The Kunitz Protease Inhibitor Form of the Amyloid Precursor Protein (KPI/APP) Inhibits the Proneuropeptide Processing Enzyme Prohormone Thiol Protease (PTP)

COLOCALIZATION OF KPI/APP AND PTP IN SECRETORY VESICLES*

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Proteolytic processing of proenkephalin and proneuropeptides is required for the production of active neurotransmitters and peptide hormones. Variations in the extent of proenkephalin processing in vivo suggest involvement of endogenous protease inhibitors. This study demonstrates that “protease nexin 2 (PN2),” the secreted form of the kunitz protease inhibitor (KPI) of the amyloid precursor protein (APP), potently inhibited the prohormone processing enzyme known as prohormone thiol protease (PTP), with a \( K_{i, app} \) of 400 nM. Moreover, PTP and PN2 formed SDS-stable complexes that are typical of kunitz protease inhibitor interactions with target proteases. In vivo, KPI/APP (120 kDa), as well as a truncated form of KPI/APP that resembles PN2 in apparent molecular mass (110 kDa), were colocalized with PTP and (Met)enkephalin in secretory vesicles of adrenal medulla (chromaffin granules). KPI/APP (110–120 kDa) was also detected in pituitary secretory vesicles that contain PTP. In chromaffin cells, calcium-dependent secretion of KPI/APP with PTP and (Met)enkephalin demonstrated the colocalization of these components in functional secretory vesicles. These results suggest a role for KPI/APP inhibition of PTP in regulated secretory vesicles. In addition, these results are the first to identify an endogenous protease target of KPI/APP, which is developmentally regulated in aging and Alzheimer’s disease.

Peptide neurotransmitters and hormones are synthesized as protein precursors that require proteolytic processing to generate active neuropeptides. Proneuropeptide precursors are routed from the rough endoplasmic reticulum to Golgi apparatus and secretory vesicles (1, 2). The presence of proneuropeptides and processed peptide products within secretory vesicles indicates that corresponding proteases for proneuropeptide processing are largely present within such vesicles. Secretory vesicles of adrenal medulla, known as chromaffin granules, have been widely used as a model system for studying neuropeptide biosynthetic enzymes (2–4). These vesicles contain several neuropeptides, including high levels of enkephalin opioid peptides (5–7) and neuropeptide Y (8), as well as galanin (9), somatostatin (7), and others.

In our studies of proneuropeptide processing enzymes in chromaffin granules, the major proenkephalin processing enzyme was identified as a novel cysteine protease known as prohormone thiol protease (PTP)¹ (2, 10–14). PTP cleaves proenkephalin at dibasic and monobasic basic residues (Arg and Lys) to generate enkephalin-related peptides that are present in adrenal medulla in vivo (12, 13, 15, 16). Proenkephalin processing in chromaffin granules is also mediated, to a lesser degree, by the subtilisin-like prohormone convertase 1 (PC1) and PC2 enzymes (17) and by a 70-kDa aspartyl protease resembling the pituitary proopiomelanocortin-converting enzyme (18).

It is known that the extent of proenkephalin processing in adrenal medulla, in vivo, is limited, because only 10% of (Met)enkephalin exists as completely processed pentapeptide, and approximately 90% of enkephalins in this tissue are present as high molecular mass intermediates of 10–25 kDa (19, 20). However, in vitro processing of proenkephalin by PTP proceeds completely, with extensive conversion of proenkephalin into peptide products. These observations suggest that endogenous protease inhibitors of PTP are present in vivo to limit the extent of proenkephalin processing.

