Secretion of Yeast Aspartic Protease 3 Is Regulated by Its Carboxy-Terminal Tail: Characterization of Secreted YAP3p†

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ABSTRACT: Yeast aspartic protease 3 (YAP3p), a basic-residue specific proprotein processing enzyme, was shown to be a membrane-associated protease. The membrane association of YAP3p was demonstrated to be through a glycophosphatidylinositol anchor situated in the carboxy terminus of the enzyme. Carboxy-terminal truncation of YAP3p by 37 amino acids resulted in secretion of YAP3p into the growth medium. Western blot analysis after sodium dodecyl sulfate−polyacrylamide gel electrophoresis showed two secreted forms of YAP3p with apparent molecular masses of ~180 and ~90 kDa. YAP3p has an isoelectric point of ~4.5 as determined by isoelectric focusing gel electrophoresis. Treatment of YAP3p with endoglycosidase H reduced the size of both forms of the protein to ~65 kDa, consistent with the presence of 10 potential N-linked glycosylation sites in the deduced amino acid sequence of this protein. Removal of the N-linked sugars did not affect the enzymatic activity of YAP3p. Analysis of the effect of temperature on the stability and the rate of enzymatic activity of YAP3p showed that the enzyme retained 100% of its activity when incubated for 1 h at 37 °C, while incubation at 50 °C for 1 h resulted in ~80% loss of activity. The dependence of activity on temperature demonstrated a calculated Q_{10} of 1.95.

Prohormones and proneuropeptides undergo specific endoproteolytic cleavages by prohormone processing enzymes at paired and/or mono, basic residue sites to release biologically active peptides (Mains et al., 1990; Lindberg, 1991; Loh et al., 1993; Seidah et al., 1993). Identification of the prohormone processing enzymes in mammals has been difficult and greatly facilitated by studies on yeast. An enzyme in yeast, named Kex2p, which is classified as a subtilisin-like serine protease, has been characterized genetically and biochemically as the enzyme responsible for the paired-basic residue specific processing of pro-α-mating factor (MF) (Julius et al., 1984; Fuller et al., 1986). In conjunction with Kex1p, a carboxypeptidase B-like enzyme in yeast, and a dipeptidyl aminopeptidase (Julius et al., 1983; Bourbonnais et al., 1991), Kex2p generates the mature mating pheromone. Recently, mammalian homologues of Kex2p have been cloned by PCR[1] using primers designed from the Kex2 gene. These enzymes known as the prohormone convertases (PCs) have been shown to process a number of mammalian prohormones at specific paired and mono basic residue sites (Bennett et al., 1992; Breslin et al., 1993; Rehemutulla et al., 1993; Decroly et al., 1994; Dupuy et al., 1994; Friedman et al., 1994).

In addition to Kex2p, *Saccharomyces cerevisiae* express an aspartic protease, yeast aspartic protease 3 (YAP3p), that was cloned, on the basis of its ability to process pro-α-MF correctly at paired basic residues, in a Kex2-deficient mutant (Egel-Mitani et al., 1990). Similarly, transfection of anglerfish prosomatostatin II (aPSS II) into yeast resulted in the cloning of YAP3 on the basis of the ability of its gene product to generate somatostatin-28 (SS-28) from transfected aPSS II by a cleavage at the mono basic residue, Arg^{73} (Bourbonnais et al., 1993).

Analysis of the nucleotide sequence of the YAP3 gene reveals that it encodes a 569 amino acid aspartic protease (Figure 1A) with 10 potential N-linked glycosylation sites and a putative zymogen activation site similar to that of prorenin (Inagami et al., 1983). The presence of a serine/threonine-rich domain in this enzyme indicates a potential for O-linked glycosylation and/or phosphorylation. Additionally, a putative yeast glycophosphatidylinositol (GPI) membrane-anchoring consensus sequence (aa 541−548, TSTSSKRN) (Nouffer et al., 1991) is present in the carboxy terminus (C-terminus) that may be responsible for the association of YAP3p with membranes of the yeast cell, observed by Olsen (1994) and Bourbonnais et al. (1994).

Specificity studies show that YAP3p can cleave the basic residues not only of pro-α-MF (Egel-Mitani et al., 1990) but also of a number of other prohormones such as proopiomelanocortin (POMC) (Azaryan et al., 1993) and aPSS I and aPSS II (Cawley et al., 1993; Bourbonnais et al., 1994). Thus, YAP3p belongs to a unique class of aspartic

† The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

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[1] Abbreviations: PCR, polymerase chain reaction; YAP3p, yeast aspartic protease 3 protein; GPI, glycophosphatidylinositol; PMSF, phenylmethanesulfonyl fluoride; AEBSF, [4-(2-aminoethyl)benzenesulfonyl fluoride]; PBS, phosphate-buffered saline; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ACTH, adrenocorticotropin hormone; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; PLC, phospholipase C; LPH, lipotropin; BSA, bovine serum albumin; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid.

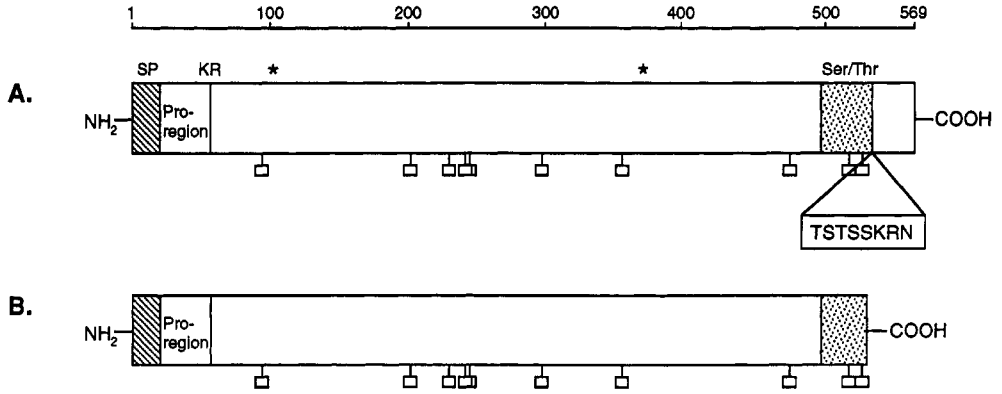

FIGURE 1: Schematic representation of full-length YAP3p (**A**) and C-terminally truncated YAP3p (**B**) protein. SP = signal peptide, KR = putative Lys-Arg zymogen activation site, Ser/Thr = serine/threonine-rich domain, * = active-site aspartic residues, □ = potential N-linked glycosylation sites. TSTSSKRN (aa 541−548) = putative consensus GPI anchoring site, similar to Gas1p (Nouffer et al., 1991).

