Cloning and Characterization of a Cysteine Proteinase from Saccharomyces cerevisiae*

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We have isolated a gene from Saccharomyces cerevisiae that encodes a protein homologous to the mammalian cysteine proteinase bleomycin hydrolase. Sequence comparison between the yeast and rabbit proteins indicates an amino acid identity of 41.5% over 277 residues and a similarity of 78.3% when conservative substitutions are included. The apparent mass of the yeast protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis is 47 kDa, although sequence analysis indicates two potential initiator methionines that suggest calculated masses of either 51 or 55 kDa. The protein is nonessential in yeast as haploid mutants disrupted at several positions along the open reading frame remain viable. Furthermore, these mutants do not exhibit any readily observable growth defects under varying conditions of temperature, nutrients, osmotic strength, or exogenous bleomycin. However, the purified protein does exhibit marked hydrolytic activity toward the substrate arginine 4-methyl-7-coumarylamide (K_m = 12.8 μM, V_max = 2.56 μmol mg^-1 h^-1), and yeast cells engineered to express this protein at higher levels maintain increased resistance to bleomycin compared to wild-type cells. Because this protein represents the first example of a cysteine proteinase identified in yeast, we have named it Yec1 (yeast cysteine proteinase).

As in higher eukaryotes, the various activities can be categorized as either cytosolic, vacuolar, or as components of the secretory pathway (13). Included within these compartments are examples of serine (Kex1, CypY) and Zn^{2+} (Cps) carboxypeptidases (14–16), aspartic (PrA) and serine (PrB, Kex2) endoproteinases (17–19), and Zn^{2+} (ApI) and Co^{2+} (ApCo) aminopeptidases (20, 21). Analogous to mammalian proteases, these enzymes function in both pheromone and zymogen precursor processing (19, 22) and general protein degradation pathways (23). However, unlike mammalian proteolytic systems, no cysteine proteinases have yet been identified in yeast.

Previously, we reported the isolation and partial biochemical characterization of a group of cysteine calcium-dependent phospholipid-binding proteins from the yeast S cerevisiae (24). Here we report the cloning and further characterization of one member (Y3) of this family, which is a yeast homolog of mammalian bleomycin hydrolase. This protein resembles both structurally and functionally members of the thiol-dependent class of cellular endoproteinases. Since it also represents the first example of such a cysteine proteinase in yeast, we have chosen to rename it accordingly as Ycp1 (yeast cysteine proteinase).

MATERIALS AND METHODS

Reagents and Media—All molecular biology reagents were used according to the manufacturer's written instructions, except where noted. Restriction and modifying enzymes were from Promega. DNA sequencing kits (Sequenase™) were from United States Biochemical. Cloning kits (Lambda Zap II™) were from Stratagene. All random primer-labeling reagents were from IBL. Bovine brain phospholipids (B-1502), Brij 35, bleomycin (1.87 μg/ml), N-Mec, and Arg-N-Mec were obtained from Sigma. Oligonucleotides used as probes were synthesized at the UVA Sequencing Facility. [γ-32P]ATP (3,000 Ci/mmol), [α-32P]CTP (5,000 Ci/mmol), and [α-35S]DATP (500 Ci/mmol) ribosiotides were purchased from Du Pont-New England Nuclear.

Bacteria were grown in LB supplemented with appropriate antibiotics (25). Yeast cells were grown on rice media (YPD) containing 1% Bacto yeast extract (Difco), 2% Bacto-peptone, and 2% glucose, or on minimal media (SD) containing 0.6% yeast nitrogen base (without amino acids) and 2% glucose, supplemented for auxotrophic requirements when necessary (26). Calcium-free medium was prepared as SD above, substituting MgCl2 for CaCl2 in the composition of yeast nitrogen base.

Bacterial and Yeast Strains—The bacterial host XL1-Blue™ (recA1, lac-rayEm, gal, thi, hsdR17 supE44, relA1, F' proAB lacIq lacZAM15, Tn10) (Stratagene) was used for the propagation and maintenance of all plasmids described. Strain C600 hfl (supE44, relA1, mcrA, hflAI50:TnlO) was used as a host for recombinant λ phage in all library screenings and titers. strain Mini-Tn3 (mTn3) mutagenesis of selected DNA constructs was performed as described (27) using bacterial strains RDP146 (F' recA1, Δlact-pro, rpsE) and NS2142Sm (F' recA, λ-cmr, rpsL). All

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The abbreviations used are: N-Mec, 7-amino-4-methylcoumarin; BLM, bleomycin; Arg-N-Mec, arginine 4-methyl-7-coumarylamide; ORF, open reading frame; SDS, sodium dodecyl sulfate.

