Specificity and Kinetic Studies on the Cleavage of Various Prohormone Mono- and Paired-basic Residue Sites by Yeast Aspartic Protease 3*

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The specificity and relative efficiency of cleavage of mono- and paired-basic residue processing sites by YAP3p was determined in vitro for a number of prohormone substrates: human ACTH1–39, bovine proinsulin, porcine cholecystokinin 33, cholecystokinin (CCK) 13-33, dynorphin A(1-11), dynorphin B(1-13), and amidorphin. YAP3p generated ACTH1–15 from ACTH1–39. It cleaved proinsulin at the paired-basic residue sites of the B-C junction as well as the C-A junction. Leu-enkephalin-Arg and Leu-enkephalin-Arg-Arg were generated from dynorphin A and dynorphin B, respectively. YAP3p generated Met-enkephalin-Lys-Lys from amidorphin showing that cleavage by this enzyme can occur at a lone pair of Lys residues. CCK33 was cleaved at Lys23 and Arg9, each containing an upstream Arg residue at the P6 and P5 position, respectively. Km values were between 10^{-4} and 10^{-5} M for the various substrates, with the highest affinity exhibited for the tetraspecific site of ACTH1–39 (1.8 \times 10^{-5} M). The tetraspecific residue site of ACTH1–39 was cleaved with the highest relative efficiency (k_cat/K_m = 3.1 \times 10^6 M^{-1} s^{-1}), while that of the monobasic site of CCK13–33 and the paired-basic site of proinsulin B-C junction, were cleaved less efficiently at 4.2 \times 10^4 M^{-1} s^{-1} and 1.6 \times 10^5 M^{-1} s^{-1}, respectively.

Yeast cells express an alternate enzyme, an aspartic protease, YAP3p, encoded by the YAP3 gene, which can process pro-o-mating factor at paired-basic residues, when this prohormone is overexpressed in a mutant yeast strain lacking the KEX2p serine protease (1). While the substrate that YAP3p is overexpressed in a mutant yeast strain lacking the KEX2p serine protease (1). While the substrate that YAP3p

The abbreviations used are: POMC, pro-opiomelanocortin; ACTH, adrenocorticotropic hormone; CCK, cholecystokinin; PSS, proopiomelanocortin; HPLC, high performance liquid chromatography; RIA, radioimmunoassay; PTH, parathyroid hormone; PAGE, polyacrylamide gel electrophoresis; CLIP, corticotropin-like intermediate lobe peptide; Boc-t-butyloxycarbonyl; dyn, dynorphin; PC, prohormone convertase.

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EXPERIMENTAL PROCEDURES

Substrates and Enzyme

Prohormone Substrates—Porcine cholecystokinin 33 (CCK33) was provided by Victor Mutt (Stockholm, Sweden). Cholecystokinin 13-33 and ACTH1–39 were purchased from Bachem California (Torrence, CA).
Porcine dynorphin A(1–11), dynorphin B(1–13), and amidorphin were purchased from Peninsula Laboratories Inc. (Belmont, CA). The proinsulin B-C junction peptide consisting of the following sequence, FFYPKARREVEGQP, was custom-synthesized by Peptide Technologies, Gaithersburg, MD. Bovine proinsulin was a generous gift from Drs. Jan Markussen and Knud Vad, Novo Nordisk (Bagsvaerd, Denmark).

