathepsin L for prohormone processing

The cleavage specificities of cathepsin L for cleavage between and at the NH_2-terminal side of the dibasic residue sites indicate that peptide intermediates possess basic residue extensions at their NH_2-termini. These findings indicate the necessity for a subsequent aminopeptidase step to remove Arg and Lys residues from

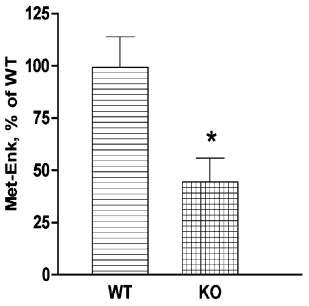

Figure 7 Enkephalin levels in brain are reduced in cathepsin L knockout mice.
Cathepsin L knockout mice showed decreased levels of (Met)enkephalin in brain. (Met)enkephalin was measured by radioimmunoassay, which does not recognize the proenkephalin precursor. Increases in relative levels of proenkephalin were also demonstrated (Yasothornsrikul et al., 2003). The asterisk (*) indicates statistical significance with $p<0.05$, by two-tailed *t*-test.

NH_2-termini of peptide intermediates (Figure 2). Indeed, Arg/Lys aminopeptidase activity is present in chromaffin granules, detected by cleavage of Arg-↓MCA and Lys-↓MCA substrates (Yasothornsrikul et al., 1998). Arg/Lys aminopeptidase in chromaffin granules (Hook and Eiden, 1984) converts Arg-(Met)enkephalin to (Met)enkephalin. Arg/Lys aminopeptidase activity is also present within pituitary secretory vesicles (Gainer et al., 1984). The Arg/Lys aminopeptidase may be related to aminopeptidase B (AP-B) that possesses specificity for basic residues (Cadel et al., 1997; Balogh et al., 1998). The presence of AP-B in secretory vesicles from chromaffin cells (chromaffin granules) has been demonstrated by immunoelectron microscopy (Hook and Cohen, unpublished results). Overall, these findings illustrate the presence of Arg/Lys aminopeptidase in neuropeptide-containing secretory vesicles.

Implications of the cathepsin L and Arg/Lys aminopeptidase pathway for prohormone processing

The cathepsin L and Arg/Lys aminopeptidase pathway for prohormone processing provides an alternative pathway to insure cellular production of essential peptide neurotransmitters and hormones. Thus, the cathepsin L and Arg/Lys aminopeptidase pathway provides an explanation for the presence of presumably modest levels of neuropeptides in *fat*/*fat* mice that lack carboxypeptidase E/H activity, due to a mutation in the CPE/H gene (Naggert et al., 1995; Che et al., 2001). The *fat*/*fat* mice survive and peptide hormone and neurotransmitter systems are functional at a level that allows essential physiological systems to operate (Fricker and Leiter, 1999). In the absence of active CPE/H, production of neuropeptides can potentially be achieved with the cathepsin L and Arg/Lys aminopeptidase processing pathway (Figure 2). Thus, processing of proprotein precursors to active peptide neurotransmitters and hormones can proceed independently of the PC1/3, PC2 (combined with other PC enzymes), and CPE/H pathway. Thus, cells possess alternative proteolytic mechanisms to insure adequate synthesis of essential neuropeptides required for physiological functions.

Newly identified carboxypeptidases also indicate alternative enzymes for this exopeptidase step. Since the *fat*/*fat* mice survive with many intact physiological systems, results suggested that CPE/H may not be the only carboxypeptidase available for proprotein processing, the related carboxypeptidase D has been identified that may provide adequate carboxypeptidase activity for production of neuropeptides in the absence of carboxypeptidase E/H (Dong et al., 1999; Fricker and Leiter, 1999; Varlamov et al., 1999). Thus, prohormone processing may also utilize multiple carboxypeptidases to accomplish the required processing of prohormones to active neuropeptides that are critical for cellular functions.

Summary

These results demonstrate the novel role for secretory vesicle cathepsin L in prohormone processing. Moreover, recent studies show that cathepsin L undergoes trafficking in the secretory pathway that results in cathepsin L as a resident protein of mature secretory vesicles, a major site for pro-neuropeptide processing (Kuliawat et al., 1997). Ongoing research indicates a role for cathepsin L in the production of multiple neuropeptides. It will be of interest to understand the biological roles of secretory vesicle cathepsin L in peptide neurotransmission and peptide hormone actions.

Arg/Lys aminopeptidase is then required for complete processing of peptide intermediates generated by cathepsin L. The specificity of cathepsin L for cleavage at the NH_2-terminal sides of dibasic residues, and between the two dibasic residues, results in peptide intermediates that require removal of these basic residue extensions at NH_2-termini. Cleavage between two dibasic residues results in peptide products that also require removal of COOH-terminal basic residues by carboxypeptidase E/H to generate the final neuropeptide.

The distinct cathepsin L and Arg/Lys aminopeptidase pathway for prohormone processing, combined with the PC enzyme and carboxypeptidase E/H pathway, provides an alternative route for proteolytic processing that generates active peptide neurotransmitters and hormones.

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