A High-Affinity Inhibitor of Yeast Carboxypeptidase Y Is Encoded by *TFS1* and Shows Homology to a Family of Lipid Binding Proteins

Anette W. Bruun,[‡] Ib Svendsen,[§] Susanne O. Sørensen,[‡,||] Morten C. Kielland-Brandt,[‡] and Jakob R. Winther*[,‡]

Departments of Yeast Genetics and Chemistry, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark

Received May 30, 1997; Revised Manuscript Received December 11, 1997

ABSTRACT: A 25−kDa inhibitor of the vacuolar enzyme carboxypeptidase Y from *Saccharomyces cerevisiae* has been characterized. The inhibitor, I_c, binds tightly with an apparent K_i of 0.1 nM. Consistent with a cytoplasmic localization, I_c is soluble and contains no sequences which could serve as potential signals for transport into the endoplasmic reticulum. Surprisingly, I_c is encoded by *TFS1*, which has previously been isolated as a high-copy suppressor of *cdc25-1*. *CDC25* encodes the putative GTP exchange factor for Ras1p/Ras2p in yeast. In an attempt to rationalize this finding, we looked for a physiological relationship by deleting or overexpressing the gene for carboxypeptidase Y in a *cdc25-1* strain. However, this did not change the phenotype of this mutant strain. I_c is the first member of a new family of protease inhibitors. The inhibitor is not hydrolyzed on binding to CPY. It has fairly high degree of specificity, showing a 200-fold higher K_i toward a carboxypeptidase from *Candida albicans* which is highly homologous to carboxypeptidase Y. The *TFS1* gene product shows extensive similarity to a class of proteins termed "21−23-kDa lipid binding proteins", members of which are found in several higher eukaryotes, including man. These proteins are highly abundant in some tissues (e.g., brain) and have in general been found to bind lipids. Considering their homology to I_c, it is tempting to speculate that they may also be inhibitors of serine carboxypeptidases.

Macromolecular protease inhibitors are found in many extracellular fluids. These include inhibitors which have a number of fairly well defined in vivo protease targets. Furthermore, inhibitors are found for most of the major classes of proteases. The best characterized ones are the serine protease inhibitors belonging to the Kunitz and serpin families (Bode & Huber, 1992). In recent years a number of cytoplasmic protease inhibitors have been identified. They have in some cases been found to be important on a systemic scale. For instance, mutations in cystatin B, an in vitro inhibitor of certain cysteine proteases, will cause progressive myoclonus epilepsy in homozygous individuals (Pennacchio et al., 1996). In general, however, little is known about the cellular functions of cytoplasmic protease inhibitors.

In yeast, the vacuole is the main depository for proteases and other hydrolases, and this compartment is in many respects equivalent to the mammalian lysosome. The most abundant endoproteases, proteinase A (an aspartate protease) and proteinase B (a subtilisin-type protease), are inhibited by cytoplasmic inhibitors which have been subjected to biochemical investigation (Bünning & Holzer, 1977) and sequence determination (Biederman et al., 1980). The structural genes have been cloned and sequenced, and both inhibitors have been found to be very small proteins (68 and 74 amino acid residues, respectively). Although they may play a role in regulation of general protein degradation in the cell during starvation (Schu & Wolf, 1991; Schu et al., 1991), their cellular function and mechanism of action remain somewhat obscure. I_c,[1] the inhibitor of carboxypeptidase Y (CPY), is substantially larger (about 25 kDa; Matern et al., 1974), but until now it has only been characterized to a very limited extent.

In this work, we have purified I_c and we present the first sequence and a biochemical characterization of a macromolecular inhibitor of a serine carboxypeptidase. The gene encoding I_c predicts an amino acid sequence with homology to a unique class of proteins found in many tissues of higher eukaryotes.

EXPERIMENTAL PROCEDURES

Enzymes and Assays. Carboxypeptidase Y (CPY) was purified as described previously (Johansen et al., 1976) and kindly supplied by K. Breddam, Carlsberg Laboratory. This preparation was >98% pure. The CPY homologue (CAC) from *Candida albicans* was expressed from the *GAL1* promoter in a plasmid similar to that used for expression of CPY (pGPR7; Nielsen et al., 1990). This plasmid was introduced into yeast strain TSY10-7D (*MAT***a** Δ*prc1::LEU2*

* Corresponding author. Phone: +45 3327 5282. Fax: +45 3327 4765.

[‡] Department of Yeast Genetics.

[§] Department of Chemistry.

[||] Present address: Danisco Biotechnology, Langebrogade 1, DK-1001 Copenhagen K, Denmark.

[1] Abbreviations: CPY, carboxypeptidase Y; DTT, dithiothreitol; FA, furylacryloyl; GdmCl, guanidinium chloride; I_c, CPY inhibitor; IPTG, isopropyl β-D-thiogalactoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RP-HPLC, reverse-phase high-pressure liquid chromatography; TFA, trifluoroacetic acid.

S0006-2960(97)01286-5 CCC: $15.00
Published on Web 02/20/1998

vpl1-1 ura3-52 leu2-3,112), and CAC was prepared from the culture supernatant as described for CPY (Nielsen et al., 1990).