proteases that have specificity for paired and single basic residues. Two vertebrate prohormone processing enzymes with similar specificity have been classified as aspartic proteases, proopiomelanocortin converting enzyme (PCE, E.C. 3.4.23.17) (Loh et al., 1985) and somatostatin-28 generating enzyme (Mackin et al., 1991). However, YAP3p is the only member of this class of enzymes that has been cloned.

Further study of the physical properties of YAP3p will lead us to a better understanding of the physiological role YAP3p may play in yeast, and as a model of this subclass of aspartic proteases, YAP3p will provide important and useful information for the future identification and characterization of putative mammalian homologues. In addition, since the specificity of YAP3p renders it a potentially commercially useful enzyme for processing recombinant prohormones *in vitro*, the ability to engineer a secreted form of the enzyme is desirable to facilitate the large-scale purification of the enzyme.

In this study we show that the carboxy terminus of YAP3p plays an important role in determining the membrane association of this enzyme in yeast cells via its GPI anchoring site. We have engineered and expressed a secreted, active form a YAP3p by removal of the C-terminal domain and characterized it with respect to its stability, pH optimum, size, glycosylation state, and isoelectric point.

EXPERIMENTAL PROCEDURES

YAP3 Constructs. Full-length (1.8 kb) and C-terminally truncated (1.6 kb) YAP3 cDNA fragments were engineered by PCR with a *Bam*HI site and a *Pst*I site at the 5′ and 3′ end, respectively. The truncated YAP3 fragment (1.6 kb) was engineered with a stop codon immediately upstream of the *Pst*II site. These two fragments were subcloned into the pEMBLyex4 vector (Baldari et al., 1987), and the resulting vectors were named pYAP3 (1.8 kb) and pYAP3LC (1.6 kb), respectively. Transformation and induction of the yeast strain BJ3501 with these two vectors were performed as described previously (Azaryan et al., 1993). Expression of the pYAP3LC clone results in an enzyme that is devoid of the putative consensus GPI anchoring site (Figure 1B).

Assays for YAP3p Enzymatic Activity. (1) YAP3p specifically cleaves adrenocorticotropin hormone ($ACTH^{1-39}$) at the tetra basic residue site (Lys^{15}—Lys^{16}—Arg^{17}—Arg^{18}) to release the products $ACTH^{1-15}$ and N-terminally extended corticotropin-like intermediate lobe peptide ($CLIP^{16-39}$) (Azaryan et al., 1993). An aliquot of enzyme was incubated with 10 μg of $ACTH^{1-39}$ for 30 min at 37 °C in 0.1 M sodium citrate, pH 4.0. The reaction was stopped with glacial acetic acid (10% final) and the products were analyzed by reverse-phase HPLC as described previously (Azaryan et al., 1993). The product, $ACTH^{1-15}$, was quantitated by integration (PE Nelson Model 1020 integrator) of the area under the peak and comparison with a standard curve generated with standard $ACTH^{1-14}$. As a control experiment, a similar reaction was set up in the presence of pepstatin A (10^{-4} M), (Sigma, St. Louis, MO) which is a specific inhibitor of aspartic proteases. (2) A rapid assay using custom iodinated human β-lipotropin (^{125}I-β_h-LPH) as substrate was performed exactly as described in Azaryan et al. (1993). Briefly, an aliquot of enzyme was incubated with ~20 000 cpm of ^{125}I-β_h-LPH for 30 min at 37 °C in 0.1 M sodium citrate, pH 4.0. The reaction was stopped by addition of BSA (0.1% final) and cold TCA (10% final) and, after vortexing, was allowed to precipitate on ice for 30 min. After centrifugation at 10000*g* for 10 min, an aliquot of the supernatant was counted in a γ-counter to determine the presence of TCA-soluble products.

Secretion of YAP3p Activity. Yeast cells transformed with either pYAP3 or pYAP3LC were grown to an optical density (OD) at 600 nm of ~1.6 in glucose-selective medium (Azaryan et al., 1993) at 30 °C, harvested, and resuspended in galactose-selective medium. The cells were allowed to continue to grow for 25 h in this induction medium. Aliquots (0.9 mL) were removed from each suspension at 0, 0.5, 1, 2, 3, 5, 6, 7, 9, 11, 12.5, and 25 h after the point of resuspension in galactose medium. The growth rates of each clone were monitored by OD at 600 nm and found to have parallel growth curves. The 0.9 mL aliquots were centrifuged at 2000*g* for 10 min to sediment the cells, and 40 μL of the supernatant (growth medium) from the 1-, 5-, 7-, 9-, 11-, and 12.5-h time points and 10 μL from the 25-h time point were assayed for YAP3p activity by the $ACTH^{1-39}$ assay. The yeast cell pellets from the 25-h time point were resuspended in 100 μL of water and 2 μL of AEBSF (100 mg/mL) and freeze-thawed six times, followed by three 1-s sonication bursts (Converter, Branson Sonic Power Co., Danbury, CT) on ice. The suspension was centrifuged at 10000*g* for 20 min and the supernatant was saved. The membranes were washed with 50 μL of water, and after centrifugation, this supernatant was added to the first and the combined soluble extract was saved for YAP3p activity

determination. The membranes were resuspended in 100 μL of water. Ten microliters of the soluble extract and 0.17 μL (5 μL of a 1:30 dilution) of the membrane suspension were assayed for YAP3p enzymatic activity by the $ACTH^{1-39}$ assay. Total activity in the medium, soluble extract, and membrane extract of each clone from the 25-h time point was corrected for total volume and expressed as a percentage of the overall total activity obtained from the 0.9 mL aliquot. All extractions were carried out at 4 °C.