Many enkephalin- and neuropeptide-containing regions in the brain (21–23) contain the kunitz protease inhibitor form of the amyloid precursor protein (KPI/APP) in normal (24–29) and Alzheimer’s disease brains (25, 30). However, the endogenous target protease inhibited by KPI/APP has not yet been elucidated, although it is known that KPI/APP functions as a protease inhibitor (31, 32). The KPI-containing forms of APP are represented by APP-770, APP-751, and the appican core protein APP-733 (770, 751, and 733 amino acids in length, respectively), which are generated by alternative splicing of the APP gene product (24–27). The KPI-containing forms of APP (24–27), as well as the non-KPI APP-695 (33, 34), represent precursors of the \( \beta \)-amyloid peptide that is present in normal brain (28, 29, 35) and that accumulates in amyloid plaques of Alzheimer’s disease brains (36–39). Studies have demon-

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¹ The abbreviations used are: PTP, prohormone thiol protease; PN2, protease nexin 2; KPI, kunitz protease inhibitor; APP, amyloid precursor protein; PC, prohormone convertase; SRM, standard release medium; PAGE, polyacrylamide gel electrophoresis.
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strated that KPI/APP (40) and the non-KPI form of APP (41) are axonally transported from neuronal cell bodies to nerve terminals; these results suggest localization of KPI/APP within secretory vesicles that undergo axonal transport to nerve terminals. The secreted form of KPI/APP is protease nexin 2 (PN2) (30, 42), which represents a cleavage product of KPI/APP when KPI/APP is cleaved within the β-peptide domain (30, 43). These observations implicate a role for KPI/APP in regulating protein processing within the regulated secretory vesicle pathway of neurons.

The presence of both the proenkephalopeptide processing enzyme PTP (2, 12–14) and the KPI/APP protease inhibitor (40) in neuroendocrine secretory vesicles suggests interactions of PTP and KPI/APP in vivo. The cleavage specificity of PTP for basic residues (Arg or Lys) (12, 13), and the identical P1 Arg residue within the reactive site domain of KPI/APP (40) and the non-KPI form of APP (41) predicts that the KPI form of APP can inhibit PTP.

**EXPERIMENTAL PROCEDURES**

**In Vitro Inhibition of PTP by the Secreted Form of KPI/APP. Protease Nexin 2, and the KPI Domain—**Purified PTP, PN2, and recombinant KPI were prepared for in vitro inhibition assays of PTP activity. PTP was purified from chromaffin granules isolated from bovine adrenal medulla as described previously (12). Protease nexin 2 was purified as described previously (45); recombinant KPI was expressed in yeast and purified as described previously (46).

For inhibition assays, purified PTP (3 ng, 5 nM) was preincubated with protease nexin 2 (5–50 nM) or recombinant KPI (1–80 μM) in PTP assay buffer (described previously (12, 13)) at 4 °C for 15 min. PTP activity was assayed by monitoring the conversion of [35S]hCysteine and [35S]methionine (100 μCi/ml) in PTP assay buffer (described previously (12, 13, 14)) with the 22C11 monoclonal antibody (1:500 dilution, Boehringer Mannheim) that recognizes an NH2-terminal epitope of APP.

**Isolation of Chromaffin Granules for Electron Microscopy, and Fractionation into Soluble and Membrane Components—**For electron microscopy, chromaffin granules were isolated by centrifugation of fresh bovine adrenal medulla homogenate and subjected to discontinuous sucrose gradient centrifugation and separated into soluble and membrane components, as described previously (12, 13, 47).

In addition, ultracentrifugation of chromaffin granules on a sequential centrifugation of sucrose gradient of 2–2.10 M sucrose was performed (as described previously (47)) to assess co-purification of KPI/APP with enkephalin-containing granule fractions, determined by measuring (Met)enkephalin by radioimmunoassay, as described previously (13, 14). KPI/APP in the chromaffin granule fractions was determined by Western blots with the R7 antiserum that recognizes the KPI domain (48, 49), performed as described previously (14).

**Preparation of Secretory Vesicles from Posterior Pituitary—**To further assess the localization of KPI/APP to secretory vesicles, neuropeptide-containing secretory vesicles of bovine posterior pituitary (50) were purified by metrizamide gradient ultracentrifugation, as described previously (15).