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yeast strains used in this study are listed in Table I.

Nucleic Acid Techniques—All DNA manipulations were performed essentially as described (25). For library construction yeast genomic DNA (strain YP3) was cut with either EcoRI or XbaI, and the resulting fragments between 0.1-11 kilobases were isolated by electrophoresis. This DNA was then ligated into the recombinant vector to produce a yeast genomic DNA library. Both libraries were transformed into the appropriate yeast strain and Southern hybridizations were performed on Zeta-bind™ nylon membranes (Cuno) in buffer H (1% bovine serum albumin (Sigma, Fraction V), 0.1 mg/ml herring sperm DNA, 0.5 mM NaH2PO4 (pH 7.0), 1% SDS, 1 mM EDTA) and then washed twice successively with buffer A (0.5% bovine serum albumin, 5% SDS, 40 mM NaH2PO4 (pH 7.0), 1 mM EDTA) and then buffer B (5% SDS, 40 mM NaH2PO4, 1% SDS, 1 mM EDTA) at 50-65 °C. Lower stringency washes were performed in buffer A at 35 °C. Probes used for hybridization were either end-labeled (oligonucleotides) with [γ-32P]dATP or random-primed (double-stranded DNA) with [α-32P]dCTP to specific activities of 1.3-1.8 × 10⁹ cpm/mmol and 4.8-7.2 × 10⁶ cpm/μg, respectively.

Nucleotide sequencing was performed in both directions by the dideoxy chain termination method (28) using [α-32P]dATP (29). Template DNA was the M13 phage derivative pBluescript KS™ (Stratagene) excised in vitro from plaque-purified Lambda Zap II™ clones. Exonuclease III-generated nested deletions of each plasmid were performed prior to sequencing. Aliquots of transformation electrocompetent on 8% acrylamide gels (4:1 acrylamide/bis) containing 8.3 M urea at 45 watts constant power. Restriction maps of all isolated inserts are shown in Fig. 2.

Yeast Manipulations—Yeast DNA isolations, transformations, crosses, and genetic manipulations were performed essentially as described (26). Diploid cells were sporulated in 0.3% potassium acetate (27) and 4053-5-4) parents and then selecting for homologous recombinations. Yeast strains used in this study are listed in Table I.

Nucleotide sequencing was performed using the dideoxy chain termination method (28) using [α-32P]dATP (29). Template DNA was the M13 phage derivative pBluescript KS™ (Stratagene) excised in vitro from plaque-purified Lambda Zap II™ clones. Exonuclease III-generated nested deletions of each plasmid were performed prior to sequencing. Aliquots of transformation electrocompetent on 8% acrylamide gels (4:1 acrylamide/bis) containing 8.3 M urea at 45 watts constant power. Restriction maps of all isolated inserts are shown in Fig. 2.

Yeast Cysteine Proteinase—Yeast cysteine calcium-dependent membrane-binding probes were purified, digested, and the resulting peptides sequenced as previously described (24). Two peptide fragments from the 47-kDa protein (YV) were used to construct both unique and partially degenerate oligonucleotide probes as shown in Fig. 1. In order to minimize degeneracies, the sequences of all three probes were based on most frequent codon usage data compiled for S. cerevisiae (32). Each oligonucleotide was end-labeled and used to screen 80-100,000 pfu of an EcoRI fragment-containing yeast genomic DNA library. As shown in Fig. 2, a single 3.4-kilobase EcoRI fragment (clone 47A) that hybridized with all three probes was excised from the plasmid pBluescript™ KS-. Subsequent digestion with exonuclease III-generated nested deletions used for sequencing. However, complete sequencing of clone 47A revealed that approximately 0.5 kilobase of 5'-coding sequence was absent. Therefore, DNA fragments from the 5' end of clone 47A were labeled