Phospholipase C Treatment of Yeast Membranes. Membranes from yeast cells transformed with pYAP3 (full length) were prepared as described above. Aliquots (5 μL) of the membranes were digested with phospholipase C (PLC, phosphotidylinositol-specific, Sigma, St. Louis, MO) as follows: 0.25 unit of PLC in 0.1 M Tris-H Cl, pH 7.4, containing 1 mM PMSF and 5 mM EDTA, overnight at 37 °C. In a parallel control experiment, membranes were incubated with boiled PLC. After incubation, the reaction mixtures were microcentrifuged at 10000*g* for 20 min and the supernatants were assayed by the $ACTH^{1-39}$ assay. In a similar experiment, both the membranes and supernatant were assayed after PLC treatment to determine the percentage of total YAP3p activity released from the membranes.

Specificity, Temperature, and pH Studies of Secreted YAP3p Activity. Induced yeast (20 h), transformed with pYAP3LC, were harvested by centrifugation. The YAP3p present in the medium was assayed by the $ACTH^{1-39}$ in the presence and absence of pepstatin A, as described above, to determine the specificity of the secreted form. The YAP3p in the medium was partially purified by batch processing with concanavalin A−(ConA−) Sepharose beads (Pharmacia, Uppsala, Sweden) described previously (Cawley et al., 1993). The ConA eluate was desalted on disposable PD-10 desalting columns (Pharmacia, Uppsala, Sweden) with 50 mM ammonium bicarbonate/0.02% Tween 20 and lyophilized. The lyophilized enzyme was reconstituted with water and stored at −20 °C until analyzed. The stability of YAP3p activity to temperature was assessed by preincubating identical aliquots of partially purified secreted YAP3p for 1h at 4−70 °C in 0.1 M sodium citrate, pH 4.0, without substrate. Activity was then assayed by the $ACTH^{1-39}$ assay at 37 °C. The dependence of YAP3p activity on temperature was tested using the $ACTH^{1-39}$ assay at 4, 23, 37, 50, 60, and 70 °C. The dependence of YAP3p activity on pH was tested with the ^{125}I-β_h-LPH assay by incubating equal aliquots of YAP3p with substrate at different pHs. The buffer system used was citric acid/Na_2HPO_4, giving a pH range of 2.3−7.6.

Calculation of Q_{10}. The $ACTH^{1-15}$ product generated at 4, 23, and 37 °C was plotted as a function of temperature. The exponential curve fit of these points, according to the Arrhenius relationship (rate = $Ae^{-Ea/RT}$), generated a line with $r^2 = 0.996$ and an equation which was used to calculate Q_{10}.

Sephadex G-75 Gel Filtration of Secreted YAP3p. ConA-purified YAP3p was applied to a Sephadex G-75 gel-filtration column coupled to an FPLC system (Pharmacia, Uppsala, Sweden) equilibrated in 20 mM sodium phosphate buffer, pH 7.0, 150 mM NaCl, 1 mM DTT, and 0.01% triton X-100. Half-milliliter fractions were collected and assayed by the rapid ^{125}I-β_h-LPH assay. An identical aliqout of YAP3p was treated with endoglycosidase H (endo H, Sigma, St. Louis, MO, 0.01 unit in 0.05 M sodium phosphate buffer containing 2 mM PMSF, pH 6.0) at 37 °C for ~12 h and analyzed in the same manner.

Generation of Antiserum against YAP3p. A fusion protein consisting of maltose binding protein (MBP) fused to yeast aspartic protease 3 (YAP3) was contructed according to the kit procedure of New England Biolabs, Inc., Beverly, MA. Transfected bacteria, expressing the fusion protein, were solubilized by boiling in 1% SDS and the protein was fractionated by SDS−PAGE. The ~110-kDa fusion protein band was excised from the gel, minced, and prepared for immunization of rabbits at Hazelton Biotechnologies, Inc., Vienna, VA, essentially as described previously (Verbalis et al., 1991). For boosts 6−10, YAP3p, purified from the soluble extract of induced BJ3501 cells, was used (Azaryan et al., 1993). The resulting antiserum, MW283, immunodepleted YAP3p enzymatic activity by 63% compared to preimmune serum (data not shown), indicating that an antiserum specific for YAP3p had been generated. A higher percentage depletion may have been obtainable by varying the conditions of the immunodepletion reaction, such as enzyme to antibody ratio. However, it is also possible that association of YAP3p molecules with itself or other proteins may mask some of the epitopes, rendering it difficult to achieve 100% immunodepletion.

Western Blotting. Partially purified secreted YAP3p was divided into four equal aliquots. One aliquot was treated with endo H, as described above, and then all samples were boiled in 5% β-mercaptoethanol and 2% SDS for 5 min, run on a Tris/glycine precast 12% polyacrylamide gel (Novex, San Diego, CA) in denaturing conditions, and transferred to nitrocellulose for immunostaining using standard SDS−PAGE and Western blotting techniques. The lanes were probed with primary antibody (MW283), preimmune antiserum, or MW283 antiserum preabsorbed with 10 μg of MBP−YAP3p fusion protein. All antisera were at a dilution of 1:10 000 with 1× PBS containing 0.1% Tween 20 and 1.5% normal goat serum. Antigen detection was visualized using the alkaline phosphatase ABC kit and procedure from Vector Laboratories, Inc., Burlingame, CA.

Isoelectric Point Determination of Secreted YAP3. An aliquot of the partially purified YAP3p was run on a precast isoelectric focusing gel, pH range 3−7, according to the manufacturer (Novex, San Diego, CA). The protein was transferred to nitrocellulose and a Western blot was performed using YAP3p antiserum MW283. Isoelectric focusing protein standards (Bio-Rad Laboratories, Hercules, CA) were run in parallel, transferred to nitrocellulose, and stained by Aurodye (Amersham Life Sciences, Arlington Heights, IL).