In addition, pituitary homogenates were prepared from fresh bovine pituitary in 25 mM histidine-HCl, pH 6.5, with a mixture of protease inhibitors (10 μM chymostatin, 10 μM pepstatin A, and 10 μM leupeptin). KPI/APP in purified posterior pituitary secretory vesicles and homogenates of bovine pituitary were analyzed by Western blots with the R7 antiserum. Protein content of samples was determined by the method of Lowry et al. (52).

**Primary Cultures of Chromaffin Cells and Cosecretion of KPI Immunoreactivity with PTP and (Met)enkephalin—**Primary cultures of chromaffin cells were prepared from fresh bovine adrenal medulla and plated in fibronectin-coated plates (Falcon 6-well plates) at 2.0 × 10⁶ cells/well, as described previously (14). To assess secretion, cells were incubated in medium containing [35S]cysteine and [35S]methionine (100 μCi/ml) (53) and 22C11 mAb (1:500 dilution, Boehringer Mannheim) for 24 h prior to KCl induction of secretion. Cells were washed and preincubated for 15 min at 37 °C with SRM buffer (described previously (14)) with or without calcium. SRM without calcium included 2.2 mM MgCl₂, (to replace 2.2 mM CaCl₂) and 2.0 mM EGTA. Cells were incubated for 15 min at 37 °C with fresh SRM buffer in the presence or absence of calcium, or with 50 mM KCl in SRM buffer in the presence or absence of calcium. The media were collected with addition of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 μM chymostatin, 10 μM leupeptin, 10 μM pepstatin A, and 1 μM E64c), and concentrated (Millipore Biomax 10K concentrator) with buffer exchange consisting of 50 mM citric acid, pH 6.0, 50 mM NaCl, 1 mM EDTA, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.5 mM chymostatin, 0.5 mM leupeptin, 0.5 mM pepstatin A and 1 μM E64c). Immunoprecipitation of KPI in the medium with R7 antibody (29) (R7 IgGs at 1:100 final dilution purified by protein A-Sepharose chromatography) was performed as described previously (53).

To assess secretion of (Met)enkephalin and PTP, cells were incubated for 15 min at 37 °C with fresh SRM buffer with or without calcium, or with 50 mM KCl in SRM buffer with or without calcium. The medium was collected with addition of protease inhibitors (as above); brought to pH 6.0 by addition of an equal volume of 100 mM sodium phosphate, pH 6.0, 50 mM NaCl, and 1 mM EDTA; and concentrated with a Millipore Ultrafree-15 centrifugal filter (Biomax-10K NMWL membrane). The medium was analyzed for (Met)enkephalin by radioimmunoassay, as described previously (14), and was subjected to anti-PTP (1:500 dilution of antisera) Western blots, by the chemiluminescent ECL method (according to the manufacturer’s protocol, Amersham Pharmacia Biotech).

**RESULTS**

**The PN2 Form of KPI/APP Inhibits PTP—**The proenkephalopeptide processing enzyme known as PTP represents the major proenkephalin processing enzyme in enkephalin-containing secretory vesicles of adrenal medulla (2, 12–14). PTP in vitro converts proenkephalin into smaller enkephalin-containing peptide fragments that resemble enkephalin peptide products in vivo (12, 13, 15). However, the extent of proenkephalin processing in adrenal medulla in vivo is limited (19), suggesting that endogenous protease inhibitors inhibit proenkephalin processing. The presence of the KPI form(s) of APP, the APP-751 and APP-770 forms, in enkephalin- and neuropeptide-containing brain regions (21–23, 28, 29) suggests possible inhibition of PTP by KPI/APP. In addition, the parallel P1 Arg residue within the reactive site loop of the KPI domain and the parallel cleavage specificity of PTP at Arg or Lys residues (31) was subjected to anti-PTP (1:500 dilution of antisera) Western blots, by the chemiluminescent ECL method (according to the manufacturer’s protocol, Amersham Pharmacia Biotech).