Carboxypeptidase activity was routinely assayed spectrophotometrically using FA-Phe-Phe-OH as substrate. In the standard assay 20 μL of carboxypeptidase sample was added to 0.2 mM FA-Phe-Phe-OH, 50 mM Mes, 1 mM EDTA, and 2.5% (v/v) methanol, pH 6.5, in 1 mL total. Cleavage of the substrate led to a decrease in absorption, which was monitored continuously at 337 nm at 25 °C. To quantitate CPY in complex with I_c, the assay was performed in the presence of 1.3 M GdmCl (Merck). This concentration of GdmCl instantly splits the I_c−CPY complex, but CPY remains stable during the assay. GdmCl reduces the CPY activity by 60%. The amount of inhibitor bound can conveniently be calculated from the difference in CPY activity in the presence and absence of GdmCl. For measurement of free inhibitor, the sample was mixed with a fixed amount of CPY (normally at 0.3 μM in 20 μL total). After incubation at 25 °C for 5−10 min, the CPY activity was measured. In all experiments involving dilute samples, tubes were coated by exposure to 0.1% Tween 20 (Sigma).

Protein concentration was routinely estimated during purification by measurement of A_{280}. A specific absorption of 1.45 A_{280} L g^{-1} was used for CPY (Johansen et al., 1976). The extinction coefficient of I_c of 0.88 A_{280} L g^{-1} was calculated from the sequence according to Gill and von Hippel (1989).

Initial Preparation of I_c and Sequence Determination. Step 1: Preparation of Crude Yeast Extract. Lysis of yeast cells was carried out essentially as described by Breddam and Beenfeldt (1991). Three-hundred grams of packed commercial bakers' yeast (Danisco Distillers Ltd.) was mixed with 7.5 mL of 1-hexanol and stirred at 5 °C for 60 min. Lysis was subsequently achieved by addition of 750 mL of water. The pH was adjusted to 8.0−8.5 by addition of 1.5 M Tris-base, and the suspension was stirred at room temperature for 18 h. Cell debris was removed by centrifugation, and crude protein was prepared from the cleared extract by a 55−75% ammonium sulfate cut. The precipitate was dissolved in 20 mM Tris-HCl, pH 7.7, and desalted on a 250-mL G50 Sephadex column equilibrated with the same buffer. Fractions exhibiting CPY activity in the presence of GdmCl were pooled.

Step 2: Ion-Exchange Chromatography. The desalted extract was applied to 100 mL of Q-Sepharose in a 2.6 cm i.d. column. After a wash with 100 mL of 0.1 M KCl and 20 mM Tris-HCl, pH 7.7, the column was developed with a 400-mL linear salt gradient to 0.5 M KCl and 20 mM Tris-HCl, pH 7.7. Active fractions eluted at around 0.3 M KCl.

Step 3: Reverse-Phase HPLC and Sequencing. Reverse-phase HPLC was carried out on a Waters HPLC, model 510, using a 5−60% linear acetonitrile gradient in 0.1% TFA with detection at 216 nm. The column used was Vydac C4. Amino acid sequencing was performed on an Applied Biosystems sequencer, model 470A, equipped with an on-line PTH amino acid analyzer, model 120 A, according to the manufacturer's instructions. Determination of the molecular mass of peptides was carried out on a Finnigan Lasermat 2000 MALDI-TOF mass spectrometer. The peptide sequences were found to correspond to a previously identified gene, *TFS1* (Robinson & Tatchell, 1991). This was confirmed by assay of inhibitory activity in the strain KT1075 (*MAT*α *leu2 his3 ura3-52* Δ*tfs1::HIS3*) and its isogenic parent KT1078 (*MAT*α *leu2 his3 ura3-52*) (kindly provided by Dr. K. Tatchell).

Purification of I_c for Biochemical Characterization. I_c was purified from yeast strain M3116 (*MAT***a** *ura3-52 leu2-3,112 his3-*Δ*200* Δ*prc1-bam-bam* Δ*pep4-1137*; this work) transformed with a high copy number plasmid (pKT1067) with the *TFS1* gene, encoding I_c (kindly provided by Dr. K. Tatchell). Cells were grown on selective medium (SC-ura; Sherman, 1991) until early stationary phase and harvested by centrifugation. Because of the absence of endogeneous CPY, no I_c complex was formed. The *pep4* deletion results in a lack of vacuolar protease activities. The free inhibitor is stable in extracts from this strain.

The inhibitor was liberated from the cells by the following freeze−thaw procedure. Cells were washed with 1/10 of the culture volume buffer, harvested by centrifugation, and resuspended in 1/100 volume buffer (1 mM DTT, 1 mM EDTA, and 20 mM Tris, pH 8.3). They were then subjected to three cycles of freeze−thaw using a dry ice/ethanol bath and a 40 °C water bath. The permeabilized cells were removed by centrifugation to give a clear, slightly yellow supernatant, which was adjusted to pH 8.3 using Tris-base. The supernatant was subsequently heat-treated by pumping it through a 150 cm long, 1 mm (i.d.) polyethylene tube coiled in an 80 °C water bath at a rate of 5 mL/min. The resulting precipitate was removed by centrifugation, and 1/3 volume of 4 M ammonium sulfate was added to the supernatant. The sample was loaded onto a 5-mL Phenyl Sepharose column (Pharmacia) and washed with 1 M ammonium sulfate, 20 mM Tris-HCl (pH 8.3), and 1 mM DTT until A_{280} had reached <0.05. The column was eluted with a linear salt gradient from 1 to 0 M ammonium sulfate. The protein eluted at approximately 0.6−0.7 M ammonium sulfate and was stable in this buffer. Just prior to use, I_c aliquots were desalted on a NAP-5 column (Pharmacia).

