

Full Length Research Paper

The Lap3p aminopeptidase is not solely responsible for bleomycin resistance in *Candida albicans*

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Detoxification of the glycopeptide bleomycin is mediated by bleomycin hydrolase, a cysteine aminopeptidase identified in a variety of organisms. The opportunistic fungal pathogen *Candida albicans* is known to exhibit increased resistance to bleomycin when compared to other yeast. Presented here is the cloning of the *C. albicans* Lap3p aminopeptidase, predicted by sequence identity to be the *Candida* form of bleomycin hydrolase. *C. albicans* Lap3p is functionally capable to replace the *Saccharomyces cerevisiae* Lap3p *in vivo*. Furthermore, the *Candida* enzyme was found to function as a cysteine aminopeptidase *in vivo*. It is shown here that upon introduction into a *lap3* deletion strain of *S. cerevisiae*, the *C. albicans* Lap3p aminopeptidase does not significantly alter the response of *Saccharomyces* to bleomycin. These results suggest that *C. albicans* Lap3p does not function as the sole factor involved in bleomycin detoxification, and may require an accessory protein or co-factor in order to efficiently mediate this process in *Candida*. This study provides the first evidence of a functional description of the *C. albicans* Lap3p cysteine aminopeptidase, and provides the foundation for further mechanistic studies of the role of this protein in the cellular processes of *Candida*.

Key words: *Candida albicans*, bleomycin detoxification, Lap3p aminopeptidase, cysteine protease.

INTRODUCTION

Candida albicans is a diploid yeast that is the most common fungal pathogen of humans (Wey et al., 1988; Pfaller, 1989; Beck-Sague et al., 1993). *Candida* species typically reside as commensals in humans, and are found

in over 50% of the population (Antley and Hazen, 1988). In immunocompromised patients however, mild to aggressive opportunistic infections can manifest from an imbalance of colonization. Coincident with this increase in

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Abbreviations: BCA, Bicinchoninic acid; E-64, *trans*-Epoxy succinyl-L-leucyl-amido(4-guanidino)butane; H-Arg-AMC, H-arginine-7-amino-4-methylcoumarin 2HCl; H-Cit-AMC, H-citrulline-7-amino-4-methylcoumarin 2HBr; YEP, yeast extract/peptone; YEPD, yeast extract/peptone/glucose; YEP-Gal, yeast extract/peptone/galactose; Ura (-), synthetic culture medium lacking uracil; PMSF, phenylmethanesulfonyl fluoride; EDTA, disodium ethylenediaminetetraacetic acid; DTT, dithiothreitol; WCE, whole cell extract.

pathogenicity is the transition of *C. albicans* from an innocuous yeast form to a more problematic mold form (Antley and Hazen, 1988). The transition of *C. albicans* from budding yeast to mold is induced by specific environmental and host factors (Muhlschlegal et al., 1998; Calderone and Fonzi, 2001; Hube, 2004). Invasive fungal infections such as candidemia results in high health care costs, and a mortality rate that approaches 50% (Wey et al., 1988). Effective treatment options for candidiasis are hindered by the lack of knowledge regarding the mechanistic basis of pathogenicity. Therefore it is imperative to more comprehensively understand fundamental biological processes of *C. albicans*.