The ability of KPI/APP to inhibit PTP was assessed in in vitro assay that tested the ability of PN2, the secreted form of KPI/APP that contains the KPI domain, to inhibit purified PTP. PN2 effectively inhibited PTP activity by 30–60% at PN2 concentrations of 100–500 nM. PTP activity was determined by measuring the conversion of recombinant [35S]enkephalin precursor to trichloroacetic acid-soluble radioactivity that represents small [35S]-peptide products (Fig. 1a). The concentration-
dependence curve indicated a $K_{\text{app}}$ of 400 nM, calculated by the equation 
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\frac{[I]}{(1 - a)} = K_{\text{app}}\frac{1}{[a]} + [E] 
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(31). Examination of PTP activity by autoradiography of SDS-PAGE gels showed that PN2 inhibited PTP cleavage of the enkephalin precursor, as well as processing of a 23-kDa intermediate (Fig. 1b). In addition, PN2 prevented production of a small 10-kDa product.

In contrast to nearly full-length KPI/APP represented by PN2, the 57-residue KPI domain itself was a significantly less effective inhibitor by nearly 2 orders of magnitude (Fig. 1b). Inhibition of PTP by 30–60% required micromolar concentrations (10–100 μM) of KPI, compared with the nanomolar levels (100–500 nM) of PN2 required for a similar degree of PTP inhibition. The KPI domain inhibited PTP with a $K_{\text{app}}$ of approximately 70 μM, in contrast to the more potent inhibition by PN2 with a $K_{\text{app}}$ of 400 nM. Similar to PN2, the KPI domain inhibited processing of the enkephalin precursor and a 23-kDa proenkephalin-derived intermediate (Fig. 2b).

Members of the kunitz protease inhibitor family possess a reactive site loop that interacts with the target protease to form an inhibitor and target protease complex (44). Formation of PTP and PN2 complexes were demonstrated by SDS-PAGE under nonreducing conditions (Fig. 3). PN2 (at 100, 200, and 400 ng) was incubated without or with PTP (1.5 ng), representing molar ratios of PN2/PTP of approximately 9, 18, and 35, respectively; reactions were analyzed by Western blots with the 22C11 antibody, which recognizes an NH$_2$-terminal domain of KPI/APP (54). The PN2/PTP complex was detected on nonreducing SDS-PAGE gels as a high molecular mass band of approximately 150 kDa, compared to uncomplexed PN2 with an apparent molecular mass of 110–130 kDa.

Localization of KPI/APP Forms in Secretory Vesicles of Adrenal Medulla (Chromaffin Granules) and Pituitary—The localization of PTP to secretory vesicles of the adrenal medulla, known as chromaffin granules, indicates that in vivo inhibition of PTP by KPI/APP requires colocalization of KPI/APP within the same organelle. Isolation of highly purified, intact chromaffin granules was demonstrated by electron microscopy (Fig. 4). The isolated chromaffin granules retain their integrity and morphology during purification, and possess morphology similar to chromaffin granules observed by electron microscopy in

![Fig. 1. PN2 inhibits PTP. a, inhibition of PTP processing of 35S-enkephalin precursor to acid-soluble radioactivity. Inhibition of PTP was assessed by the ability of PN2 to inhibit PTP activity, assayed by measuring the conversion of 35S-(Met)enkephalin precursor to trichloroacetic acid-soluble radioactivity. b, inhibition of PTP cleavage of 35S-enkephalin precursor. 35S-enkephalin precursor (35 kDa) was incubated without PTP (lane 1), with PTP (lane 2), and with PTP in the presence of PN2 (50 nM) (lane 3). Reactions were subjected to SDS-PAGE and autoradiography.](image-url)