Production and Purification of Recombinant I_c from Escherichia coli. TFS1 was expressed in *E. coli* using the IPTG-induced T7 RNA/promoter−polymerase system (Tabor & Richardson, 1985). Expression cassettes were constructed by polymerase chain reaction (PCR). A His-tagged version was prepared in which a methionine codon followed by six histidine codons was inserted at the start of the coding region using primer oAB1-D (CCCGGGCATATGCACCACCAC-CACCACCACATGAACCAAGCAATAGACTTCGC). The downstream end was primed with oAB11U (GGTACCGTC-GACCTATTTCGTTTCCGCATAGAAGAAATTGG). The PCR product was inserted into *Nde*I and *Sal*I sites in pT7-7 (Tabor et al., 1985) to give plasmid pAB608. When cultures had grown to an OD_{600} of 0.5, expression was induced using 1 mM IPTG for 4 h at 37 °C. The recombinant protein was extracted by freeze−thaw using 10 mM Tris, 1 mM EDTA, and 1 mM DTT as extraction buffer, essentially as described by Johnson and Hecht (1994). The histidine-tagged inhibitor, 6xHis-I_c, was purified from the freeze−thaw supernatant in one step by Ni-chelate chromatography, eluting the protein with a linear gradient of imidazole (Novagen Inc., Madison). Imidazole was removed from active fractions by dialysis. A nontagged version of I_c was also prepared and found to be in a freeze−thaw-extractable form (as judged by SDS−

PAGE), but essentially no activity could be recovered even after partial purification of the protein (data not shown).

Characterization of Inhibitory Properties. Both the native and recombinant forms of I_c were characterized using the equation for a tight-binding inhibitor (Williams & Morrison, 1979):

$$v_i = v_{max} \frac{\sqrt{(K_{i(app)} - E_t + I_t)^2 - (4E_tK_{i(app)})} - (K_{i(app)} - E_t + I_t)}{2E_t} \quad (1)$$

where E_t is the total enzyme concentration, I_t is the total inhibitor concentration, and $K_{i(app)}$ is the apparent K_i. To determine the $K_{i(app)}$, steady-state rates for the hydrolysis of FA-Phe-Phe were measured at fixed concentrations of CPY and substrate with various concentrations of I_c. Plots of initial rate, v_i, versus concentration of I_c were fitted according to eq 1.

Refolding and Thermostability of CPY in the Presence of I_c. The denaturation and renaturation of CPY in 6 M GdmCl were carried out essentially as described by Winther and Sørensen (1991). Renaturation was achieved by dilution of the denaturant to 0.1 M in 50 mM Mes, 1 mM EDTA, and 0.9 M ammonium sulfate, pH 6.5. The final concentration of CPY was 0.05 mg/mL, and I_c was present at a concentration of 0.04 mg/mL in the renaturation mixture, equivalent to a 3-fold molar excess. Activity half-life was determined at 55 °C in 50 mM Mes and 1 mM EDTA, pH 6.5, with a CPY concentration of 0.023 mg/mL and an I_c concentration of 0.02 mg/mL. Samples were withdrawn and assayed in the presence of 1.3 M GdmCl as described above.

RESULTS

Amino Acid Sequence of I_c. In the purification procedure previously described (Matern et al., 1974) the complex between CPY and I_c was dissociated early in the purification by treatment of the crude extract with cold acetone. This treatment was reported to specifically precipitate and inactivate CPY, as well as the other vacuolar proteases (proteinase A and proteinase B) that are normally responsible for degradation of I_c. In our hands, this selective precipitation was difficult to reproduce; the recovery of inhibitor was usually very poor. As an alternative to this procedure, we chose to purify the I_c−CPY complex and only later separate I_c from CPY. As starting material we used commercial bakers' yeast. Purification of the complex was particularly convenient since the I_c−CPY complex can be followed by measurement of CPY activity in the presence of 1.3 M GdmCl, which dissociates the complex. Assay in the absence of GdmCl reveals the amount of uninhibited CPY.

In a crude extract typically >95% of the CPY activity is inhibited; however, at pH lower than 5.5 the inhibitory acitivity is efficiently degraded. Thus, in the initial steps of the purification, pH was maintained above 8.0 to avoid the degradation of I_c by proteinase A or proteinase B. The yield of the complex after ammonium sulfate precipitation and desalting on G50 was typically 50−60%. The I_c−CPY complex bound very well to Q-Sepharose, eluting at about 300 mM KCl. Thus, this was a very efficient purification step and, apart from a strong band which was most likely CPY, active fractions from this step contained only a few major bands on SDS−PAGE (Figure 1A). The material from the Q-Sepharose chromatography was further purified by RP-HPLC on a C4 column. At the concentrations of acetonitrile and TFA used, CPY and I_c are dissociated. The major peak showed the N-terminal sequence of CPY, while material from the other peak was blocked (Figure 1B). After treatment of the material from this peak with trypsin, peptides were separated by HPLC on a C18 column. Sequencing of several of these (underlined in Figure 1C) disclosed identity with the amino acid sequence encoded by the *TFS1* gene. Two reports on the DNA sequence of this gene were in conflict with respect to the C-terminus (Tripp et al., 1989; Robinson & Tatchell, 1991). Amino acid sequencing of a peptide distinguishing the two DNA sequences (double-underlined in Figure 1C) confirmed that the sequence published by Robinson and Tatchell (1991) was correct. One peptide was found to be N-terminally blocked, suggesting that this was the N-terminus of I_c. Determination of its molecular mass by spectroscopy on MALDI-TOF gave 1972.9 Da, compatible with the mass of an N-terminal peptide CH_3CO-MNQAIDFAQASIDSYKK, which is 1972.6. This strongly suggests that the inhibitor is modified by N-terminal acetylation. Analysis of inhibitory activity in strains deleted for or overproducing *TFS1* confirmed that I_c is indeed encoded by *TFS1*.