**RESULTS**

Cloning, Sequence Analysis, and Mapping of YCP1—Yeast cysteine calcium-dependent membrane-binding probes were purified, digested, and the resulting peptides sequenced as previously described (24). Two peptide fragments from the 47-kDa protein (YV) were used to construct both unique and partially degenerate oligonucleotide probes as shown in Fig. 1. In order to minimize degeneracies, the sequences of all three probes were based on most frequent codon usage data compiled for S. cerevisiae (32). Each oligonucleotide was end-labeled and used to screen 80-100,000 pfu of an EcoRI fragment-containing yeast genomic DNA library. As shown in Fig. 2, a single 3.4-kilobase EcoRI fragment (clone 47A) that hybridized with all three probes was excised from the plasmid pBluescript™ KS-. Subsequent digestion with exonuclease III-generated nested deletions used for sequencing. However, complete sequencing of clone 47A revealed that approximately 0.5 kilobase of 5'-coding sequence was absent. Therefore, DNA fragments from the 5' end of clone 47A were labeled

**FIG. 1.** Ycp1 peptide and oligonucleotide probe sequences. Over- and underlined peptide sequences were used to construct the corresponding degenerate and non-degenerate probes, respectively. All oligonucleotide are written 3'→5' (left to right). Calculated degeneracies appear in parentheses beside each probe name.

**FIG. 2.** Restriction/disruption map of the YCP1 gene. The complete YCP1 gene is shown above its corresponding clones used for sequencing. Approximate positions of the LEU2 and URA3 mTn3 disruptions are indicated below the open reading frame (boxed) as their respective yeast strain numbers (NK822 and NK829). R, EcoRI; F, PstI; H, HindIII; S, SacI; Sc, SacII; B, BamHI.
potential initiator methionines exist (*) with numbering beginning at the first in-frame ATG (1449-base pair ORF that encodes a potential 483-amino-acid polypeptide). The YCP1 ORF appears to be uninterrupted as it does not contain any consensus sequences for intron splicing (33). There are two potential initiator methionine “ATG” codons in the YCP1 sequence, but only the second one (AAGAATGTC) is flanked by nucleotides that satisfy the consensus rules for initiation of translation in yeast (A/YAA/TAAATGTC) (34). If one assumes that this second methionine represents the correct translational start site, then the calculated Mr of the resulting polypeptide chain is 52,076 Da, in closer agreement to the apparent mass of 47 kDa (as determined by SDS-gel electrophoresis) than the 55,471-Da value obtained using the first ATG codon. Attempts to resolve this issue by protein microsequencing were hindered by a blocked NH2 terminus. The 5' flanking region upstream of both ATGs contains putative “CAAT” and “TATA” boxes that conform well to published consensus sequences (35). Furthermore, this region is rich in A + T content (61% over 450 nucleotides), suggesting that the Ycp1 gene product may be highly expressed (36). This contention is supported by the observation that a 5' pyrimidine-rich stretch in YCP1 is immediately followed by a “CAAG” box, two elements commonly found in the promoter region of highly transcribed yeast genes (35). The 3' flanking region possesses over 71% A + T content (250 nucleotides) and contains both potential polyadenylation (AATAAA) (37) and transcription termination (TATG...T) (38) signals. Interestingly, this 3' region encoded 130 residues of the NH2 terminus of Kex2, a calcium-dependent serine protease required for processing of the a-factor precursor polypeptide (19). Therefore, the YCP1 gene is located on the left arm of chromosome XIV, placing its termination codon exactly 494 base pairs upstream of the ATG start codon of KEX2 (see Fig. 3). Southern analysis indicates that YCP1 is a single-copy gene.

The inferred amino acid composition of the Ycp1 protein indicates that it possesses a net negative charge (average pI = 6.1, using either initiator methionine) in disagreement with its basic behavior during two-dimensional gel electrophoresis (estimated pI = 8.0) and chromatography on Mono-Q (24). The hydrophobicity profile of the Ycp1 protein is unparalleled (data not shown), revealing a uniform distribution of both hydrophilic and hydrophobic domains throughout its entire length. Furthermore, no putative membrane-spanning segments could be identified. The sequence of the Ycp1 protein does contain two potential N-linked glycosylation sites, but since this protein is routinely isolated from yeast cytosol, it is doubtful that they are used. When the amino acid sequence of Ycp1 was compared against those of known calcium and phospholipid-binding proteins, no sequence similarities were obvious, indicating that Ycp1p does not contain either “EF-hand” or “endoxin-fold” -like structural domains (39, 40). Direct sequence comparison between the Ycp1 protein and another protein (Y2) isolated from yeast cytosol in those of peptides Y3A and Y3B. In the 3'-flanking region, potential polyadenylation signals are underlined, and the KEX2 ATG codon is boxed at nucleotide position 2600.