![Fig. 2. The KPI domain is a less effective inhibitor of PTP. a, inhibition of PTP processing of 35S-enkephalin precursor to acid-soluble radioactivity. Inhibition of PTP by recombinant KPI domain was tested by monitoring PTP-mediated conversion of 35S-enkephalin precursor to trichloroacetic acid-soluble radioactivity. b, inhibition of PTP cleavage of 35S-enkephalin precursor. 35S-enkephalin precursor was incubated without PTP (lane 1), with PTP (lane 2), and with PTP in the presence of KPI (40 μM) (lane 3). Reactions were subjected to SDS-PAGE (12% polyacrylamide) and autoradiography.](image-url)
Chromaffin granules contain KPI/APP, based on Western blots with the R7 and R1 antibodies (48, 49) that recognize the KPI domain and the COOH-terminal region of APP, respectively (Fig. 5a). Western blots with the R7 antiserum showed the presence of full-length KPI/APP of 110–120 kDa in chromaffin granules (Fig. 5b). KPI/APP was present in both the soluble and membrane components of these granules. The same 110–120-kDa band was detected by the R1 antiserum that recognizes the COOH-terminal region of APP (Fig. 5c). These results indicate the presence of full-length KPI/APP that includes the COOH-terminal domain of KPI/APP. These findings are consistent with earlier results indicating the presence of full-length APP in chromaffin granules (53, 56, 57); however, it was not previously known whether KPI/APP represented the chromaffin granule APP. It is noted that KPI/APP of 110–120 kDa in chromaffin granules detected on reducing SDS-PAGE gels differs slightly from analyses of PN2 of 110–130 kDa (lanes 2 and 3) on a nonreducing SDS-PAGE gel.

Because chromaffin granules possess protein components common to other neuroendocrine secretory vesicles, the presence of KPI/APP was analyzed in bovine pituitary and, specifically, in secretory vesicles isolated from posterior pituitary, which also contain PTP and several neuropeptides, including vasopressin and oxytocin (50). Pituitary homogenate, which represents primarily anterior pituitary, contains KPI/APP forms of approximately 115 and 120 kDa, as detected on Western blots with the R7 antiserum (Fig. 7, lane 1). Secretory vesicles of posterior pituitary also contain KPI/APP as a 110-kDa form, the molecular mass of which appears consistent with that of PN2, a truncated form of KPI/APP. Slight variations in the apparent molecular masses of KPI/APP bands suggest differences in their proteolytic processing. Overall, the presence of KPI/APP forms in PTP-containing secretory vesicles of adrenal medulla and pituitary suggest that KPI/APP inhibition of PTP can occur in vivo within neurosecretory vesicles.

Co-secretion of KPI Immunoreactivity with PTP and (Met)enkephalin from Chromaffin Cells—The colocalization of KPI/APP in chromaffin granules with PTP and (Met)enkephalin predicts that KPI immunoreactivity should be cosecreted with PTP and (Met)enkephalin. Secretion of vesicular 35S-proteins (labeled with [35S]cysteine and [35S]methionine) from chromaffin cells in primary culture was induced by KCl depolarization. Immunoprecipitation of R7 immunoreactivity in the secretion medium and analysis by autoradiography of SDS-PAGE gels demonstrated KCl-induced secretion of 56- and 45-kDa KPI-containing APP fragments (Fig. 8a). It appears that the KPI/APP of 110–120 kDa in chromaffin granules may be converted to truncated 56- and 45-kDa KPI-containing fragments upon secretion into the extracellular medium. KCl-induced secretion of KPI-containing fragments required Ca2+ in the medium, because secretion was reduced when Ca2+ was omitted. The Ca2+—dependent secretion of KPI/APP fragments indicates release from regulated secretory vesicles, which undergo exocytosis in response to stimulated membrane depolarization (60).

Importantly, cosecretion of KPI/APP fragments with PTP and (Met)enkephalin from chromaffin cells was demonstrated (Fig. 8, b and c). Secretion of PTP and (Met)enkephalin were clearly induced by KCl depolarization in a Ca2+—dependent manner. It is noted that a low basal level of KPI/APP fragment and (Met)enkephalin secretion was observed; the lower sensitivity of the PTP Western blot assay did not allow detection of possible basal secretion. Importantly, co-secretion provides strong evidence for the colocalization of KPI/APP, PTP, and (Met)enkephalin in functional secretory vesicles. These results support the hypothesis of in vivo regulation of PTP and KPI/APP, because PN2 is known to lack a 8–10-kDa segment located at the COOH terminus of KPI/APP (120 kDa) (30, 43). KPI/APP and (Met)enkephalin were detected in identical fractions (fractions 5–14), indicating the localization of KPI/APP with (Met)enkephalin-containing in chromaffin granules.