Proteolysis is known to play a vital role in cellular metabolic processes. The *Saccharomyces cerevisiae* YCP1 (*LAP3*) gene is a cysteine aminopeptidase originally isolated based on a genetic approach in which mutant cells possessing a decreased ability to hydrolyze an aminopeptidase substrate were screened (Kambouris et al., 1992; Enekel and Wolf, 1993). Subsequent work has provided evidence that Lap3p is widely distributed throughout nature, plays a role in numerous cellular processes, and has been given numerous names based on the function for which it was identified. Enekel and Wolf (1993) showed that *LAP3* was identical to *S. cerevisiae* *BLH1*, a protein showing significant sequence homology to rabbit bleomycin hydrolase. The rabbit bleomycin hydrolase is responsible for inducing resistance to the anticancer glycopeptide bleomycin through a cysteine aminopeptidase activity (Sebti et al., 1987; Sebti et al., 1989; Sebti et al., 1989). Mutants in *BLH1* (orthologous to *LAP3*) demonstrate a decreased ability to detoxify bleomycin (Enekel and Wolf, 1993). Increased bleomycin resistance is observed following introduction of the wild type *BLH1* gene in both *S. cerevisiae* (Pei et al., 1995) and mammalian cells (Wang and Ramotar, 2002). Furthermore, Xu and Johnston (1994) isolated the *GAL6* cysteine aminopeptidase (identical to *LAP3*) bound and localized to GAL4p DNA binding sites. Although *GAL6* deletion mutants are viable, Gal6p (Lap3p) is known to exert a modest negative effect on *GAL* promoter mediated transcription (Zheng et al., 1997). In humans, *BLH1* is expressed in most tissue types (Takeda et al., 1996), and functions in protection against homocysteine toxicity (Zimny et al., 2006) and progression of atopic dermatitis. A correlative association with development of astrogliosis has also been attributed to abnormalities in *BLH1* function (Montoya et al., 1998; Montoya et al., 2007). In order to provide clarity, the enzyme identified and described in the following work will be referred to as *LAP3*.

To date, the *C. albicans* Lap3p remains uncharacterized, and its function is largely inferred from sequence homology to Lap3p found in other organisms. *C. albicans* *LAP3* is a gene that is positively regulated by

Sfu1p, a transcription factor that upregulates gene expression in iron starvation conditions (Lan et al., 2004). Based on the distribution pattern of Lap3p throughout nature, as well as the varied functions attributed to this protein, we sought to identify *LAP3* in *C. albicans*. Enzyme assays utilizing specific inhibitors demonstrated the presence of cysteine aminopeptidase activity in *C. albicans*. Using the genome sequence present in the Candida Genome Database (CGD), we have amplified the putative *C. albicans* *LAP3* gene and expressed it in a *S. cerevisiae* strain deleted for the *LAP3* gene. Incorporation of the *Candida* *LAP3* gene resulted in no remarkable growth defects in *S. cerevisiae*. Additionally, cysteine aminopeptidase activity was restored in the transformants, suggesting that the *C. albicans* *LAP3* is capable of functionally replacing the equivalent gene in baker's yeast. Introduction of the *C. albicans* *LAP3* into *S. cerevisiae* did not increase the resistance to bleomycin, suggesting that Lap3p is not the sole factor involved in bleomycin detoxification in *Candida*. This work represents the first characterization of the *C. albicans* Lap3p aminopeptidase, and provides the basis for elucidating its role in *Candida*.

METHODOLOGY

Strains and culture conditions

The *Escherichia coli* Novablue cells (EMD Millipore, Billerica, MA) were used for plasmid amplification. Bacteria were cultured in Luria-Bertani (LB) medium supplemented with the appropriate antibiotics. *S. cerevisiae* were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were as follows: *Sc001* (BY4741; MATa Δ his3 Δ leu2 Δ met15 Δ ura3), Δ lap3 (YNL239W; MATa Δ his3 Δ leu2 Δ met15 Δ ura3 Δ lap3::KAN). *Candida albicans* strains used in this study, *Ca002* (Ca1001) and *Ca004* (MYA2876). Yeast strains in either rich medium (YEP) or complete synthetic yeast culture medium (ura -); 0.67% yeast nitrogen base without amino acids, 2 g/L yeast complete synthetic amino acid mix lacking uracil) supplemented with the appropriate carbon sources were grown at 30°C. Carbon sources glucose (D) and galactose (G) were prepared and sterilized separately from culture medium and added to a final concentration of 2% prior to use. Growth phenotypes of wild type and transformant strains were assessed by plating spot dilutions onto either rich medium or selective medium lacking uracil and supplemented with 200 μ g/ml Geneticin (G418; Life Technologies, Carlsbad, CA) in order to maintain the *lap3* deletion. Plates were incubated between 1-3 days at 30°C.