Fig. 3. Nucleotide sequence of YCP1. The nucleotide and deduced amino acid sequences of the YCP1 gene are shown. Two potential initiator methionines exist (*) with numbering beginning at the first in-frame ATG (1449-base pair ORF that encodes a potential 483-amino-acid polypeptide). The YCP1 ORF appears to be uninterrupted as it does not contain any consensus sequences for intron splicing (33). There are two potential initiator methionine “ATG” codons in the YCP1 sequence, but only the second one (AAGAATGTC) is flanked by nucleotides that satisfy the consensus rules for initiation of translation in yeast (A/YAA/TAAATGTC) (34). If one assumes that this second methionine represents the correct translational start site, then the calculated Mr of the resulting polypeptide chain is 52,076 Da, in closer agreement to the apparent mass of 47 kDa (as determined by SDS-gel electrophoresis) than the 55,471-Da value obtained using the first ATG codon. Attempts to resolve this issue by protein microsequencing were hindered by a blocked NH2 terminus. The 5'-flanking region upstream of both ATGs contains putative “CAAT” and “TATA” boxes that conform well to published consensus sequences (35). Furthermore, this region is rich in A + T content (61% over 450 nucleotides), suggesting that the Ycp1 gene product may be highly expressed (36). This contention is supported by the observation that a 5' pyrimidine-rich stretch in YCP1 is immediately followed by a “CAAG” box, two elements commonly found in the promoter region of highly transcribed yeast genes (35). The 3'-flanking region possesses over 71% A + T content (250 nucleotides) and contains both potential polyadenylation (AATAAA) (37) and transcription termination (TATG...T) (38) signals. Interestingly, this 3' region encoded 130 residues of the NH2 terminus of Kex2, a calcium-dependent serine protease required for processing of the α-factor precursor polypeptide (19). Therefore, the YCP1 gene is located on the left arm of chromosome XIV, placing its termination codon exactly 494 base pairs upstream of the ATG start codon of KEX2 (see Fig. 3). Southern analysis indicates that YCP1 is a single-copy gene.

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a similar fashion\(^7\) (24) also failed to reveal any significant degree of homology, indicating that both proteins may contain sequences for a novel calcium- and phospholipid-binding site(s). Two potential candidates for such a coordination site become apparent when two-dimensional matrix analysis is used to compare the sequence of the Ycp1 protein against itself. Ycp1p contains a small (21 amino acids) 2-fold internal repeat at positions 57–77 and 222–242 for which a consensus sequence can be constructed (T-LK—ADD-(L/I)(L/I)—R-Q-Q). Comparison of this sequence against the current protein databases does not reveal the presence of any other known proteins containing such a domain. At present it is not known whether these two regions are responsible for the observed calcium and phospholipid binding properties of the Ycp1 protein.

A FASTA search (41) of the Genbank protein sequence library (December, 1991) using the complete predicted Ycp1 amino acid sequence indicates that this protein is 41.5% identical to the mammalian enzyme bleomycin hydrolase isolated from rabbit lung (8). This homology further increases to 78.3% when conservative substitutions are included. The complete alignment of both sequences is shown in Fig. 4A. By using the second initiator methionine in the yeast protein, it is larger than the mammalian protein by 177 amino acids, distributed approximately equally as both NH- and COOH-terminal extensions. In particular, Fig. 4B compares a 15-residue domain in Ycp1 with a highly conserved region present in the active sites of several different mammalian cysteine proteinases, suggesting that Ycp1 is a yeast thiol-dependent endoproteinase (8).