Preparative-Scale Purification of I_c. Having identified the gene encoding I_c, we investigated three methods for preparative-scale purification of I_c: (A) production in *E. coli* of a modified I_c with an N-terminal His_6 tag; (B) production in *E. coli* of wild-type I_c; and (C) production of wild-type I_c from yeast cells overexpressing *TFS1*. Expression and purification of the His-tagged protein (procedure A) was exceedingly efficient. The protein was expressed at high levels from the T7 polymerase system and yielded a soluble protein with inhibitory activity. However, the His-tagged protein was found to inhibit CPY less efficiently than wild-type I_c, a point to be described in more detail below. We then attempted to prepare I_c with native sequence from the *E. coli* expression system (procedure B). However, although the protein was soluble, it was essentially inactive. Preparation of larger amounts of native I_c was finally achieved by overexpression of *TFS1* in a yeast strain deleted for the genes for CPY and proteinase A. I_c was efficiently and fairly specifically liberated from the cells by freeze−thawing. The subsequent heat treatment of the extract resulted in denaturation of the majority of the other proteins, which could subsequently be removed by centrifugation. The I_c yield in this step was 40−50%. Chromatography of the heat-treated supernatant on Phenyl Sepharose gave pure I_c. The I_c used in the experiments described below was taken from the pure fraction of this step and desalted just prior to use.

Characterization of Native and Recombinant Forms of I_c. If 1 molar equiv of I_c was added to CPY during an assay, the CPY activity was reduced and only reached a new equillibrium after about 3 min. To ensure equilibrium of the I_c−CPY complex, enzyme and inhibitor were preincubated at 25 °C for 5 min at 50 times the concentration of the assay. The subsequent establishment of a new equilibrium in the assay is more rapid (less than 2 min; Figure 3). These effects are only detectable at I_c/CPY ratios above 0.9. CPY was assayed at increasing concentrations of I_c, and