Cloning of *Saccharomyces* and *Candida* *LAP3*

The yeast Gateway destination expression vector pYES-DEST52 (Life Technologies, Carlsbad, CA) was subjected to site directed mutagenesis using the oligonucleotide 5'CCGGTCATCATCACCATCACCATGAGTAAAAACCGCTGAT CCTAGAGGGCC3' (Integrated DNA Technologies, Coralville, IA) in order to remove a PmeI restriction site from the 3' end of the multiple cloning site. Mutagenesis was performed using the Change-IT site directed mutagenesis kit from Affymetrix (Santa Clara, CA). Following disruption of the PmeI restriction site, a novel

PmeI site was integrated into the vector in order to create an orientation in which the inserted open reading frame would ultimately be flanked on its 3' end by a V5 epitope, followed by a protease cleavage site and finally a 6-Histidine purification tag. This was accomplished using the oligonucleotide 5'CTCGGTCTCGATTCTACGCGTGGTTTAAACACCGGTCATCAT CACCATCAC3' (Integrated DNA Technologies, Coralville, IA). The Tobacco Etch Virus (TEV) protease cleavage site was inserted into the PmeI digested pYES-DEST52 via ligation of the duplexed oligonucleotide 5'AAACCTCGAGGAAAACCTGTATTTTCAGGGCTCCTTCGGTTT 3' (Integrated DNA Technologies, Coralville, IA), and the resultant plasmid was renamed pFG91.

C. albicans LAP3 was amplified by PCR from *Ca002* genomic DNA, using the oligonucleotide pair 5'CACCATGGGTCCAACAC3'/5'AGCTAAAGCACCCATAGG3' (Integrated DNA Technologies, Coralville, IA). The LAP3 fragment was then ligated into the Gateway entry vector pENTR-D-TOPO (Life Technologies, Carlsbad, CA). The resultant plasmid was renamed pENTR-Ca LAP3. This plasmid possessed the *C. albicans* LAP3 gene flanked by the *attL* recombination sequences to facilitate its transfer into the pFG91 destination vector (which contains the corresponding *attR* acceptor sites).

pFG91 was then mixed with pENTR-Ca LAP3 and a recombination event was carried out between the 2 plasmids using the LR clonase recombination enzyme (Life Technologies, Carlsbad, CA). Following recombination the generated plasmid contained the *C. albicans* LAP3 gene immediately upstream and in frame with a V5 epitope, TEV cleavage site, and 6-Histidine tag. This plasmid was confirmed by sequencing (Genewiz, South Plainfield, NJ) and renamed pFG99.

Yeast transformations

The *S. cerevisiae* strain Δ *lap3* (ATCC #YNL239W), which is deleted for LAP3, was transformed with pFG99 in order to address the possibility of functional replacement of the *S. cerevisiae* LAP3 gene with the orthologous gene from *C. albicans*. Transformations were carried out by a standard protocol that utilized lithium acetate and polyethylene glycol (Ito, et al., 1983). Transformed cells were plated on selective media and allowed to grow at 25°C for four to eight days.

Whole cell extracts and enzyme assays

Fungal whole cell extracts from *Ca002*, *Sc001*, and *Sc Δ lap3*, along with strains transformed with an empty vector or pFG99 were generated as described previously (Xu and Johnston, 1994) and quantitated by absorbance at 562 nm using the BCA Protein Assay kit (Pierce, Rockford, IL). Measurement of cysteine aminopeptidase activity in whole cell extracts was performed by modification of the protocol described in Xu and Johnston (1994). Briefly, 50 μ g WCE was mixed with 25 μ l 4X assay buffer (200 mM KH₂PO₄, 200 mM NaHPO₄, pH 7.5, 8 mM ethylenediaminetetraacetic acid (EDTA), 40 mM DTT), and pre-warmed to 30°C. Sterile water was used to bring the final reaction volume to 100 μ l. The reaction was initiated upon the addition of 5 μ l of 0.5 mM H-Arg-7-amino-4-methylcoumarin hydrochloride (H-Arg-AMC; Bachem, Torrance, CA) or H-Citrulline-7-amino-4-methylcoumarin hydrobromic acid (H-Cit-AMC; Bachem, Torrance, CA). Reactions were performed in triplicate and allowed to progress at 30°C for 2 h.