**Construction of YCP1 Mutants and Growth Characteristics**—As a first step toward characterizing the in vivo function of the Ycp1 protein, the ORF of its isolated gene was disrupted using mini-Tn3 mutagenesis (27). Specifically, the 3.4-kilobase EcoRI fragment of clone 47A (containing the COOH-terminal 60% of Ycp1p) was ligated into the corresponding site of the plasmid vector pHS68 (27). This construct was then used as the target of transposon-mediated mutagenesis in bacterial strain RDP146. After resolution of all cointegrates, a bank of plasmids containing either LEU2 or URA3 disruptions of the insert was established. From this bank several individual disruptions were characterized by restriction analysis, and two (one LEU2 and one URA3) containing mTn3 sequences near to the 5′ end of the YCP1 insert were excised and transformed into the diploid strain 5899. Individual transformants were selected on appropriate media and characterized by Southern hybridizations of total genomic DNA. Clones showing one wild-type and one disrupted copy of the YCP1 locus were sporulated, and tetrads were dissected. For both disruptions, four viable spores were obtained with all heterozygous markers segregating 2:2. That each marker cosegregated with its corresponding disruption was verified by Southern hybridization of genomic DNA isolated from individual haploid progeny. These results indicate that under normal growth conditions Ycp1p is nonessential.

For the purpose of making valid comparisons with other yeast mutants defective in calcium- and phospholipid-binding proteins, it was desirable to have all ycp1 alleles placed within a common genetic background. Therefore, each gene disruption was retransformed into the haploid strains 4053-5-2 and 4053-5-4 and again, the resulting constructs were verified by Southern hybridization analysis of restriction enzyme-digested total genomic DNA. These mutants (NK822-NK823) were used in all subsequent experiments; their respective genotypes are listed in Table I, and the approximate positions of corresponding disruptions are shown in Fig. 2.

Since under normal growth conditions YCP1 is a nonessential gene, studies were initiated in order to better characterize the underlying molecular defect(s) in ycp1 haploid disruption mutants. At the light microscopic level, the disruption strains NK822-NK823 all showed normal cellular morphologies. Furthermore, none of these mutants exhibited either a cold- or temperature-sensitive phenotype at the four different temperatures examined (15, 23, 30, and 37 °C). As shown in Fig. 5, the growth rates in either complete (normal) or minimal (stressed) medium indicate that under both types of environmental conditions the growth of ycp1 strains is indistinguishable from isogenic wild-type.

Because the Ycp1 protein was isolated as a calcium-dependent membrane-binding protein, the sensitivity of ycp1 mutants to varying external calcium concentrations was also examined. The results indicated that for external calcium concentrations between 0–300 mM, the growth properties of

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**Table I**

**Relevant yeast strains**

| Strain   | Genotype                                      |
|----------|-----------------------------------------------|
| NK 822-3 | MATa, trpl-1, leu2-3, ura3-5, his7 (ypl1:URA3) |
| NK 822-15| MATa, trpl-1, leu2-3, ura3-5, his7 (ypl1:URA3) |
| NK 823-2 | MATa, trpl-1, leu2-3, ura3-5, his7 (ypl1:LEU2) |
| NK 823-4 | MATa, trpl-1, leu2-3, ura3-5, his7 (ypl1:LEU2) |
| 4053-5-2 | MATa, trpl-1, leu2-3, ura3-5, his7 (ypl1:LEU2) |
| 4053-5-4 | MATa, trpl-1, leu2-3, ura3-5, his7 (ypl1:LEU2) |

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\(^7\) N. G. Kambouris, D. J. Burke, and C. E. Creutz, submitted for publication.
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**Fig. 5. Growth curves.** Comparison of the growth characteristics of *ycpl* and isogenic wild-type strains in complete (panel A) or minimal (panel B) media. □, wild-type (4053-5-2); □, *ycpl* (NK823-2).

**Fig. 6. Lineweaver-Burk plot of Ycplp hydrolase activity.** The ability of the Ycpl protein to hydrolyze the substrate Arg-N-Mec was examined as described under “Results.” From the graph, $K_m = 12.8 \mu M$ and $V_{max} = 2.56 \mu mol/mg/h$.

these mutants are qualitatively indistinguishable from isogenic wild-type strains (data not shown).