Yeast Aspartic Protease 3—Transformed yeast, strain BJ3501 containing the YAP3 gene, was induced to express YAP3p activity as described previously (3). The YAP3p enzymatic activity that was secreted into the growth media was partially purified by concanavalin A affinity chromatography (4). For the kinetic experiments (Figs. 2 and 3), YAP3p was purified further by DEAE-Sepharose anion exchange chromatography, desalted, lyophilized, reconstituted with water, and stored at −20°C until use. One unit of YAP3p activity is expressed as the amount of enzyme that generates 0.18 μg of ACTH1-15 from 10 μg of ACTH1-39 in a 100-μl 0.1 M sodium citrate buffer, pH 4.0, and 37°C for 30 min. In this preparation (1 unit of YAP3p = 0.5 fmol), it was determined that no other proteolytic activity was present by the following criteria. 1) Growth media or concanavalin A-purified growth media from both untransformed and transformed/uninduced yeast cells were found to have no proteolytic activity when assayed by the sensitive 125I-hACTH1-39–Lipotropin assay. In this assay, activity was measured by the ability of a protease to generate trichloroacetic acid-soluble counts/min from 10 μg of 125I-hACTH1-39–Lipotropin. 2) Pepstatin A, which is a specific inhibitor of aspartic proteases, completely inhibited the secreted proteolytic activity from the galactose-induced transformed yeast. 3) Using ACTH1-39 as substrate, the two products generated by YAP3p which have been identified previously by HPLC and amino acid sequencing (3), as ACTH1-15 and CLIP16-39, were stable over time. There were no anomalous cleavages in the presence of pepstatin A, indicating the absence of any other proteolytic activity including carboxypeptidase or aminopeptidase activity in the enzyme preparation.

Generation and Identification of Products Generated by YAP3p

Cholecystokinin 33—One hundred ng of CCK33 (26 pmol) was incubated with 0.07 pmol (140 units) of YAP3p in 100 μl of 0.1 M sodium citrate, pH 4.0, for 1, 2, 5, and 8 h at 37°C in the presence and absence of 3.7 × 10−5 M pepstatin A (ICN Biomedicals Inc., Aurora, OH). Substrate alone and enzyme alone were incubated in parallel, as controls. The reaction was stopped by addition of 50 μl of 0.1 M HCl and immediately frozen on dry ice until analysis. Products were separated from the substrate by DEAE-Sephadex A-25 ion exchange resin and then quantitated by radioimmunoassay (RIA) with an antibody that recognizes CCK8, CCK12, and CCK33 (5). The products were then further analyzed by Sephadex G-50 gel filtration chromatography, and each product was analyzed by RIA. The CCK8-sized peak of immuno-reactivity from the Sephadex G-50 column was analyzed further by HPLC separation followed by RIA as described previously (5).

ACTH1-39, Dynorphin A, Dynorphin B, Rimorphin, Amidorphin, Proinsulin, Proinsulin B-C junction Peptide, and Cholecystokinin 33—Ten μg of each peptide (2-9 nmol) were incubated with enzyme (0.014–0.7 mmol) in 100 μl of 0.1 M sodium citrate, pH 4.0, and 37°C. The reactions were stopped by the addition of 10 μl of glacial acetic acid, and the products were separated on an LKB 2150 HPLC system using a Bio-Rad HiPore RP-318 column (5 × 250 mm). Buffer A was 0.1% trifluoroacetic acid, and buffer B was 80% acetonitrile in 0.1% trifluoroacetic acid. The gradients for each substrate are indicated in their respective figure legends. Products were monitored by absorbance at 214 nm, and individual peaks were collected for identification by amino acid sequence analysis. The peptide products from several HPLC runs were pooled and prepared for N-terminal amino acid sequence analysis by microcentrifugation in a ProSpin cartridge (Applied Biosystems, Foster City, CA.). Amino acid sequence analysis was carried out by Edman degradation using an Applied Biosystems Model 470A Protein Sequencer with an on-line phenylthiodydantoin (PTH) analyzer. Control experiments for each substrate included incubations with pepstatin A, enzyme alone, and substrate alone.

Cleavage of Native and Denatured/Reduced Proinsulin

Twenty μg of bovine proinsulin (2 mg/ml) was boiled for 5 min in 0.03 M sodium citrate, pH 4.0, and 2% (v/v) Triton X-100. After microcentrifugation to collect the condensate, dithiothreitol (20 mM final) and buffer A were added to a final volume of 30 μl. Two pmol of YAP3p was added and incubated for 1 h at 37°C after which 10 μl of a 4 × SDS sample buffer was added to the reaction mixture and then prepared for SDS-PAGE. The proteins were separated by SDS-PAGE on a 16%
in each reaction and the time incubated is indicated in the legend to Fig. 2. The kinetic assays were done in triplicate. Products were measured in centimeters of peak height when analyzed by reverse phase HPLC and detected by absorbance at 214 nm and converted to nanomoles of product generated using standard curves generated on the same HPLC column under identical gradient conditions. The product of the reaction was quantitated by comparison to a column under identical gradient conditions. The product generated using standard curves generated on the same HPLC column and detected by absorbance at 214 nm and converted to nanomoles of product. Since there are no commercially available standards for the product of dynorphin A, dynorphin B, and amidorphin, standard curves of Leu-enkephalin-Arg, Leu-enkephalin-Arg-Arg, and Met-enkephalin-Lys-Lys, respectively, were generated by quantitating the products of dynorphin A, dynorphin B, and amidorphin generated from ACTH1–14 while the products of dynorphin A, dynorphin B, and amidorphin were quantitated by standard curves of proinsulin itself. The product of proinsulin, a proinsulin intermediate, was quantitated by a standard curve of proinsulin itself.