Fluorescence of liberated AMC corresponded to aminopeptidase activity and was measured in a BioTek Synergy2 microplate reader, using excitation wavelength of 340 nm and emission wavelength of 465 nm. Specific protease inhibitors (Enzo Biosciences,

Farmingdale, NY) were incubated with WCE for 15 min prior to the start of the reactions.

Bleomycin detoxification

To assess detoxification of bleomycin by different strains, cells were grown in the appropriate medium to mid logarithmic phase, pelleted and washed in cold sterile water. Ten-fold dilutions of each strain were spotted on culture plates containing 1, 2.5 or 5 μ g/ml bleomycin (Bleocin; EMD Millipore, Billerica, MA). Plates lacking bleomycin were used as positive controls for growth. Plates were incubated at 30°C for 24-72 h (depending on colony density) and bleomycin detoxification was scored by visual inspection of the colony survival following incubation.

Additionally, sensitivity to bleomycin was measured in liquid cultures. Cells in early logarithmic phase were washed in cold water and resuspended in an equal volume of medium supplemented with 1, 2.5, or 5 μ g/ml bleomycin (Bleocin; EMD Millipore, Billerica, MA). Cells were induced with 2% galactose containing medium for 2 h, and treated with the indicated concentrations of bleomycin for 2 h. Cells were plated on selective medium, ura (-), as previously described, with the exception that glucose was the sole carbon source. Plates were scored for survivors after 2 days of growth at 30°C.

Western blot analysis

A *S. cerevisiae* Δ *lap3* strain harboring the *C. albicans* LAP3 overexpression plasmid pFG99 was used to visualize the expression pattern of Lap3p. Cells were grown to early log phase and either induced in ura (-) selective medium supplemented with 2% galactose or left uninduced in ura (-) medium with 2% glucose. Alternatively, YEP supplemented with either 2% glucose (uninduced) or 2% galactose (induced) was also used to address Lap3p protein expression.

Ten μ g whole cell extract taken from each growth condition was run on 10% Tricine SDS-polyacrylamide gels and transferred to nitrocellulose (BioRad, Hercules, CA) using submerged blotting apparatus (Ideia Scientific). *C. albicans* Lap3 was visualized by a standard immunoblot method using a mouse antibody generated against the V5 epitope (Sigma-Aldrich, St. Louis, MO).

RESULTS

C. albicans LAP3 functionally replaces LAP3 in *S. cerevisiae*

Sequence analysis of the *C. albicans* genome predicted an open reading frame encoding a putative aminopeptidase possessing significant sequence homology to the *S. cerevisiae* LAP3 gene (Candida Genome Database). The corresponding gene product, however has remained uncharacterized.

Using a SIM (Swiss Institute of Bioinformatics) amino acid sequence alignment, it was discovered that there is a 41% sequence identity (over 87% of the protein) with the *S. cerevisiae* Lap3p aminopeptidase (Figure 1). Of note is that essential active site amino acids found in *S. cerevisiae*, in particular the active site triad of Cysteine 73, Histidine 369 and Asparagine 392 (Joshua-Tor et al., 1995) are highly conserved in the *Candida* protein.