**In Vitro Bleomycin Hydrolase Activity.** Based on the observed structural similarity between mammalian BLM hydrolase and the *YCP1* gene product, we sought to determine whether the Ycpl protein possessed any cysteine proteinase activity by using a fluorimetric assay adapted from studies on the cathepsins (31). The results, presented in Fig. 6, indicate that Ycplp is able to hydrolyze the NH$_2$-terminal arginine moiety from the substrate Arg-N-Mec in a concentration-dependent fashion. Furthermore, this activity is completely inhibited by either prior heat shock of the enzyme (100 °C, 5 min) or the addition of 12.5 mM N-ethylmaleimide to the reaction mixture, suggesting that the catalytic site of the Ycpl protein contains an active sulfhydryl group.

Because Ycplp was isolated as a calcium- and phospholipid-binding protein, a series of experiments was undertaken to determine whether either of these two components could modify the observed hydrolytic activity. Kinetic studies indicated that when Arg-N-Mec was the substrate, Ycplp exhibited $V_{max} = 2.56 \mu mol/mg/h$ and a $K_m = 12.8 \mu M$, in close agreement with those values reported for rabbit pulmonary BLM hydrolase ($V_{max} = 1.9 \mu mol/mg/h, K_m = 60 \mu M$) using BLM-B$_2$ as the substrate (42). Previous studies indicated either no effect (43) or a small (30%) negative effect (44) of free calcium concentrations up to 2 mM on rabbit BLM hydrolase activity. Using Arg-N-Mec at a final concentration of 16 \mu M ($K_m$), we observed a marked reduction in the hydrolytic activity of Ycplp when 2 mg/ml phosphatidylserine vesicles were included in the assay mixture (Table II). Furthermore, this activity could be partially restored by the addition of free Ca$^{2+}$ to 1 mM. Calcium alone at this concentration exhibited only a slight inhibitory effect. These results did not appear to be due to fluorescence quenching by the additional reaction components, as neither calcium nor phospholipid alone nor in combination altered the fluorescence quantum yield of the N-Mec standard.

**In Vivo Bleomycin Hydrolase Activity.** Because BLM hydrolase has been proposed to function in protecting mammalian cells from the toxic effects of bleomycin (8), we sought to examine the bleomycin sensitivities of yeast cells altered in the expression of the *ycpl* gene. As shown in Fig. 7, cells expressing high levels of Ycplp exhibited an increased resistance to the lethal effects of bleomycin when compared to isogenic strains expressing normal levels of the protein. In contrast, *ycp1* disruption mutants displayed wild-type levels of sensitivity to the antibiotic, suggesting that in the absence of Ycplp, other proteinases may serve to complement Ycplp function.

**Table II**

| Ca$^{2+}$ (1 mM free)* | Phospholipid$^a$ | Activity $^a \times 10^8$ | \(\mu mol/ml-min\) |
|------------------------|-----------------|-----------------|-----------------|
| -                      | -               | 84.1            |                 |
| +                      | -               | 78.7            |                 |
| +                      | +               | 65.9            |                 |
| -                      | +               | 48.5            |                 |

$^a$Total Ca$^{2+} = 2.0$ mM, EGTA = 1.0 mM. $^a$Represents phosphatidylserine (60%)/phosphatidylinositol (20%) vesicles at 2 mg/ml.

*Substrate is Arg-N-Mec at 16 \mu M.

**Fig. 7. Bleomycin sensitivities of Ycp1 mutants.** Strain C7/Y6 harboring plasmid YEpDB60 (panel 1) or YEpDB60-YCP1 (panel 2) grown on SD+GAL. Strains 4053-5-2 (panel 3) and NK823-2 (panel 4) grown on YPD. Discs containing 1 mg of bleomycin each were placed in the center of the plates as described under "Results."
The bleomycins are a group of glycopeptide antitumor antibiotics isolated from the organism Streptomyces verticillus (42). Traditionally, these compounds have been used extensively in the treatment of many different human malignancies. The proposed in vivo mechanism of action of these agents involves both single- and double-stranded DNA scission, most probably resulting from the formation of a DNA-BLM-metal-oxygen quaternary complex that releases both hydroxyl and superoxide radicals (45). Much of the utility of the bleomycins as components of modern chemotherapeutic regimens derives from their apparent lack of significant bone marrow, renal, or hepatic toxicities (46). However, because some tissues exhibit increased resistance to the toxic effects of BLM (47), several investigators have attempted to isolate biochemical factors that may be responsible for altering cellular sensitivity to these agents. One such factor that has been both isolated and cloned from rabbit lungs is bleomycin hydrolase (8, 43, 44, 48). This enzyme exhibits a cysteine proteinase activity and will inactivate BLM by hydrolyzing the carboxamide bond of its B-aminoalaninamide moiety (49).