**RESULTS**

**Proteolytic Processing of Proinsulin by YAP3p—Bovine proinsulin** was cleaved by YAP3p to generate an intermediate product designated peak A in Fig. 1B, upper panel. This peak contained three N-terminal amino acid sequences, and their PTH amino acid yields are reported in parentheses. 1) FVNQHLXGSH (7.8 pmol) corresponds to the N-terminal sequence of the insulin B chain, and 2) E(V)HEGQVGAL (5.7 pmol) corresponds to the N-terminal sequence of the C peptide. The third sequence was the N-terminal Arg extended form of the C peptide (2.4 pmol). This result demonstrated that YAP3p cleaved primarily on the carboxyl side of the paired-basic site, Arg31-Arg32, of the junction between B chain and C peptide of bovine proinsulin. With prolonged incubation (36 h, data not shown), the two peaks designated peak B and peak C in Fig. 1B, upper panel, increased. These peaks, pooled from multiple reactions that had been scaled up, were subjected to complete N-terminal amino acid sequence analysis. Peak B contained two N-terminal amino acid sequences; insulin B chain, with Arg31-Arg32 extensions at the C-terminal and insulin A chain with and without N-terminal extended Arg. The yields of Arg and Gly were 61.5 and 13.5 pmol, respectively. The sequences of the A chain revealed that YAP3p cleaved the C-A junction in the middle and on the carboxyl side of the paired-basic site.

2 N. X. Cawley, H.-C. Chen, and Y. P. Loh, manuscript in preparation.
Lys$^{59}$-Arg$^{60}$. Based on the average yields of PTH-derivatives, a 4:1 ratio is calculated between the two cleavages at the bond Lys$^{59}$-Arg$^{60}$ and Arg$^{60}$-Gly$^{61}$, respectively. Peak C was identified as C peptide. Pepstatin A inhibited the generation of these products (Fig. 1B, lower panel). The $V_{\text{max}}$ for the processing of proinsulin at the B-C junction was determined to be 7.9 ± 0.4 pmol/min (Fig. 2A), and the $K_{\text{m}}, k_{\text{cat}},$ and $k_{\text{cat}}/K_{\text{m}}$ values are shown in Table I. The cleavage specificity of proinsulin B-C junction peptide by YAP3p was shown to be identical with that of proinsulin itself, cleaving preferentially on the carboxyl side of the Arg-Arg pair and in the same ratio (data not shown). This peptide was processed by YAP3p with a $V_{\text{max}}$ of 214.9 ± 26.4 pmol/min (Fig. 3) while the $K_{\text{m}}, k_{\text{cat}},$ and $k_{\text{cat}}/K_{\text{m}}$ values for the generation of these products are reported in Table I.

Cleavage of Native and Denatured/Reduced Proinsulin—Both native and denatured/reduced proinsulin were cleaved by YAP3p (Fig. 4). However, only ~14% of the denatured/reduced proinsulin (lane 3) was cleaved compared to ~34% of the native proinsulin (lane 2) relative to the negative control (lane 1). An additional control experiment using ACTH$^{1-39}$ as a substrate in these denaturing/reducing conditions demonstrated that YAP3p activity was not inhibited when compared to its activity against ACTH$^{1-39}$ in the absence of these conditions (data not shown).