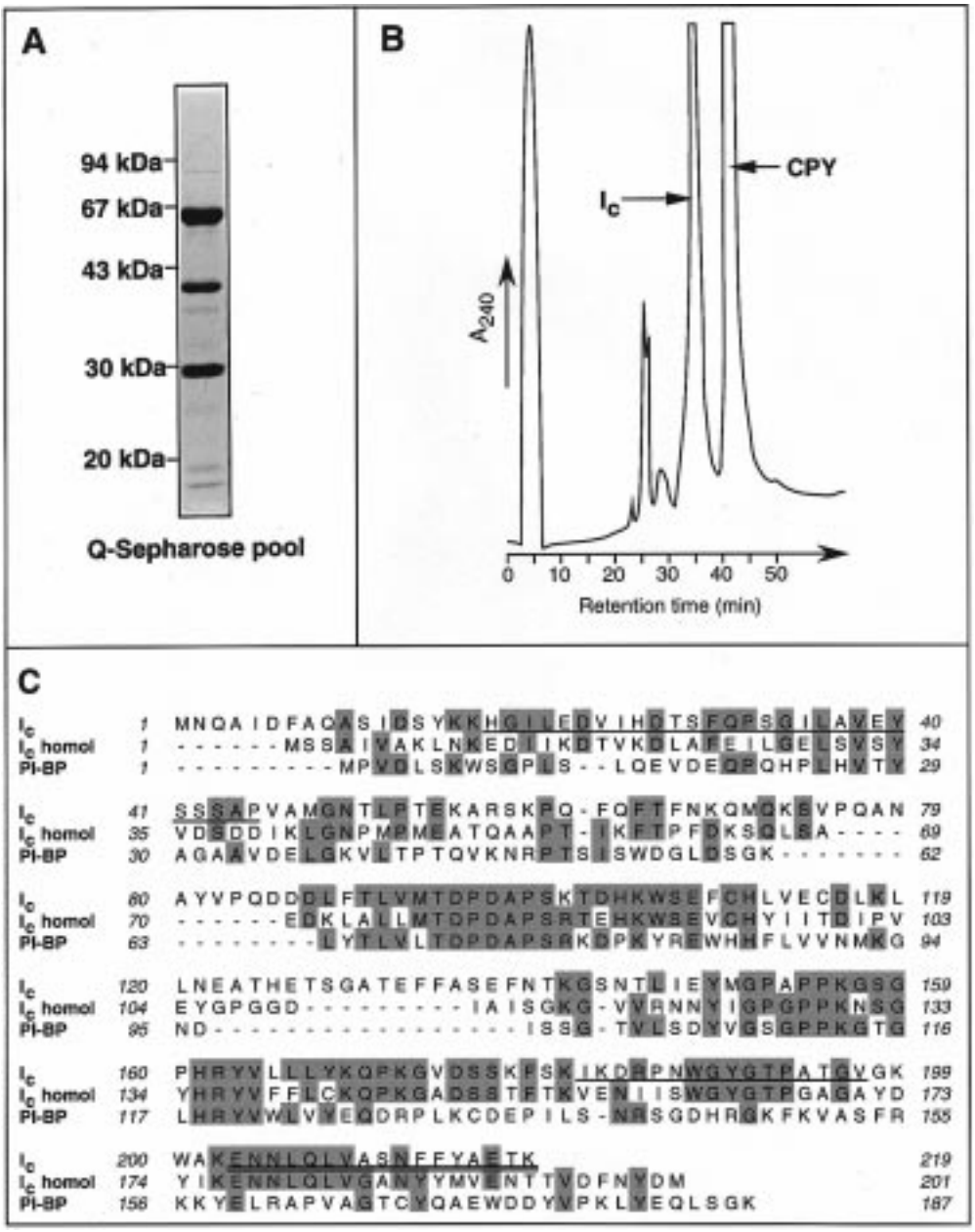

FIGURE 1: (A) Analytical SDS−PAGE of a partially purified CPY−I_c complex obtained by chromatography on Q-Sepharose (the positions of molecular mass markers are indicated). (B) Chromatogram showing the semipreparative separation of the same material by HPLC on a Vydac C4 column. The peak containing CPY was identified by its N-terminal sequence. The peak labeled I_c was subjected to tryptic digestion, separation of peptides, and sequencing. (C) Amino acid sequence of I_c. The sequences of tryptic peptides used for the identification of the gene encoding I_c are underlined. The double-underlined sequence defines the C-terminus, the sequence of which has been disputed (Tripp et al., 1989; Robinson and Tatchell, 1991). These sequences unambiguously identified *TFS1* as encoding I_c, and this identification was confirmed by gene disruption as well as overexpression. The *S. cerevisiae* genome contains a close homologue of *TFS1*, denoted "I_c homol" in the alignment. Also shown is the sequence of a member of the 21−23-kDa lipid binding proteins, the phosphatidylethanolamine binding protein from human brain (Seddiqi et al., 1994). The sequence alignment was carried out using the CLUSTAL V alignment software (Higgins et al., 1991).

fitting the data to eq 1 yielded an apparent K_i (Williams & Morrison, 1979) of 0.1 nM (Figure 4A), making I_c a high-affinity inhibitor. The specificity of I_c was investigated using the CAC enzyme, which is a CPY homologue from *C. albicans*. Here we found the K_i to be 23 nM, or more than 200-fold higher than that toward CPY, indicating a high degree of ligand specificity (Figure 4B). Lineweaver−Burk plots (not shown) indicated that the substrate did not significantly compete with the inhibitor at the concentrations used. However, since K_i is 10^6-fold below the K_m of the substrate used, this would be the case even if the inhibition were competitive. As discussed by Williams and Morrison (1979), the type of inhibition cannot be revealed by this type of analysis unless substrates with very low K_m or very high concentrations are used. Such suitable substrates are not readily avaliable.

To see whether I_c might aid the folding of CPY, a denaturation−renaturation experiment was performed. This showed that I_c could indeed enhance the refolding yield 3-fold (Figure 5). Along the same lines, it has previously been shown that the noncovalently bound propeptide stabilized CPY against heat inactivation (Winther et al., 1994).

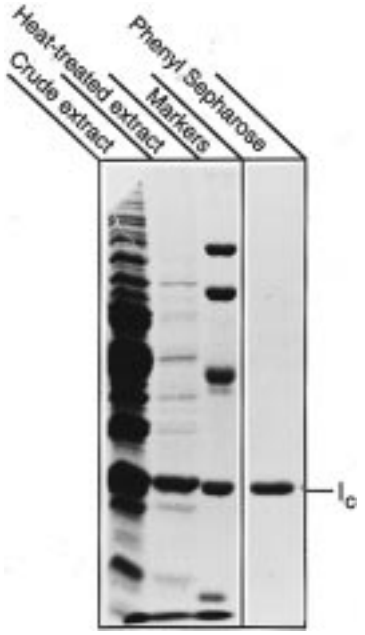

FIGURE 2: Aliquots from the 10% SDS−PAGE purification of I_c. The sample named "Crude extract" was prepared by freeze−thaw lysis of cells overproducing *TFS1*. This extract was heated briefly to 80 °C, and aggregated protein was removed by centrifugation. The supernatant was loaded onto the gel as "Heat-treated extract". "Markers" are, from the top downward, 94-, 67-, 43-, 30-, and 20-kDa molecular mass markers. "Phenyl Sepharose" indicates the fraction obtained by Phenyl Sepharose chromatography. This preparation was used in the experiments described in the present paper.

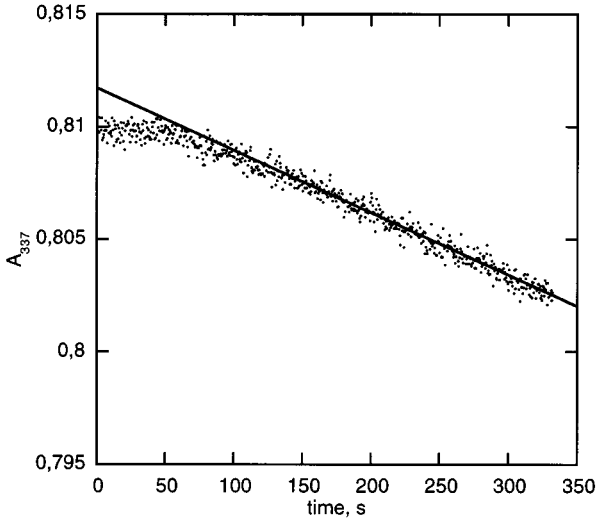

FIGURE 3: Activity of CPY at 4 nM with approximately equimolar amounts of Ic present. Hydrolysis of FA-Phe-Phe-OH results in a decrease in absorbance. The activity is followed by the reduction in A_{337} with time. After preincubation of the enzyme−substrate mixture, the CPY activity is measured by a 50-fold dilution into the assay buffer. Because of this dilution, the inhibition is initially higher; however, a new equilibrium is reached within 2−3 min as seen from the appearance of a hydrolysis rate which is linear with time. In determining the inhibition constants, time was allowed in every case for such a new equilibrium to be reached.