RESULTS

Secretion of YAP3p Activity. The presence of YAP3p activity in the medium was determined by the $ACTH^{1-39}$ assay. Figure 2 (upper panel) shows the generation of $ACTH^{1-15}$ and $CLIP^{16-39}$ from $ACTH^{1-39}$ by secreted YAP3p activity. Addition of pepstatin A, an aspartic protease inhibitor, to the reaction mixture inhibited the generation of the products by ~94% (Figure 2, lower panel), indicating that the enzymatic activity in the medium was due to YAP3p. Furthermore, no enzymatic activity was observed in the medium of transformed uninduced yeast cells. A time-course study of the secretion of YAP3p is shown in Figure 3A. A comparison of the activity in the medium between the full-length (Figure 3A, open squares) and the C-

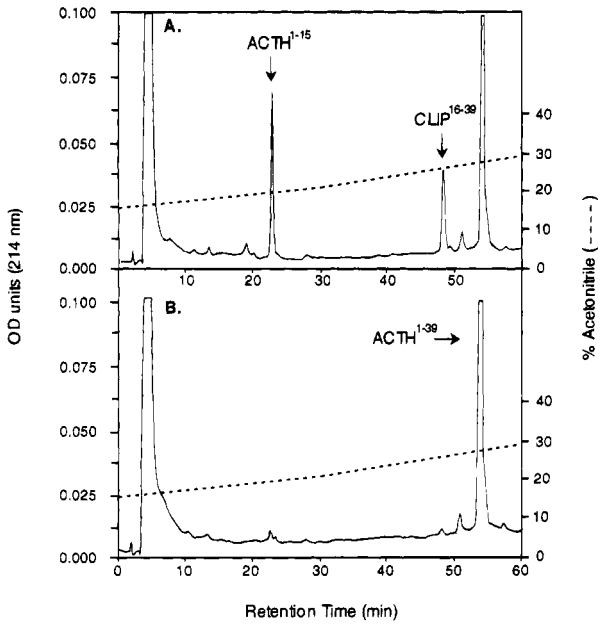

FIGURE 2: (**A**) HPLC profile of the products generated from $ACTH^{1-39}$ by secreted YAP3p. (**B**) Pepstatin A specifically inhibited the generation of these products.

terminally truncated form (Figure 3A, filled squares) of YAP3p shows that the truncated YAP3p was secreted into the growth medium while the full length was not. Other secretion time-course studies which extended past 30 h demonstrated a gradual linear secretion profile for truncated YAP3p that appeared to correlate with the growth rate of the yeast. When the growth of the yeast stopped because of nutrient limitations due to high cell density, no further increase in activity was observed in the medium (data not shown). Analysis of the enzymatic activity in the membrane and soluble extracts revealed that most of the full-length YAP3p activity (~83% of total activity, Figure 3B, filled bars) was associated with the membranes, whereas the truncated YAP3p activity was primarily secreted (~76.5% of total activity, Figure 3B, hatched bars) into the growth medium. Overnight incubation with phospholipase C of equal aliquots of membrane preparations from cells transformed with pYAP3 resulted in the release of YAP3p activity which generated 3.7 ± 0.012 μg (± SEM, n = 3) of $ACTH^{1-15}$. In contrast, membranes treated with boiled PLC yielded YAP3p activity which generated 1.5 ± 0.024 μg (± SEM, n = 3) of $ACTH^{1-15}$. This ~2.5-fold increase of YAP3p activity in the supernatant after PLC treatment of the membranes over the negative control represents release of 54% of the total (membranes plus supernatant) YAP3p activity.

Temperature and pH Studies of Secreted YAP3p Activity. Secreted YAP3p was characterized with respect to its stability and its dependence of enzymatic activity on temperature. Figure 4A shows the results of the relative stability of YAP3p to temperature. Preincubation of YAP3p at 4, 23, and 37 °C for 1 h had no measurable effect on the activity of YAP3p. However, preincubation at 50 °C for 1 h resulted in an ~80% decrease in activity, while at 60 and 70 °C, no activity remained. To determine the dependence of YAP3p activity on temperature, YAP3p was assayed directly at various temperatures, 4–70 °C. The results demonstrated a temperature optimum of between 37 and 50 °C (Figure 4B) with a calculated Q_{10} of 1.95 based on the equation y =

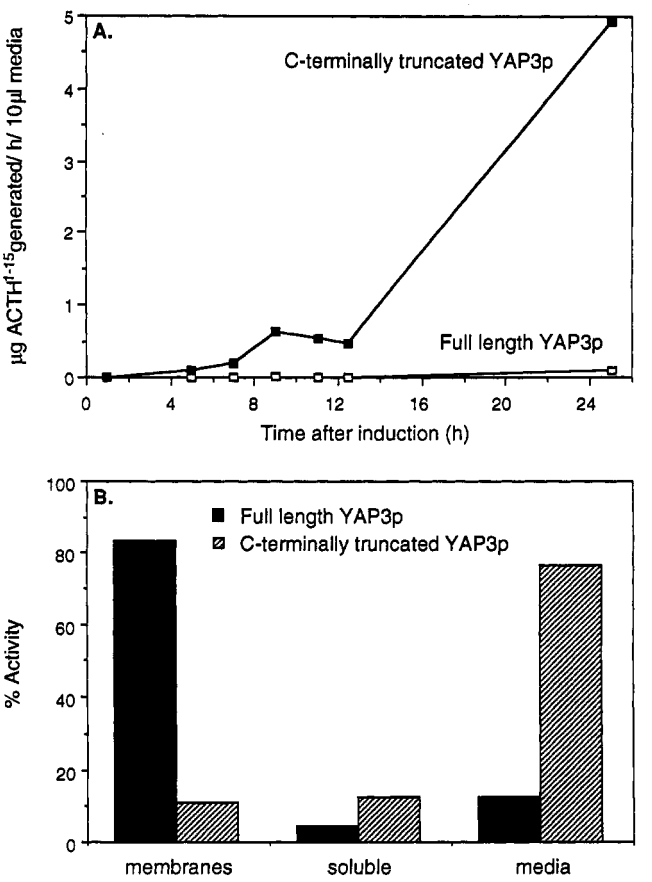

FIGURE 3: (**A**) Time course of YAP3p secretion. Aliquots of media from induced yeast that were transformed with either pYAP3 (full length) or pYAP3LC (truncated) were assayed by the $ACTH^{1-39}$ assay. YAP3p activity is expressed as micrograms of $ACTH^{1-15}$ generated per hour per 10 μL of medium. Filled squares (■) indicate the secretion profile of truncated YAP3p, whereas open squares (□) indicate the secretion profile of full-length YAP3p. (**B**) The distribution of YAP3p activity between cell membranes, soluble cellular extract and growth media for both YAP3p constructs was determined and expressed as percentage of total activity.