Proteolytic Processing of CCK33 by YAP3p—YAP3p generated CCK8-immunoreactive products from CCK33 in a time-independent manner (Fig. 5). Pepstatin A completely inhibited the generation of these products (Fig. 5). When products from the 5-h time point were separated on a Sephadex G-50 gel filtration column and assayed by RIA, two peaks of CCK8-immunoreactivity which co-eluted with CCK22 and CCK8 standards were observed (Fig. 6B). Further analysis of the CCK8-sized peak by HPLC showed an elution profile that was identical with oxidized CCK8 standards (7) (Fig. 6C), indicating that YAP3p cleaved on the carboxyl side of the monobasic Arg$^{2}$ of CCK33 to generate CCK8. To verify the cleavage by YAP3p at Lys$^{23}$ of CCK33 that would generate CCK22 (see Fig. 6A), CCK13-33 was incubated with YAP3p, and the products were analyzed by HPLC. Two homogenous products were generated (Fig. 7B, upper panel) and identified as CCK23–33 and CCK13–22, respectively, in amino acid sequence analysis, indicating cleavage by YAP3p on the carboxyl side of Lys$^{23}$. The presence of pepstatin A completely inhibited the formation of these products (Fig. 7B, lower panel). No products were detected when substrate alone or enzyme alone were incubated (data not shown). The $V_{\text{max}}$ for the cleavage of CCK13-33 was determined to be 29.2 ± 1.4 pmol/min (Fig. 2B), and the $K_{\text{m}}, k_{\text{cat}},$ and $k_{\text{cat}}/K_{\text{m}}$ values are shown in Table I.

**Table I**

Summary of the kinetic constants, $K_{\text{m}}, k_{\text{cat}},$ and $k_{\text{cat}}/K_{\text{m}}$ of YAP3p for the hydrolysis of the substrates indicated. Data used in the Lineweaver-Burk plots were obtained from triplicate initial rate experiments (see “Experimental Procedures”). S.D., standard deviation.

| Substrate                | Km μM (±SD) | $k_{\text{cat}}$ s$^{-1}$ | $k_{\text{cat}}/K_{\text{m}}$ s$^{-1}$ |
|-------------------------|-------------|---------------------------|--------------------------------------|
| ACTH$^{1-39}$           | 17.7 ± 2    | 55.3                      | 3.1 × 10$^6$                         |
| Dynorphin B (1-13)      | 79 ± 17.8   | 76.5                      | 9.7 × 10$^4$                         |
| Amidorphin              | 44 ± 11.3   | 25.1                      | 5.7 × 10$^3$                         |
| Dynorphin A (1-11)      | 136.1 ± 13.5| 49.4                      | 3.6 × 10$^4$                         |
| CCK(12-33)              | 115.3 ± 8.1 | 4.9                       | 4.2 × 10$^4$                         |
| B-C junction peptide    | 228.9 ± 31.9| 5.4                       | 2.4 × 10$^4$                         |
| Proinsulin (B-C)        | 18.5 ± 2.1  | 0.3                       | 1.6 × 10$^4$                         |
phin by YAP3p—YAP3p generated Leu-enkephalin-Arg and Leu-enkephalin-Arg-Arg from dynorphin A and dynorphin B, respectively (Figs. 8 and 9). Products generated from dynorphin A (Fig. 8B, upper panel) were identified as (R)IRPK, Leu-enkephalin-Arg-Arg (minor), and Leu-enkephalin-Arg (major), by amino acid analysis and comparison to standards that were run on the same HPLC system. In the peak identified as (R)IRPK, the ratio of this pentapeptide to its Arg truncated tetrapeptide (IRPK) was approximately 35:1 based on the yields of PTH-Arg and PTH-Ile at cycle 2 of the amino acid sequence analysis and comparison of peak heights between the peaks of Leu-enkephalin-Arg and Leu-enkephalin-Arg-Arg. This demonstrated that YAP3p preferentially cleaved between the Arg⁶-Arg⁷ pair of dynorphin A to yield mainly Leu-enkephalin-Arg. Products generated from dynorphin B (Fig. 9B, upper panel) were identified as QFKVVT and Leu-enkephalin-Arg-Arg which demonstrated a processing pattern different from that of dynorphin A because the preferred site of cleavage was on the carboxyl side of the Arg⁶-Arg⁷ pair of dynorphin B to yield Leu-enkephalin-Arg-Arg. Amidorphin was cleaved by YAP3p to generate primarily Met-enkephalin-Lys-Lys (Fig. 10B). The generation of all products by YAP3p was inhibited by pepstatin A (Figs. 8B, 9B, and 10B, lower panels), and no products were detected when substrate alone or enzyme alone were incubated (data not shown). The \( V_{\text{max}} \) for the generation of Leu-enkephalin-Arg from dynorphin A was 118.5 ± 4.4 pmol/min, Leu-enkephalin-Arg-Arg from dynorphin B was 45.9 ± 6.2 pmol/min, and Met-enkephalin-Lys-Lys from amidorphin was 30.1 ± 3 pmol/min (Fig. 2, C, D, and E) and the \( K_m \), \( k_{\text{cat}} \), and \( k_{\text{cat}}/K_m \) values for the generation of these products are reported in Table I.