Neither bovine serum albumin nor hen egg white lysozyme has such effects (Winther et al., 1994). We found that the CPY activity followed an exponential decay. The half-life at 55 °C increased from about 22 min to more than 4 h at a concentration of CPY of 0.4 μM and with 1.5 molar excess of I_c.

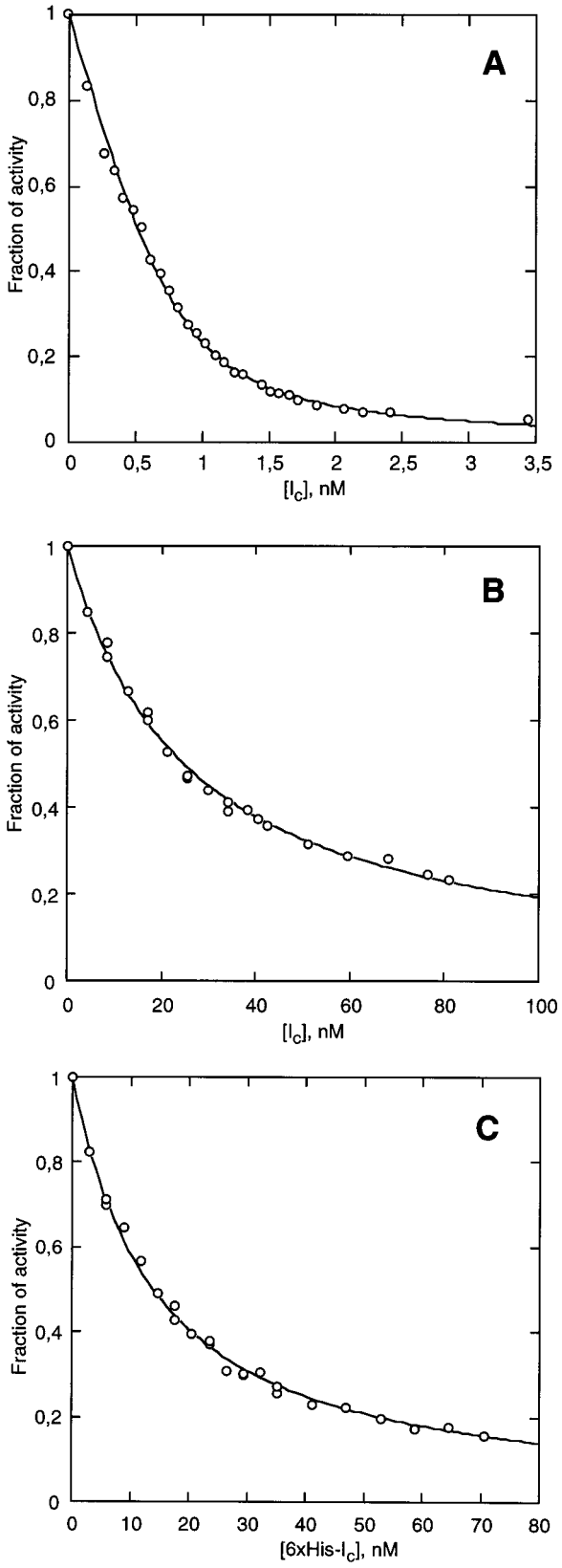

FIGURE 4: (A) Relative CPY activity at increasing concentration of I_c. The CPY concentration was 0.8 nM. (B) Relative CAC activity at increasing concentration of I_c. The CAC concentration was 2 nM. (C) Relative CPY activity at increasing concentration of the N-terminally histidine tagged I_c. A CPY concentration of 3.5 nM was used. The curves indicated on the plots are those obtained by fitting the data to eq 1 (see Experimental Procedures). The apparent K_i values were determined from these curves (see Results).

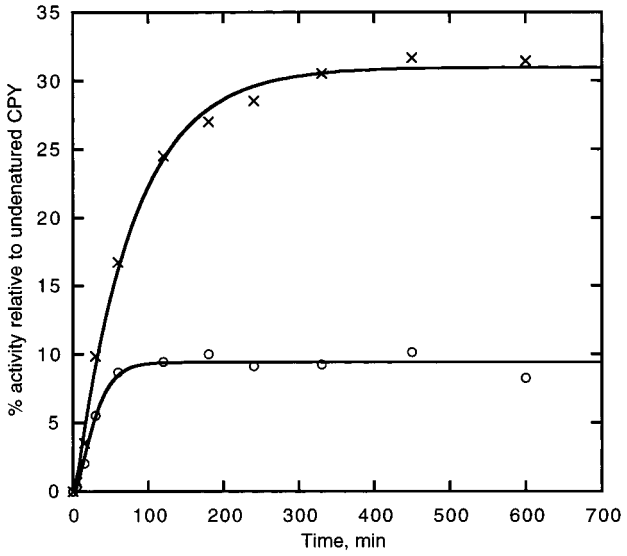

FIGURE 5: Refolding of CPY stimulated by I_c. CPY was denatured in 6 M GdmCl and subsequently refolded by 60-fold dilution of the sample in 0.9 M ammonium sulfate in the absence (○) or presence (×) of I_c. The appearance of correctly folded CPY was followed by assaying aliquots for activity in the presence of 1.3 M GdmCl. Refolding kinetics was fitted to a process involving two rate-limiting steps as described previously for this reaction (Winther et al., 1994). For experimental details see Experimental Procedures.