It is noted that two peaks of (Met)enkephalin were indicated on the multistep sucrose gradient. These results demonstrate two subpopulations of chromaffin granules of differing densities. It is apparent that KPI/APP was detected in both populations of chromaffin granules. Recent investigations of secretory vesicles (58, 59) indicate that immature secretory granules are initially generated by budding from the trans-Golgi apparatus and that these immature secretory granules undergo maturation into the more dense mature granules. The two peaks of (Met)enkephalin-containing chromaffin granules may possibly represent immature and mature secretory granules.

Fig. 3. PN2/PTP complex formation. PTP was incubated alone (lane 1) or with protease nexin 2 (PN2, 100, 200, and 400 ng in lanes 2–4, respectively) for 10 min at 37 °C, and SDS-stable complexes were assessed by SDS-PAGE without reducing agent and without heating. Samples analyzed by Western blots with the 22C11 monoclonal antibody that recognizes the NH2-terminal domain of PN2 and KPI/APP. PN2/PTP complexes were visualized as a high molecular mass band (lane 4), compared with uncomplexed PN2 of approximately 110–130 kDa (lanes 2 and 3) on a nonreducing SDS-PAGE gel.

Fig. 4. Chromaffin granules and electron microscopy. The integrity and homogeneity of isolated chromaffin granules (secretory vesicles) from bovine adrenal medulla is illustrated by electron microscopy (EM). Electron microscopy showed an average diameter of approximately 0.1 μm for these granules.

situ in chromaffin cells (55). These purified, homogeneous granules are, therefore, ideal for biochemical analyses of KPI/APP in this organelle.
DISCUSSION

The biosynthesis of peptide neurotransmitters and peptide hormones requires proteolytic processing of inactive pro-neuropeptides into smaller, biologically active neuropeptides. PTP has been identified as a major enkephalin processing enzyme in adrenal medullary secretory vesicles, known as chromaffin granules. Characterization of PTP indicates that it is involved in the production of enkephalin opioid peptides (2, 12–15). The limited extent of proenkephalin processing in vivo suggests the presence of endogenous protease inhibitors to prevent complete processing (19). Results from this study indi-
cate that the KPI-containing APP, in the form of PN2, is a potent inhibitor of PTP. The KPI domain itself, however, was less effective compared with PN2, because the $K_{\text{app}}$ for PN2 inhibition was approximately 400 nM, whereas the $K_{\text{app}}$ for KPI inhibition (70 μM) was 175-fold greater. Moreover, PTP and PN2 formed SDS-stable complexes that are typical of kunitz protease inhibitor interactions with target proteases. In addition, KPI/APP and PTP are present in isolated secretory vesicles (chromaffin granules) and are co-secreted with (Met)enkephalin from chromaffin cells. These results indicate that the KPI/APP inhibitor and PTP protease are colocalized in functional secretory vesicles that undergo Ca$^{2+}$-dependent exocytosis. These findings suggest a role for KPI/APP in the regulation of PTP during the biosynthesis of enkephalins and neuropeptides.