Proteolytic Processing of ACTH₁–₃₉ by YAP3p—Secreted YAP3p cleaved ACTH¹–₃⁹ to yield ACTH¹–₁₅ and CLIP¹⁶–₃⁹ (data not shown), products identical with that previously reported for the cleavage of ACTH¹–₃⁹ by YAP3p purified from yeast cell extracts (3). The \( V_{\text{max}} \) for the generation of ACTH¹–¹₅ by YAP3p was determined to be 33.2 ± 4.8 pmol/min (Fig. 2F), and the \( K_m \), \( k_{\text{cat}} \), and \( k_{\text{cat}}/K_m \) values are shown in Table I.
DISCUSSION

Processing of prohormones to yield active hormones occurs most commonly at paired-basic residues and to a lesser extent at specific monobasic residue sites (8–11). A number of prohormone processing enzymes capable of carrying out these specific mono/paired-basic residue cleavages have been described. They include the serine/subtilisin-like enzymes, furin and the proprotein convertases, (12–19), and representatives from the thiol(20,21) and aspartic protease classes (1,22–25). YAP3p is a member of the aspartic protease family of prohormone processing enzymes. In this study, we have analyzed a set of substrates to further define the specificity of YAP3p and the mono/paired-basic residue cleavage site motifs recognized by this enzyme. The catalytic efficiency ($k_{cat}/K_m$) of YAP3p for the cleavage of these substrates was determined.

YAP3p cleaved bovine proinsulin at the B-C junction which contains the sequence PKARRE (Fig. 1A). Cleavage occurred preferentially on the carboxyl side of the Arg-Arg pair, putting the Lys at P4, while some cleavage was observed in between, putting the Lys at P3. This preference is similar to that of furin where an Arg at P4 enhances the cleavage rate of synthetic proalbumin peptides (26) while an Arg at P3 is deleterious (27). The synthetic substrate, Boc-RVRR-methylcoumarin amide was cleaved by YAP3p (3) exclusively in between the Arg-Arg pair, rendering the assignment of the upstream Arg in this substrate in the P3 position (Table II). The absence of any cleavage on the carboxyl side of the pair of Arg residues is presumably due to the steric hindrance of the 7-amino-4-methylcoumarin moiety that would be in the S1' pocket of the active site. Anglerfish pro-somatostatin I (aPSS I), containing the sequence PRRKR (Table II), was cleaved by YAP3p, similar to the proinsulin B-C junction, primarily on the carboxyl side of the Arg-Lys cleavage site, although some cleavage in between the pair was observed (4), indicating an overall degree of tolerance but not preference by YAP3p for a basic residue in the P3 position. In an effort to determine the role that structural conformation plays in the efficiency of this reaction, we compared the ability of YAP3p to cleave native versus denatured/reduced proinsulin. YAP3p cleaved the native proinsulin better than the denatured/reduced proinsulin (Fig. 4), demonstrating that disruption of the conformation of the prohormone negatively affected the efficiency of its cleavage. When we analyzed the cleavage of the proinsulin B-C junction peptide, we found that the specificity was identical with that of the full-length prohormone itself, demonstrating that the primary sequence around the cleavage site was sufficient to dictate the specificity
but the $K_m$ was greatly increased.