$(1.46)\cdot e^{029x}$, obtained from a plot of $ACTH^{1-15}$ produced at 4, 23, and 37 °C (Figure 4C; see Experimental Procedures). The dependence of YAP3p activity on pH was also determined and shown to have a pH optimum of 4.0–4.5 (data not shown).

Characterization of the Physical Properties of Secreted YAP3p. Secreted YAP3p was found to contain a major and a minor form. Their apparent molecular masses determined by SDS–PAGE under reducing conditions followed by Western blot were ~90 kDa for the minor one and ~180 kDa for the major one (Figure 5, lane 1). No immunostaining was present in the preimmune and preabsorption controls (lanes 3 and 4, respectively). Upon treatment with endo H, both immunostained bands shifted to one band with an apparent molecular mass of ~65 kDa (Figure 5, lane 2). No immunostaining of YAP3p was apparent in the media from untransformed or transformed/uninduced cells (data not shown).

Analysis of secreted YAP3p by Sephadex G-75 gel-filtration chromatography (Figure 6) showed a peak of YAP3p activity eluting in the included volume with a calculated apparent molecular mass of ~110 kDa (open bars). YAP3p maintained its enzymatic activity after endo H treatment to remove N-linked glycosylation, eluting with an apparent molecular mass of ~60–65 kDa (filled bars).

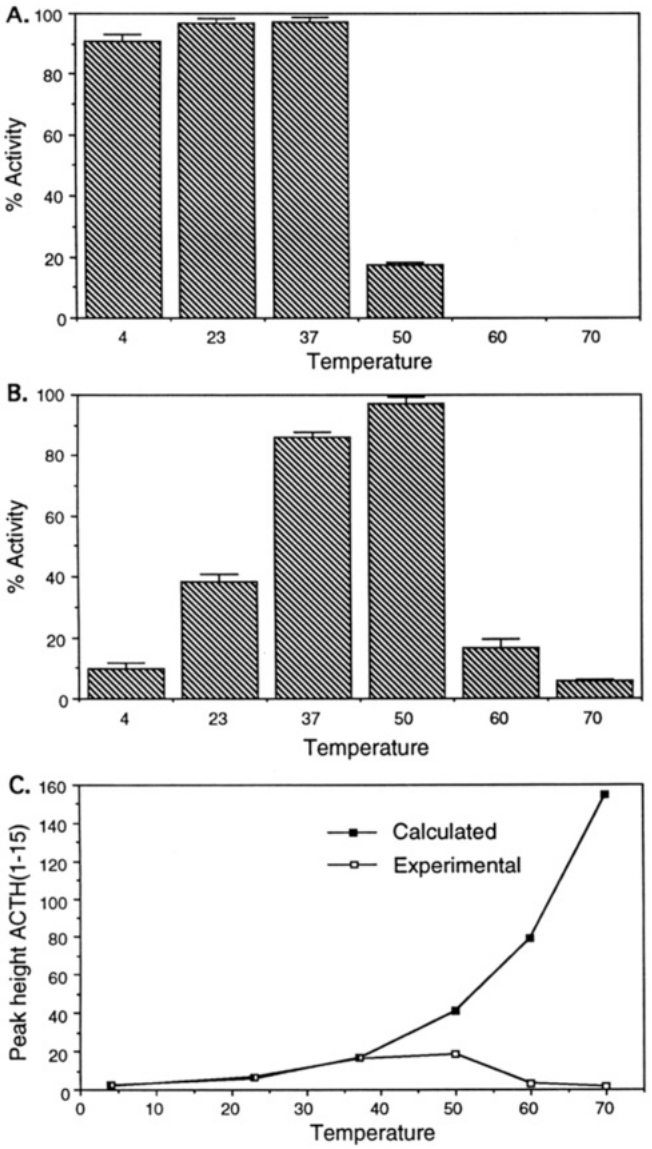

FIGURE 4: Analysis of stability and dependence of secreted YAP3p activity on temperature. (**A**) YAP3p was preincubated for 1 h at 4−70 °C and then assayed for residual activity by the $ACTH^{1-39}$ assay. (**B**) YAP3p was assayed directly at varying temperatures, 4−70 °C. The results shown in panels A and B are the mean of three experiments; the error bars show the standard error of the mean. Activity is expressed as micrograms of $ACTH^{1-15}$ generated per hour. (**C**) Comparison of the calculated and experimental activity dependence of YAP3p on temperature (see Experimental Procedures). The ordinate shows the peak height of the $ACTH^{1-15}$ product generated per 30 min in arbitrary units. The abscissa shows the temperature.

Figure 7 shows the result of a Western blot of YAP3p that was separated on an isoelectric focusing polyacrylamide gel. In comparison to standards that were run in a parallel lane, the isoelectric point (p*I*) of YAP3p was estimated to be ~4.5.