This study provides answers to two important questions. First, what endogenous protease inhibitor may regulate proneuropeptide processing? Second, what is (or are) the endogenous protease target(s) of neuronal KPI/APP? KPI/APP inhibition of PTP and their colocalization in secretory vesicles have identified KPI/APP as a natural, endogenous inhibitor of the proneuropeptide processing enzyme PTP. Furthermore, this study has answered the second question with identification of PTP as an endogenous protease target of KPI/APP. Chromaffin cells have been extensively used as a model neuronal cell system for studies of brain neurotransmitter synthesizing enzymes. The presence of KPI/APP and PTP in chromaffin granules predicted their localization in secretory vesicles of posterior pituitary. Localization to posterior pituitary indicates synthesis of KPI/APP and PTP in neuronal cell bodies of the hypothalamus (brain region), because nerve terminals of posterior pituitary originate from hypothalamic neurons. Moreover, chromaffin granules and pituitary secretory vesicles contain KPI/APP as 120- and 110-kDa forms, suggesting the presence of a truncated form of KPI/APP (110 kDa) that possesses a molecular mass similar to that of PN2. In addition, PTP is present in human and rat hippocampus, suggesting KPI/APP inhibition of PTP in brain. These results suggest that KPI and PTP interactions can occur in adrenal medulla, posterior pituitary, and certain brain regions that synthesize enkephalins and neuropeptides.

It is known that specific proteolysis of proenkephalin and many proneuropeptides is limited in vivo, which predicts that endogenous protease inhibitors regulate proneuropeptide processing enzymes. The processing of proneuropeptides at paired basic or monobasic residues (Arg or Lys) is achieved by several proneuropeptide processing enzymes: a novel cysteine protease known as PTP (12–15), subtilisin-like PCs termed PC1 and PC2 (as well as other members of the subtilisin-like protease family) (61, 62), and a 70-kDa aspartyl protease related to the yapsin family (63). Therefore, protease inhibitors of proenkephalin and proneuropeptide processing should contain a basic residue, Arg or Lys, at the P1 position of the reactive site domain of the inhibitor that will be recognized by the target protease. The Arg residue at the P1 position of KPI/APP predicts that it should be an effective inhibitor of PTP that pos-

\[3\] V. Y. H. Hook, unpublished observations.

**Fig. 7.** KPI/APP in pituitary secretory vesicles. The presence of KPI/APP in bovine pituitary homogenate (lane 1) and secretory vesicles isolated from bovine posterior pituitary (lane 2) was assessed by Western blots with the R7 antiserum, as described under "Experimental Procedures.”

**Fig. 8.** Co-secretion of KPI immunoreactivity with PTP and (Met)enkephalin from chromaffin cells. a, secretion of KPI immunoreactivity. Primary cultures of chromaffin cells were incubated in medium containing $[^{35}\text{S}]$cysteine and $[^{35}\text{S}]$methionine for 24 h before KCl-induced secretion. Secretion of $[^{35}\text{S}]$KPI immunoreactivity, performed in duplicate, was assessed by immunoprecipitation and autoradiography of $[^{35}\text{S}]$-proteins released in the cell culture medium. $[^{35}\text{S}]$-KPI immunoprecipitates were analyzed from medium of control cells with Ca$^{2+}$ and KCl-treated cells in medium without Ca$^{2+}$ (lanes 1 and 2), control cells in medium without Ca$^{2+}$ (lanes 3 and 4), KCl-treated cells with Ca$^{2+}$ (lanes 5 and 6), and KCl-treated cells in the absence of Ca$^{2+}$ (lanes 7 and 8). b, secretion of PTP. The secretion of PTP was induced by KCl and the release of PTP into the medium was assessed by Western blots with anti-PTP serum. PTP was analyzed in medium from control cells with Ca$^{2+}$ (lane 1), control cells in medium without Ca$^{2+}$ (lane 2), KCl-treated cells with Ca$^{2+}$ (lane 3), and KCl-treated cells in the absence of Ca$^{2+}$ (lane 4). c, secretion of (Met)enkephalin. (Met)enkephalin secretion induced by KCl depolarization was measured by radioimmunoassay. (Met)enkephalin was measured in medium from control (C) cells with Ca$^{2+}$, control cells in medium without Ca$^{2+}$, KCl-treated (KCl) cells with Ca$^{2+}$, and KCl-treated cells in the absence of Ca$^{2+}$, as indicated.
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Sesses cleavage specificity for Arg or Lys basic residues. Protease nexin 2, the secreted form of KPI/APP, was a potent inhibitor of PTP. SDS-stable complexes of PN2 and PTP indicate protease inhibitor and target protease interactions. However, the 57-residue KPI domain itself was not sufficient for full inhibition, because inhibition by KPI required micromolar concentrations (K_{app} of 70 μM KPI), in contrast to the nanomolar levels (K_{app} of 400 nM PN2) of PN2 that were effective for PTP inhibition. These findings suggest that interactions of KPI/APP and PTP at sites other than the P1 residue are required for inhibition of PTP by KPI/APP.