The bovine proinsulin C-A junction contains the sequence, PPQKRG,
i.e. a lone pair of basic residues. YAP3p cleaved the C-A junction of proinsulin preferentially in between the Lys-Arg pair indicating that an Arg residue in the P1 position is an acceptable position. Cleavage at this site was relatively slow compared to the B-C junction, perhaps due to the presence of the two prolines immediately upstream from the cleavage site (P3 and P4). Further support of this hypothesis is borne out by our studies with aPSS I and aPSS II (4). While aPSS I was cleaved by YAP3p at the Arg-Lys pair preferentially after the Lys to yield somatostatin 14 (Table II), the cleavage of aPSS II at the analogous Arg-Lys site was not detected, possibly due to the presence of the two prolines immediately upstream (at P5 and P6) from the expected cleavage site (Table II). The proline side chain is known to have less conformational freedom resulting in a more rigid structure around the peptide bond. This may prevent the substrate in the cleavage site region from assuming a structure that binds efficiently to the active site pocket of the aspartic protease which generally spans up to 10 amino acids (28, 29). It is noteworthy that the specificity of YAP3p for the cleavage sites of bovine proinsulin is subtly different, especially at the C-A junction, to that observed in vivo where processing occurs exclusively on the carboxyl side of both sites.

YAP3p has been shown to cleave aPSS II in vivo and in vitro at a monobasic Arg residue within a motif containing an upstream Arg at P6 (2, 4). However, in these two studies, cleavage of aPSS I, where the upstream Arg at P6 is substituted by a histidine, was observed by one group (2) and not by the other (4). This discrepancy exemplifies the differences that can be observed between in vivo and in vitro studies where the regulation of enzyme to substrate ratio dictates the efficacy of the reaction. The finding that no cleavage by YAP3p was observed when the a-mating factor leader-proinsulin fusion protein was mutated to delete the Arg at the Lys-Arg junction (1) (Table II), suggests that YAP3p does not cleave monobasic sites without an additional basic residue upstream or downstream.

To determine if YAP3p will cleave a monobasic Arg, as well as a Lys, within a motif having an upstream basic residue, CCK33 was tested as a substrate. YAP3p cleaved sulfated CCK33 at two monobasic residue sites, each containing an upstream Arg at either the P6 or P5 position (Fig. 6A and Table II). Cleavage at Arg9 generated CCK8, while cleavage at Lys23 generated CCK22 (Fig. 6, B and C). YAP3p was also shown to cleave CCK13–33 to CCK13–22 and CCK23–33 at Lys23 (Fig. 7B). These results indicate that YAP3p can recognize both a monobasic Lys or Arg apparently within a motif containing an upstream Arg. This cleavage specificity exhibited by YAP3p is similar to the CCK8 generating enzyme previously described from rat brain synaptosomes which is capable of both these cleavages (30). Based on the relative concentrations of the products generated at the 5-h time point (Fig. 6B), it appears that YAP3p cleaved preferentially at Lys23 to generate CCK22 rather than at Arg9 to generate CCK8. This result may be an indication that YAP3p prefers mono-Lys sites over mono-Arg sites, or simply that the upstream Arg in the P6 position is more favorable than in the P5 position. The presence of a
The cleavage specificity of YAP3p for the substrates tested in this and previous studies (Table I) indicates that YAP3p recognizes the following motifs: a pair of basic residues or monobasic residues with an additional upstream basic residue within the P2-P6 position. However, cleavage of some monobasic sites without an upstream basic residue may be regulated by additional amino acids in the cleavage site. Cleavage at paired basic residues can occur either between or on the carboxyl side of the pair of basic residues, the preference being substrate-dependent and likely governed predominantly by the upstream and downstream basic residues surrounding the cleavage site. However, most of the substrates studied also contain nonpolar residues in the P3 position. The importance of such residues remains unclear, but the finding that a charged residue, Arg or Lys, as mentioned before, is tolerable in the P3 position suggests that S3 does not have a strict requirement for a given type of amino acid. Cleavage at a monobasic site occurs on the carboxyl side of the basic residue. The specificity of YAP3p shows some overlap with that of the mammalian pro-opiomelanocortin converting enzyme (3, 31). Pro-opiomelanocortin converting enzyme has been shown to cleave at paired-basic residue sites of POMC, proinsulin (22, 32), and the cortin converting enzyme has been shown to cleave at paired-basic residue sites of CCK33, and pro-opiomelanocortin converting enzyme did not cleave amidorphin, both of which are cleaved at Lys-Lys pair, but ACTH1–39 has 4 additional basic residues flanking the cleavage site between P2-P6 and P2′-P6′ more efficiently than the monobasic residue motif as in CCK13–33. Moreover, having additional basic residues flanking the cleavage site between P2-P6 and P2′-P6′ enhances the affinity of binding and catalytic efficiency. This is exemplified by the decrease in K_m and increase in k_cat/K_m for ACTH1–39 versus amidorphin, both of which are cleaved at a Lys-Lys pair, but ACTH1–39 has 4 additional basic residues flanking the cleavage site. Tetrasubstitution will result in a highly efficient cleavage site for YAP3p. CCK13–33 and amidorphin were both cleaved on the carboxyl side of a Lys residue, but amidorphin was cleaved with a >10-fold higher efficiency than CCK13–33. This may be an indication of the preference by YAP3p for a basic residue in the P2 position, Lys in amidorphin, rather than P6, Arg in CCK13–33.