Does Overproduction of I_c Suppress cdc25-1 by Means of Inhibition of CPY? The genetic interaction (Robinson and Tatchell, 1991) between *TFS1* and *CDC25* and the biochemical interaction between CPY and the *TFS1* gene product, I_c, suggested that perhaps overexpression of *TFS1* suppressed *cdc25-1* by means of inhibition of CPY. If, for example, CPY activity somehow had negative effects on strains expressing the *cdc25-1* allele, then overexpression of *PRC1* might reduce the growth rate even at the permissive temperature for *cdc25-1*. To test this hypothesis, *PRC1* was overexpressed in a *cdc25-1* strain; however, an altered growth phenotype could not be detected. A putative *PRC1*−*CDC25* link could also display itself in the ability of a *prc1* strain to suppress *cdc25-1*. To test this, strains containing these two markers were crossed and spores dissected, but no suppression phenotype was observed in several independent *cdc25-1*−*prc1* double mutants. In conclusion, these experiments cannot rationalize why a high copy number of the structural gene of I_c suppresses *cdc25-1*.

DISCUSSION

CPY is the classical member of the serine carboxypeptidase families. These enzymes utilize a mechanism involving a catalytic Asp-His-Ser triad very similar to that of the serine proteases of the trypsin and subtilisin family. However, there are some differences in mechanism and pH optimum (Remington & Breddam, 1994). To our knowledge the only macromolecular inhibitor toward a serine carboxypeptidase described is I_c from yeast. We purified this inhibitor to gain insight into its structure and function. Amino acid sequencing showed that I_c is encoded by the yeast gene *TFS1* (Figure 1C), which predicts a protein with a molecular mass of 24.3 kDa and a calculated isoelectric point of 6.3. There are no potential sites for N-linked glycosylation and no hydrophobic segments that could serve as obvious membrane anchors or signal sequences. The amino acid sequencing also identified intact N- and C-terminal peptides, which showed that no posttranslational proteolysis had taken place and suggested an acetylation of the N-terminus.

It was surprising to find that *TFS1* originally was identified as a high-copy suppressor of the temperature-sensitive *cdc25-1* allele (*TFS* for 25 suppressor). *CDC25* is essential for growth and is involved in the GTP/GDP exchange of Ras1p/Ras2p in yeast [see Broach (1991) for review]. The fact that overexpression of *TFS1* suppressed specific alleles of *cdc25* (*cdc25-1* and *cdc25*−*5*), but not a *cdc25* deletion, suggested that there might be specific interactions between the gene products (Robinson & Tatchell, 1991). There are, however, no direct indications that Cdc25p and Tfs1p interact chemically or physically. Indeed, the only well-established biochemical activity of the *TFS1* gene product appears to be its inhibition of CPY. On this basis, we wished to test whether there was a genetic interaction between *prc1* and *cdc25*. By a genetic cross a Δ*prc1*−*cdc25-1* strain was constructed. If CPY activity had a role in the phenotype of the *cdc25-1* allele, then deletion of *prc1* could be expected to suppress this mutation. In a parallel approach, the *cdc25-1* strain was transformed with a high-copy plasmid containing *PRC1* to test whether this would have negative effects on the growth of cells under normally permissive conditions. In neither case were any effects observed. Taken together, these results provide no evidence that CPY inhibition should be responsible for the *cdc25-1* suppressor phenotype of *TFS1*. It is possible that the suppression is the result of induction of a general stress response.

I_c belongs to a family of proteins with fairly high sequence similarity. The closest homologue of I_c is found in the yeast genome. This homologue is a putative protein encoded from an open reading frame with unknown function (YLR179C) and has extensive identity (37%) throughout the length of the proteins (Figure 1B). Perhaps more interestingly, I_c is homologous to a group of proteins broadly termed as "21−23-kDa proteins" which are found in various tissues in higher eukaryotes (Schoentgen and Jolles, 1995). The function of these proteins is unknown. However, they have been subjected to fairly extensive biochemical characterization, and it has been shown that they bind lipids and possess a putative nucleotide binding site (Bernier & Jolles, 1984). The identities between I_c and the human phosphatidyl-ethanolamine binding protein are mainly located within two regions, residues 90−110 and 148−170, respectively (Figure 1C).

Addition of increasing amounts of I_c to a fixed amount of CPY gives a reduction in CPY activity similar to that of active site titration (Figure 4A) and an apparant K_i of 0.1 nM. Unlike some serine protease inhibitors, I_c is not hydrolyzed upon binding to CPY, since it is recovered in intact form after dissociation from the enzyme with GdmCl. It is interesting that the inhibition of CAC is much weaker (Figure 4B) even though this enzyme has 74% sequence identity with CPY. This indicates a fairly high degree of specificity and suggests an evolutionary significance of the high affinity between I_c and CPY. It is also interesting that an N-terminal histidine tag reduces the inhibition drastically (Figure 4C). This might indicate that the N-terminus is involved in binding to the enzyme. It was impossible to prepare an active form of I_c without a histidine tag in *E.*

coli even though it was in a soluble form. One could speculate that the N-terminal acetylation found in the native I_c is important for function and that its absence to some extent can be compensated by the presence of a histidine tag.