In an earlier paper we reported the isolation of several calcium-dependent phospholipid-binding proteins from the cytosol of S. cerevisiae (24). Here we present evidence suggesting that one member of this group of proteins (previously referred to as Y3, henceforth called Ycp1) is a cysteine proteinase which shares many of the characteristics of mammalian bleomycin hydrolase (42, 43).

Structurally, the Ycp1 protein exhibits over 41.5% amino acid sequence identity with bleomycin hydrolase isolated from rabbit lungs (8). With conservative substitutions this homology increases to greater than 78%. Direct comparison of a 15-residue domain surrounding the active site of bleomycin hydrolase with the corresponding region in Ycp1 indicates over 90% sequence identity. This domain also comprises the catalytic site of several other well-known thiol-dependent pro- teases such as papain and various cathepsins. Importantly, each of these proteins includes an invariant cysteine residue within its active site which is also present in Ycp1p. Outside of this domain the sequence homology between Ycp1p and human papain, cathepsin H, and cathepsin L is insignificant (50).

Functionally, both BLM hydrolase and Ycp1p exhibit similar kinetic parameters, albeit using different compounds as substrates. Comparison of the activity of human cathepsin H (31) with Ycp1p using saturating concentrations of Arg-N-Mec as the substrate indicates that the yeast protein routinely possesses 30% of the total mammalian protein activity on a per μg basis (0.16 nmol μg−1 min−1 versus 0.05 nmol μg−1 min−1), respectively. This is not unreasonable considering that during the purification of our protein, no specific steps were taken to insure the preservation of thiol-dependent enzymatic activity. At present the exact role of bleomycin hydrolase in vivo remains unknown. In mammalian tissues many well-known thiol-dependent proteinases have been shown to function as prohormone-processing enzymes in secretory tissues (9, 10, 31, 32). In S. cerevisiae, no such cysteine endoproteases have yet been reported, indicating that Ycp1p represents the first example of what may be a family of yeast cysteine proteinases. In support of this possibility, one explanation for the observed wild-type sensitivity of ycp1 mutants to the toxic effects of bleomycin could be the result of functional complementation by similar proteases within the cell. Indeed, when the fluorometric assays described here are performed on crude yeast cytosolic extracts, we routinely observe high levels of N-ethylmaleimide-sensitive activity that remain undetectable regardless of the cystosol source: wild-type or ycp1 cells (data not shown).

S. cerevisiae possesses several different and well-characterized proteolytic systems that can be categorized as either cytosolic, vacuolar, or components of the secretory pathway (12, 13). Although we do not know the exact subcellular distribution of the Ycp1 protein, its classification as a thiol proteinase should not necessarily restrict it to any one of these compartments. On the other hand, it is interesting that YCP1 should be so closely linked to KEX2, a gene whose product is a serine endoproteinase dependent on calcium for processing of the α-factor precursor polypeptide (19). If Ycp1 does function as part of a proteolytic system in yeast, then its observed calcium-dependent membrane binding behavior may serve both to localize the protein to a specific subcellular organelle, and to provide a means of regulating its enzymatic activity. Of related importance, the activity of most proteinases remains low in log-phase cultures, increasing more than 100-fold when cells approach the stationary plateau (12). If this condition reflects an alteration in the overall abundance of these proteins, then it would help to explain our biochemical observation that commercial cake preparations of baker’s yeast consistently yield the greatest amounts of the Ycp1 protein (24).

The effects of bleomycins on yeast have been recently examined with the isolation and partial characterization of several bleomycin-sensitive (bms) and resistant (bmr) mutants from S. cerevisiae (53). Although several of these combined mutants appear to involve defective DNA repair mechanisms, a few could potentially be altered in either drug uptake or metabolism. It will be interesting to determine whether any of these mutants represent defects in the YCP1 gene.

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