DISCUSSION

Full-length YAP3p was shown not to be secreted but to be primarily associated with yeast cell membranes. Removal of the last 37 amino acids of YAP3p, which included a putative GPI consensus anchoring sequence and a domain of hydrophobic residues, caused YAP3p to be secreted into the medium (Figure 3A). Although the last 17 amino acids of YAP3p have a very hydrophobic character, this segment

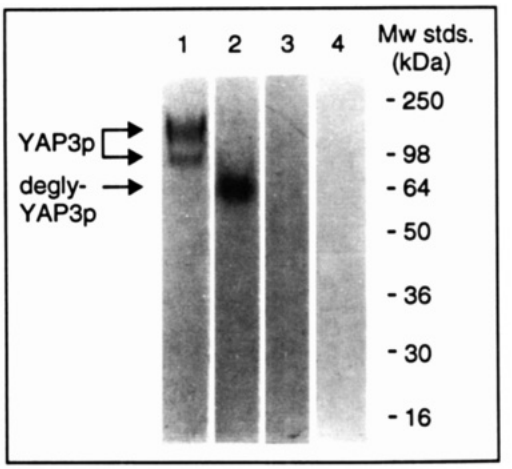

FIGURE 5: Western blot analysis of secreted YAP3p using antiserum MW283. Identical aliquots of partially purified YAP3p were loaded in each lane, except that the sample in lane 2 was treated with endo H (see Experimental Procedures) prior to the SDS−PAGE. Lanes 1 and 2, probed with YAP3p antiserum; lane 3, probed with YAP3p preimmune antiserum; and lane 4, probed with YAP3p antiserum preabsorbed with YAP3p fusion protein. Protein standards used were Novex Seeblue prestained standards (Novex, San Diego, CA), molecular mass range 250−6 kDa. All proteins were run in reducing conditions.

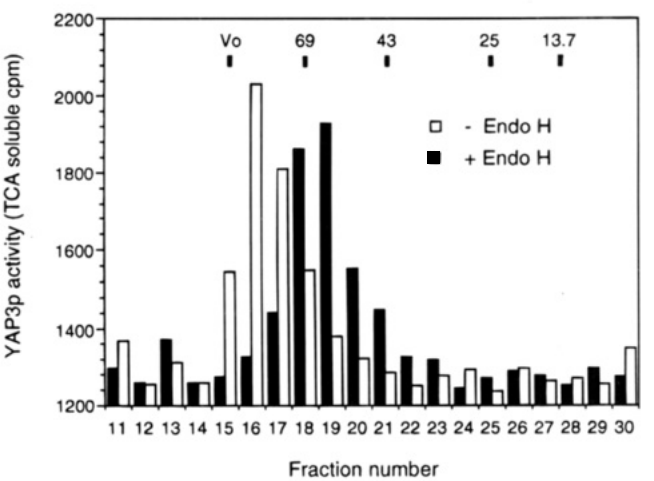

FIGURE 6: Sephadex G-75 gel filtration activity profile of endo H-treated (■) and non-endo H-treated (□) secreted YAP3p. Gel filtration standards were V_o = dextran blue (2000 kDa), BSA (69 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and RNase (13.7 kDa).

is generally not considered to be long enough to span a membrane and thus association with the membrane is unlikely to be through an interaction with this domain. The consensus GPI sequence of YAP3p (aa 541−548) is similar to that previously characterized for another yeast protein, Gas1p (Nouffer et al., 1991). However, until now this has remained only a putative membrane anchoring site of YAP3p. Our results, showing the release of YAP3p activity from the membranes by phosphatidylinositol-specific phospholipase C, provide evidence for the membrane association of YAP3p via this GPI binding site. A 54% release of total activity from the membranes was observed, but this in fact may be an underestimation due to the possibility that some YAP3p may not have been accessible to the PLC. A low amount of YAP3p activity was detected in the supernatant of the negative control (i.e., incubation of membranes with boiled PLC) even in the presence of PMSF and EDTA, suggesting that this basal release of YAP3p from the

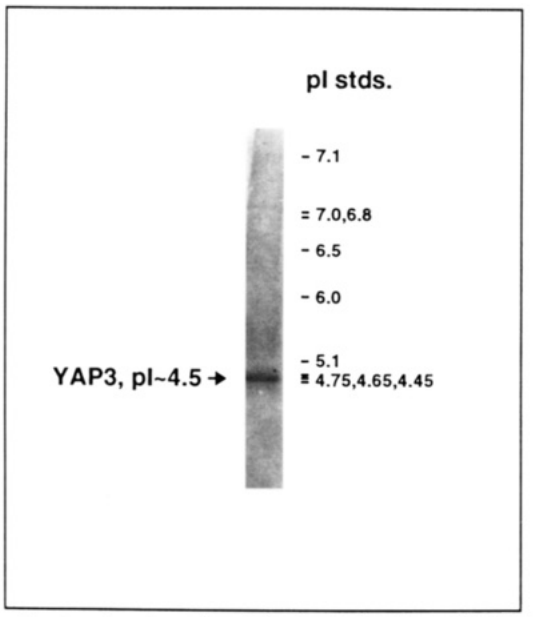

FIGURE 7: Isoelectric point (p*I*) determination of YAP3p by isoelectric focusing polyacrylamide gel electrophoresis. The p*I* standards are shown and YAP3p is indicated by the arrow.

membranes is not due to the action of serine or thiol proteases or metalloproteases on amino acid residues near the GPI anchoring site. It is also unlikely to be due to proteolytic cleavage by YAP3p itself or other yeast aspartic proteases since they are not active at pH 7.4. Most likely, other enzymes that can cause dissociation of the membrane structure such as membrane-bound lipases or glycosidases may be responsible for the low basal release. However, our results clearly demonstrate that treatment of the membranes with PLC causes significant release of YAP3p activity above the basal level.

The generation of a GPI binding site occurs in the endoplasmic reticulum (ER) (Doering et al., 1990). The mechanism involves specific C-terminal cleavage of the protein to be anchored, presumably after the Asn of the GPI consensus sequence. Thus, wild-type YAP3p is probably naturally C-terminally truncated at Asn^{548} and is associated with the membrane through this C-terminal amino acid. Additionally, recognition for a protein to be anchored by this mechanism is facilitated by the C-terminal hydrophobic region, since site-directed mutagenesis of a hydrophobic residue to an Arg in the C-terminus hydrophobic domain of Gas1p, which is normally GPI-anchored, resulted in the loss of membrane anchoring of this protein and subsequent secretion (Nouffer et al., 1991). While other processing proteases, such as furin and Kex2p, also appear to be membrane-associated via the C-terminus (Brenner & Fuller, 1992; Molloy et al., 1992), the association of these enzymes appears to be via their transmembrane domain rather than by GPI binding since they lack the appropriate consensus sequence.