The kunitz protease inhibitor family typically inhibits serine proteases (44). However, protease nexin 2 inhibition of the cysteine protease PTP demonstrates cross-class inhibition; that is, inhibition of a target protease that differs in protease class compared with the protease inhibitor. The parallel P1 residues for KPI/APP and PTP provide an hypothesized mechanism for PTP and KPI/APP interactions. Several other important examples illustrate cross-class inhibition of proteases by serpins (serine protease inhibitors) with P1 residues that mimic the cleavage specificities of target proteases. The cowpox virus crmA gene encodes a serpin that inhibits the cysteine protease interleukin-converting enzyme (64), as well as interleukin-converting enzyme-related caspases involved in apoptosis (65, 66). In addition, the squamous cell carcinoma antigen serpin inhibits the cysteine proteases cathepsins K, L, and S (67). It is hypothesized that cross-class interactions involve recognition of the P1 residue of the serpin by target proteases that possess cleavage specificity for the same P1 residue within its substrate.

Importantly, the colocalization of PTP and KPI/APP within secretory vesicles of adrenal medulla, known as chromaffin granules, is consistent with KPI/APP inhibition of PTP in vivo. Western blots of KPI/APP, detected by an antibody that recognizes the KPI domain (R7 antiserum), showed that it is present in soluble and membrane components of chromaffin granules. The presence of full-length KPI/APP was demonstrated by recognition of the 110–120-kDa KPI-containing band by an antibody directed toward the COOH-terminal region (R1 antibody) of APP. These results indicate that previous identification of APP in chromaffin granules includes the KPI form of APP (53, 56, 57). Importantly, functional colocalization of KPI/APP with PTP and Met-enkephalin in regulated secretory vesicles was demonstrated by their cosecretion from chromaffin cells upon membrane depolarization induced by KCl, in a Ca^{2+}-dependent manner. It is well known that exocytosis of the contents of regulated secretory vesicles occurs in a Ca^{2+}-dependent manner (60).

The presence of KPI/APP and PTP in regulated secretory vesicles of adrenal medulla suggested that they are likely to be present in neuronal secretory vesicles that are axonally transported from neuronal cell bodies to nerve terminals, where Ca^{2+}-dependent secretion of vesicular contents occurs. Indeed, the presence of KPI/APP (Fig. 7) and PTP2 in posterior pituitary indicates the synthesis of KPI/APP and PTP in neurons of the hypothalamus (of the brain), the axons of which terminate in the posterior pituitary. Secretory vesicles in hypothalamic neurons are transported along axons to nerve terminals in the posterior pituitary. Recent studies have demonstrated axonal transport of KPI/APP in neuronal retinal ganglion cells (42). The findings from this study provide evidence for the colocalization of KPI/APP and PTP in secretory vesicles of neuroendocrine cells.

Results from this study demonstrate a role for KPI/APP in the regulation of PTP in proenkephalin and proenkephalin biosynthesis. It is proposed that KPI/APP tonically inhibits PTP in vivo to influence the extent of proenkephalin and proenkephalin processing. Interactions of KPI/APP and PTP in vivo are supported by findings that they are normal components of neurosecretory vesicles. Under abnormal conditions, detection of changes in levels of expression of KPI/APP and certain neuropeptides in Alzheimer’s disease (68, 69) suggest that the role of KPI/APP in proenkephalin processing should be investigated in disease conditions. It will be important in future studies to elucidate how KPI/APP and PTP interactions may modify physiological production of active enkephalins and neuropeptides that mediate neurotransmission.

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