From the present studies it would appear that YAP3p can cleave all the motifs recognized by the subtilisin-like serine proteases, PC1/3, PC2, and furin. PC1/3 has been shown to cleave paired-basic residue sites (36–38) and at a monobasic residue with an upstream Arg in the P4 position (39), while PC2 cleaves only at paired-basic residues (15, 19). Furin, on the other hand, prefers a paired-basic residue motif with an additional upstream Arg residue at the P4 position, although the basic residue at P2 appears not to be essential (12, 39–42).

| Substrate                | Product                  | k_cat/K_m (M⁻¹s⁻¹) |
|--------------------------|--------------------------|--------------------|
| Prostasins               | Cleaved B-C junction     | 1.44E-4 ± 3.4E-4   |
| Prostasins               | Cleaved C-A junction     | 1.44E-4 ± 3.4E-4   |
| Prostasins B-C junction  |                          | 1.44E-4 ± 3.4E-4   |
| CCK13–33                 |                          | 4.2E-5 ± 1.2E-5    |
| CCK13–22 and CCK23–33    |                          | 4.2E-5 ± 1.2E-5    |
| Dynorphin A 1–11         |                          | 4.2E-5 ± 1.2E-5    |
| Dynorphin B 1–13         |                          | 4.2E-5 ± 1.2E-5    |
| Amidorphin               |                          | 4.2E-5 ± 1.2E-5    |
| N-POMCIV(2)              |                          | 4.2E-5 ± 1.2E-5    |
| Proinsulin(22, 32)       |                          | 4.2E-5 ± 1.2E-5    |
| Pro-opiomelanocortin     |                          | 4.2E-5 ± 1.2E-5    |
| Dynorphin(30)            |                          | 4.2E-5 ± 1.2E-5    |
| Dynorphin B 1–13         |                          | 4.2E-5 ± 1.2E-5    |
| Dynorphin A 1–11         |                          | 4.2E-5 ± 1.2E-5    |

Amino acid sequence of residues surrounding the cleavage sites are indicated by the single-letter amino acid code. The preferred scissile bond cleaved by YAP3p is aligned between the −1 and +1 position. Data from published literature are indicated by reference number. * cleavage by YAP3p not detected.

E. C. Ledgerwood, S. O. Brennan, N. X. Cawley, Y. P. Loh, and P. M. George, manuscript submitted.
Future kinetic studies on the cleavage of these substrates by PC1 and PC2 will be important in assessing whether these enzymes cleave the same substrates with different efficiencies and the role the structural conformation plays in dictating efficiency of cleavages, independent of the enzyme. Such kinetic information will also be important in determining the relative role the aspartic proteases and the subtilisin-like serine processing enzymes play in cleaving various prohormones, since both families of proteases can be found in the same endocrine cells, e.g. the presence of pro-opiomelanocortin converting enzyme, PC1, and PC2 in the pituitary intermediate lobe cells (22, 43).

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