The cellular localization of the membrane-associated YAP3p has not been determined directly, but it has been deduced to be associated on the extracellular side of the plasma membrane on the basis of the following observations. (1) YAP3 was initially cloned on the basis of the ability of its gene product to process pro-α-mating factor *in vivo*, demonstrating that it was localized to a compartment in the yeast secretory pathway (Egel-Mitani et al., 1990). (2) Making spheroplasts by treatment with zymolase, or glass-bead lysis of yeast cells followed by alkali extraction of cell wall mannoproteins, demonstrated that YAP3p sedimented with the spheroplasts upon centrifugation and was not released under the alkaline extraction conditions, indicating that the YAP3p was not associated with the yeast cell wall (Olsen, 1994). (3) Since the pH optimum of YAP3p was determined to be at pH 4.0−4.5, it is highly unlikely that YAP3p is functionally active in and therefore localized to the endoplasmic reticulum (ER) or cis or medial Golgi compartments. (4) In an elegant experiment by Olsen (1994), a fusion protein containing the first 150 amino acids of carboxypeptidase Y (CPY) and invertase was constructed. The CPY sequence caused the fusion protein to be sorted to the yeast vacuole, resulting in the absence of extracellular invertase activity. When a pro-α-mating factor cleavage site was engineered at the junction between the two proteins, invertase activity was detected extracellularly as a result of its processing by Kex2p in the Golgi. This demonstrated that sorting to the vacuole takes place in a compartment distal to the Kex2p action, most probably in the trans Golgi network (TGN). However, in a Kex2-deficient mutant expressing high levels of YAP3p, which is capable of cleaving the pro-α-mating factor cleavage site, the fusion protein was sorted to the vacuole. This indicated that YAP3p was localized to a compartment distal to the TGN, i.e., in either the secretory vesicles or plasma membrane. Any YAP3p that is associated within these secretory vesicles will ultimately be localized to the extracellular side of the plasma membrane after exocytosis. Immunoelectron microscopy would be necessary to directly confirm this localization of YAP3p.

The secreted form of truncated YAP3p was overexpressed and characterized with respect to its physical properties. The predicted molecular mass of the truncated YAP3p is ~49 kDa, based on the removal of the signal peptide, putative propeptide region, and the last 37 amino acids of the protein. Previously, recombinant truncated YAP3p, purified from the cellular extract of yeast cells, was determined to be a ~70 kDa glycoprotein (Azaryan et al., 1993). In this study, the secreted truncated forms of YAP3p were characterized by Western blot as hyperglycosylated enzymes of ~90 and ~180 kDa. Since the ~180 kDa band was rather broad, it may contain a heterogenous population of differentially hyperglycosylated forms of YAP3p. The possibility exists that the ~180 kDa form of YAP3p is a dimer of the 90 kDa form. However, since the protein sample had been boiled in buffer containing 2% SDS and 5% β-mercapto-ethanol prior to SDS−PAGE, this seems highly unlikely. Upon treatment with endoglycosidase H, both bands shifted to one band of ~65 kDa. The residual size difference between the ~65 kDa form and the predicted molecular mass is probably due to O-linked glycosylation. There was no evidence for the ~70 kDa cellular form of YAP3p being secreted. These results suggest that the biosynthetic pathway of YAP3p involves glycosylation to a ~70 kDa form followed by hyperglycosylation to ~90 and ~180 kDa forms prior to secretion. The phenomenon of hyperglycosylation is not unusual in yeast. For example, another aspartic protease from yeast, Bar1, is ~300% larger than the expected size due to hyperglycosylation (MacKay et al., 1991). Surprisingly, the secreted forms of YAP3p deter-

mined by SDS−PAGE as ~180 and ~90 kDa proteins were found in the included volume (apparent molecular mass ~110 kDa) and not the void volume of the Sephadex G-75 gel-filtration column. This experiment has been repeated several times using different enzyme preparations with the same result, suggesting that the molecular shape may have a significant influence on the retention of the hyperglycosylated YAP3p on this column independent of molecular mass. In addition, while the conditions of the run were such that ionic interactions were minimized (see Experimental Procedures), the inclusion of YAP3p may also be the result of nonspecific binding of the sugars on the protein with the Sephadex beads.

A comparison of the secreted YAP3p and that of the cellular component, previously described, shows that there is no evident difference in specificity as observed by the $ACTH^{1-39}$ assay (Azaryan et al., 1993). Similarly, the dependence of this activity on pH showed only a slightly broader pH profile than that obtained for the cellular form. Both had a pH optimum between 4.0 and 4.5. The stability of secreted YAP3p at different temperatures show that this enzyme follows a typical heat inactivation profile, going from 100% to 0% activity when preincubated for 1 h between 37 and 60 °C (Figure 4A). However, the ability of YAP3p to generate more product at 50 °C than at 37 °C in a 30 min incubation (Figure 4B) is an indication that the increase in the rate of enzymatic activity, due to the increased temperature, $Q_{10} = 1.95$ (Figure 4C), is faster than the rate of heat inactivation. This may be important when considering a function for YAP3p *in vivo*. Although the function of YAP3p *in vivo* is still unknown, it is conceivable that it is related to stress response, in that it may get induced in a heat-shock manner. Upon closer scrutiny of the promoter region of the YAP3 gene, putative heat shock elements are evident that may be involved in this response in yeast (Olsen, 1994). Since yeast normally grow at an optimum temperature of ~30 °C, the ability of a heat-induced enzyme to function satisfactorily at the higher temperature may have a profound effect on the viability of the cell. Although YAP3 was previously shown to be a nonessential gene under normal conditions (Bourbonnais et al., 1993), it would be interesting to determine the capacity of its function in yeast at elevated temperatures. In addition to heat stability, partially purified YAP3p from the growth medium was found to withstand two consecutive lyophilizations which did not result in any detectable loss of enzymatic activity (unpublished data). The lyophilized powder was stable indefinitely. Furthermore, YAP3p enzyme that has been stored for 8 months at −20 °C appears to be stable.