Ca Lap3p	64	LSKWEDDFKSQTKNLLAQNALAKNAIVDVIAKNSVGKQSLKDRYLFNITVDTIGSPAHLN
Sc Lap3p	10	INSWNKEFQSDLTHQLATTVL-KNYNADDALLNKTRLQK-QDNRVFNFTVSTDSTP--VT
		* * * * * * * * * * * * * *
Ca Lap3p	124	NQKSSGR CWIF ASSNVLRTHVIKKNYNLKEDDFQLSQSYLYFYDKLEKANFFLENIEDTSS
Sc Lap3p	66	NQKSSGR CWLFA ATNQLRLNLVLSLELNKE--FELSQAYLFFYDKLEKANYFLDQIVSSAD
		***** * * * * * * * * * * * * * * * * * *
Ca Lap3p	184	EDLDSRLISYLFSSNPVNDGGQWDMIVNLVKNYGVVNEVFPDNAQSTNSS-KLNYVVTEK
Sc Lap3p	124	QDIDSRLVQYLLAAPTEDGGQYSMFLNLVKKYGLIPKDLYGDLPTYSTTASRKWNSLLTTK
		* *
Ca Lap3p	243	LREYGLKLRSLIA-KDAPKNVISSFKASAIAIKSIYKTIALALGTPP-KPTDEFLEWFEIDKD
Sc Lap3p	184	LREFAETLRTALKERSADDSIIIVTLREQMREIFRLMSLFMDIPPVQPNEQFTWEYVDKD
		*** ** * * * * * * * * * * * * * * * * * *
Ca Lap3p	301	GKYKSFKTNPLDFYKTHVRFDASEHFSLIHDPNEYNKLYTVERLNNIFGGKPIEYINLE
Sc Lap3p	244	KKIHTIKSTPLEFASKYAKLDPSTPVSLINDPRHPYGKLIKIDRLGNVLGGDAVIYLNVD
		* *
Ca Lap3p	361	IDEIKQVAIKMLKDNEPVFFGSDVGKFSKSGILDTTAYDYSTAFDFSLDITKSQRLKV
Sc Lap3p	304	NETLSKLVVKRLQNNKAVFFGSHTPKFMDKKTGVMDIELWNYP-AIGYNLPQQKASRIRY
		* *
Ca Lap3p	421	GSSQ MT HAM VITGVHIDPQTNKPVRWKIE NS WG EDSG QKGWFMMTDEWFDEYVFQIVTNK
Sc Lap3p	363	HESL MT HAM LIITGCHVDETSKLPRLRYR VEN SWG KDSG KDGLYVMTQKYFEEYCFQIVVDI
		* *
Ca Lap3p	481	KYSGKKAYDIWKS--KEFNTPPYDPMGALA
Sc Lap3p	423	NELPKELASKFTSGKEEPIVLPWIWDPMGALA
		* *

Figure 1. Amino acid sequence analysis of *Candida albicans* Lap3p. Comparison of the *S. cerevisiae* and *C. albicans* Lap3p amino acid sequences was carried out using the SIM amino acid sequence alignment program (Swiss Institute of Bioinformatics). Data shown represents 87% of the *C. albicans* Lap3p amino acid sequence in which there is 41% identity to the well-characterized *S. cerevisiae* Lap3p sequence. Amino acids denoted with asterisks (*) indicate highly conserved amino acids between the 2 proteins. Enlarged amino acids are those found in the active site triad of the *S. cerevisiae* protein, and represent those amino acids necessary for peptidase activity. Note the highly conserved nature of the *Candida* amino acid sequence at these positions, as well as in the regions surrounding the active site triad (in bold).

Therefore, sequence analysis strongly suggests that the *Candida* Lap3p is orthologous to the *Saccharomyces* Lap3 protein.