CPY is synthesized as a zymogen, in which the propeptide efficiently inhibits the enzyme. The propeptide is also important for the folding of CPY, increasing both the yield and the rate of the refolding reaction in vitro (Winther & Sørensen, 1991; Winther et al., 1994). The chaperone-like activity was seen even when the propeptide was not covalently linked to the enzyme, but only in the presence of a fairly high concentration of ammonium sulfate (Winther et al., 1994). The propeptide appears to inhibit the enzyme not by covering the active site cleft but rather by distorting the enzyme structure (Sørensen & Winther, 1994). We reasoned that I_c might interact with CPY in much the same way that the propeptide does. We find it interesting that by two criteria I_c performed similarly to the propeptide. I_c enhances the refolding yield by more than a factor of 3 (Figure 5). This suggests that the inhibitor binds to a folding intermediate, the further folding of which is promoted by binding. The increase in denaturation half-life at 55 °C from 12 min to over 4 h is consistent with such a model (Winther et al., 1994). However, in this case stabilization could also be explained by binding of I_c to folded CPY.

It is not clear why so many cytoplasmic inhibitors toward endogeneous proteases have evolved and are found, not only in yeast, but also in plants and animals. It is commonly argued that they are necessary to prevent the potentially damaging effect of "leakage" from the proteolytic compartments such as the vacuole. However, there are several arguments against such a view. One of the important functions of the vacuole is to maintain osmotic and electrolytic stability of the cell, in addition to many other functions involving small molecules. A leaky vacuolar membrane would result not only in release of proteases and other hydrolases into the cytoplasm but also a potentially much more damaging and rapid formation of an equilibrium of salts and pH. There is no evidence that such leakage should be a common phenomenon or, indeed, that a cell should be able to survive it. Furthermore, most of the inhibitors are found in near equimolar amounts with their cognate proteases, which would hardly be necessary in cases of minor "leakages". A more likely function is perhaps in regulation of proteolysis under conditions of autophagocytosis, when cytoplasmic content enters the vacuole for degradation (Takeshige et al., 1992). In any event, the low K_i would suggest that the inhibition is the result of a specific evolutionary process.

In conclusion, we have identified a new type of carboxypeptidase inhibitor and assigned this function to a member of proteins which have not been previously assigned to any specific cellular function. In view of the extensive sequence similarity to I_c, it is tempting to speculate that the 21−23-kDa lipid binding proteins are also inhibitors of specific cellular serine carboxypeptidases.

ACKNOWLEDGMENT

We are most grateful to Kelly Tatchell for providing strains and plasmids. Klaus Breddam is thanked for supplying pure carboxypeptidase Y. We also thank Ann-Sofi Steinholz for preparing photographs.

REFERENCES

Bernier, I., & Jolles, P. (1984) *Biochim. Biophys. Acta 790*, 174−181.
Biedermann, K., Montali, U., Martin, B., Svendsen, I., & Ottesen, M. (1980) *Carlsberg Res. Commun. 45*, 225−235.
Bode, W., & Huber, R. (1992) *Eur. J. Biochem. 204*, 433−451.
Breddam, K., & Beenfeldt, T. (1991) *Appl. Microbiol. Biotechnol. 35*, 323−329.
Broach, J. R. (1991) *Curr. Opin. Genet. Dev. 1*, 370−377.
Bünning, P., & Holzer, H. (1977) *J. Biol. Chem. 252*, 5316−5323.
Gill, S. C., & von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319−326.
Higgins, D. G., Bleasby, A. J., & Fuchs, R. (1991) *Comput. Appl. Biosci. 8*, 189−191.
Johansen, J. T., Breddam, K., & Ottesen, M. (1976) *Carlsberg Res. Commun. 41*, 1−14.
Johnson, B. H., & Hecht, M. H. (1994) *Bio/Technology 12*, 1357−1360.
Matern, H., Hoffmann, M., & Holzer, H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4874−4878.
Nielsen, T. L., Holmberg, S., & Petersen, J. G. L. (1990) *Appl. Microbiol. Biotechnol. 33*, 307−312.
Pennacchio, L. A., Lehesjoki, A.-E., Stone, N. E., Willour, V. L., Virtaneva, K., Miao, J., D'Amato, E., Ramirez, L., Faham, M., Koskiniemi, M., Warrington, J. A., Norio, R., de la Capelle, A., Cox, D. A., & Myers, R. M. (1996) *Science 271*, 1731−1734.
Remington, S. J., & Breddam, K. (1994) *Methods Enzymol. 244*, 231−248.
Robinson, L. C., & Tatchell, K. (1991) *Mol. Gen. Genet. 230*, 241−250.
Schoentgen, F., & Jolles, P. (1995) *FEBS Lett. 369*, 22−26.
Schu, P., & Wolf, D. H. (1991) *FEBS Lett. 283*, 78−84.
Schu, P., Suarez Rendueles, P., & Wolf, D. H. (1991) *Eur. J. Biochem. 197*, 1−7.
Seddiqi, N., Bollengier, F., Alliel, P. M., Perin, J. P., Bonnet, F., Bucquoy, S., Jolles, P., & Schoentgen, F. (1994) *J. Mol. Evol. 39*, 655−660.
Sherman, F. (1991) *Methods Enzymol. 194*, 3−21.
Sørensen, S. O., & Winther, J. R. (1994) *Biochim. Biophys. Acta 1205*, 289−293.
Tabor, S., & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074−1078.
Takeshige, K., Baba, M., Tsuboi, S., Noda, T., & Ohsumi, Y. (1992) *J. Cell Biol. 119*, 301−311.
Tripp, M. L., Bouchard, R. A., & Piñon, R. (1989) *Mol. Microbiol. 3*, 1319−1327.
Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol. 63*, 437−467.
Winther, J. R., & Sørensen, P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9330−9334.
Winther, J. R., Sørensen, P., & Kielland-Brandt, M. C. (1994) *J. Biol. Chem. 269*, 22007−22013.

BI971286W