In summary, we have engineered a carboxy-terminally truncated form of YAP3p that is secreted from yeast cells and easily harvested. This truncated YAP3p enzyme appears to be identical in specificity to full-length YAP3p (Figure 3A,B, $ACTH^{1-39}$ assay) and capable of cleaving various prohormone substrates at specific mono/dibasic residue motifs (Azaryan et al., 1993; Cawley et al., 1993). The temperature dependence and stability of secreted YAP3p and the ability to store the enzyme for an indefinite period in a lyophilyzed form without losing enzymatic activity renders this enzyme highly useful commercially for the *in vitro* production of hormones and neuropeptides from recombinant precursors, such as proinsulin. Secreted YAP3p has recently been purified to apparent homogeneity with a yield of ~0.3 μg of YAP3p/g of wet yeast (Cawley et al., manuscript in preparation) which is 2−3-fold higher than that achievable with yeast cell extract (Azaryan et al., 1993). The functionality of YAP3p at higher temperatures found in this study suggests that this enzyme may play an important role in yeast cells *in vivo* under heat-induced stress.

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REFERENCES

Azaryan, A. V., Wong, M., Friedman, T. C., Cawley, N. X., Estivariz, F. E., Chen, H. C., & Loh, Y. P. (1993) *J. Biol. Chem. 268*, 11968−11975.

Baldari, C., Murray, J. A., Ghiara, P., Cesareni, G., & Galeotti, C. L. (1987) *EMBO J. 6*, 229−234.

Bennett, D. L., Bailyes, E. M., Nielsen, E., Guest, P. C., Rutherford, N. G., Arden, S. D., & Hutton, J. C. (1992) *J. Biol. Chem. 267*, 15229−15236.

Bourbonnais, Y., Germain, D., Latchinian-Sadek, L., Boileau, G., & Thomas, D. Y. (1991) *Enzyme 45*, 244−256.

Bourbonnais, Y., Ash, J., Daigle, M., & Thomas, D. Y. (1993) *EMBO J. 12*, 285−294.

Bourbonnais, Y., Germain, D., Ash, J., & Thomas, D. Y. (1994) *Biochimie 76*, 226−233.

Brenner, C., & Fuller, R. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 922−926.

Breslin, M. B., Lindberg, I., Benjannet, S., Mathis, J. P., Lazure, C., & Seidah, N. G. (1993) *J. Biol. Chem. 268*, 27084−27093.

Cawley, N. X., Noe, B. D., & Loh, Y. P. (1993) *FEBS Lett. 332*, 273−276.

Decroly, E., Vandenbranden, M., Ruysschaert, J.-M., Cogniaux, J., Jacob, G. S., Howard, S. C., Marshall, G., Kompelli, A., Basak, A., Jean, F., Lazure, C., Benjannet, S., Chretien, M., Day, R., & Seidah, N. G. (1994) *J. Biol. Chem. 269*, 12240−12247.

Doering, T. L., Masterson, W. J., Hart, G. W., & Englund, P. T. (1990) *J. Biol. Chem. 258*, 15261−15273.

Dupuy, A., Lindberg, I., Zhou, Y., Akil, H., Lazure, C., Chretien, M., Seidah, N. G., & Day, R. (1994) *FEBS Lett. 337*, 60−65.

Egel-Mitani, M., Flygenring, H. P., & Hansen, M. T. (1990) *Yeast 6*, 127−137.

Friedman, T. C., Loh, Y. P., & Birch, N. P. (1994) *Endocrinology 135*, 854−862.

Fuller, R., Brake, A., & Thorner, J. (1986) *Microbiology*, 273−278.

Inagami, T., Chang, J. J., Dykes, C. W., Takii, Y., Kisaragi, M., & Misono, K. S. (1983) *Fed. Proc. 42*, 2729−2734.

Julius, D., Blair, L., Brake, A., Sprague, G., & Thorner, J. (1983) *Cell 32*, 839−852.

Julius, D., Brake, A., Blair, L., Kunisawa, R., & Thorner, J. (1984) *Cell 37*, 1075−1089.

Lindberg, I. (1991) *Mol. Endocrinol. 5*, 1361−1365.

Loh, Y. P., Parish, D. C., & Tuteja, R. (1985) *J. Biol. Chem. 260*, 7194−7205.

Loh, Y. P., Beinfeld, M. C., & Birch, N. P. (1993) in *Mechanisms of intracellular trafficking and processing of proproteins* (Loh, Y. P., Ed.) pp 179−224, CRC Press, Boca Raton, FL.

MacKay, V. L., Armstrong, J., Yip, C., Welch, S., Walker, K., Osborn, S., Sheppard, P., & Forstrom, J. (1991). In *Structure and Function of the Aspartic Proteinases* (Dunn, B. M., Ed.) pp 161−172, Plenum Press, New York.

Mackin, R. B., Noe, B. D., & Spiess, J. (1991) *Endocrinology 129*, 1951−1957.

Mains, R. E., Dickerson, I. M., May, V., Stoffers, D. A., Perkins, S. N., Ouafik, L. H., Husten, E. J., & Eipper, B. A. (1990) *Front. Neuroendocrinol. 11*, 52−89.

Molloy, S. S., Bresnahan, P. A., Leppla, S. H., Klimpel, K. R., & Thomas, G. (1992) *J. Biol. Chem. 267*, 16396−16402.

Nouffer, C., Jeno, P., Conzelmann, A., & Riezman, H. (1991) *Mol. Cell. Biol. 11*, 27−37.

Olsen, V. (1994) A putative prohormone convertase from *Saccharomyces cerevisiae*. Characterization of biosynthesis and cellular localization of the YAP3 gene product, M.S. Thesis, University of Copenhagen, Denmark.

Rehemtulla, A., Barr, P. J., Rhodes, C. J., & Kaufman, R. J. (1993) *Biochemistry 32*, 11586−11590.

Seidah, N. G., Day, R., Marcinkiewicz, M., & Chretien, M. (1993) *Ann. N.Y. Acad. Sci. 680*, 135−146.

Verbalis, J. G., Hoffman, G. E., Rosenbaum, L. C., Nilaver, G., & Loh, Y. P. (1991) *J. Neuroendocrinol. 3*, 267−272.

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