To address its cellular functions in fungi, the *C. albicans* *LAP3* gene was cloned into a Gateway Destination (Life Technologies, Carlsbad, CA) yeast expression vector. The resultant inducible expression plasmid (pFG99) was introduced into a *Saccharomyces*

strain deleted for *LAP3* (Δ *lap3*), and verified for protein production by western blot analysis (Figure 2C). Transformation of pFG99 (*CaLAP3*) into Δ *lap3* demonstrated no measurable effects on yeast growth on a rich culture medium (Figure 2A). Similar results were obtained when transformants were selected on a uracil deficient culture medium, which was used to ensure expression of the *C. albicans* *LAP3* gene present on

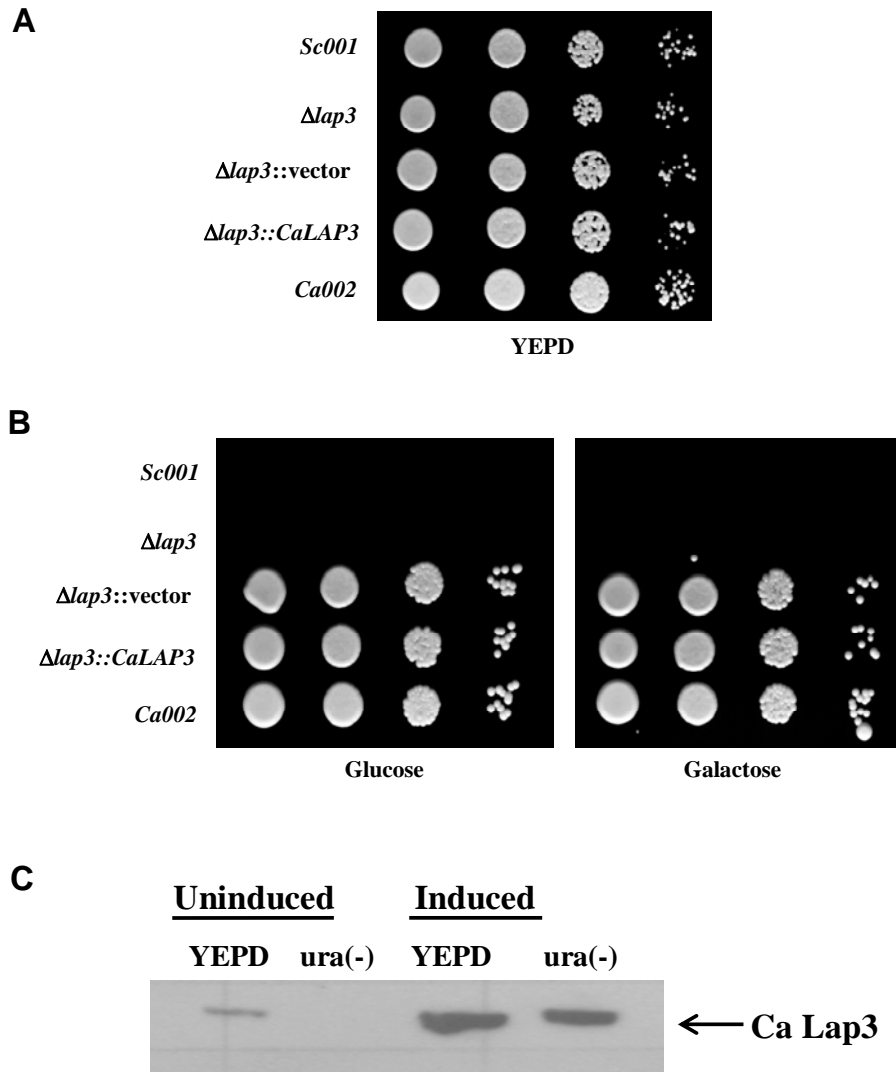


Figure 2. *Candida albicans* LAP3 serves as a functional equivalent of the *Saccharomyces cerevisiae* LAP3 gene. *S. cerevisiae* deleted for the LAP3 ($\Delta lap3$) gene were transformed with either empty vector or an expression vector containing *C. albicans* LAP3. Following selection of transformants, cells were spotted in 10-fold serial dilutions on rich medium (A), or synthetic medium lacking uracil (B) supplemented with 200 μ g/ml Geneticin. Untransformed $\Delta lap3$ cells, along with wild type *S. cerevisiae* (*Sc001*) and *C. albicans* (*Ca002*) were included on each plate. Panel B demonstrates representative data from spot dilutions on medium supplemented with either glucose or galactose. Plates were maintained at 30°C for two days prior to assessing growth patterns. (C) Western blot analysis was carried out in order to visualize the expression of *C. albicans* Lap3p in *S. cerevisiae* wild type and mutant strains. $\Delta lap3::pFG99$ cells were either grown for 2 h in uninducing conditions (2% glucose) or induced by washing the cell pellet and resuspending the cells in culture medium supplemented with 2% galactose. WCE generated from each cell pellet were used as the input in SDS polyacrylamide gel electrophoresis. Following transfer to nitrocellulose, the expression of *C. albicans* Lap3p in *S. cerevisiae* was observed using an anti-V5 epitope antibody (Sigma-Aldrich, St. Louis, MO).

pFG99 (Figure 2B). Additionally, upon overexpression of the *CaLAP3* (Figure 2B, galactose), growth patterns of the transformed strains appeared to be unaffected.

Lap3p orthologs are found in a number of eukaryotes (Sebti et al., 1987; Ferrando et al., 1996; Takeda et al., 1996; Schwartz et al., 1999) and are known to exhibit

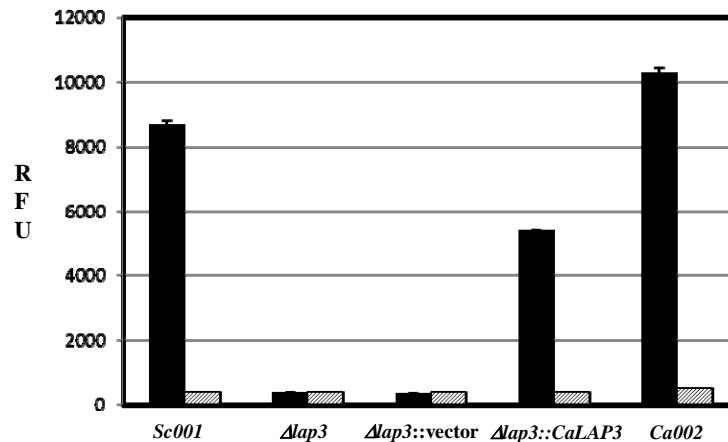


Figure 3. Enzyme activity of *C. albicans* Lap3p in *S. cerevisiae*. Cleavage of a fluorogenic cysteine aminopeptidase substrate was measured by incubating H-Arginine-AMC with 50 μg whole cell extracts from Sc001, Ca002, Δlap3 and Δlap3::CaLAP3 strains. The yeast strain Δlap3::vector was used as a control against transformation induced activation of peptidase activity in the lap3 deletion strain. Cross-hatched bars represent WCE that were treated with the cysteine protease specific inhibitor E-64 for 5 min prior to initiation of the reaction. Reactions were conducted at 30°C for 2 h. Experiments were repeated 3 times, and results reflect the specific hydrolysis of the fluorogenic substrate in relative fluorescence units (RFU).

aminopeptidase activity against the fluorogenic substrate H-Arg-AMC, and to a lesser extent H-Citrulline-AMC. As demonstrated in Figure 3, a whole cell extract from a *S. cerevisiae* strain containing the *Candida* LAP3 gene as the only source of the Lap3p aminopeptidase exhibited activity against H-Arg-AMC comparable to the activity of wild type *Saccharomyces* and *Candida* strains. Importantly, transformation of the Δlap3 strain with the empty vector did not significantly affect aminopeptidase activity against H-Arg-AMC. Incubation of the yeast whole cell extracts with the cysteine protease specific inhibitor E-64 resulted in significant decrease of Lap3p activity in whole cell extracts. Similar results were obtained using the H-Cit-AMC substrate (data not shown), suggesting that the *Candida* Lap3p enzyme possesses general aminopeptidase properties similar to other characterized Lap3p orthologs.

***Candida* Lap3p functions as a member of the cysteine aminopeptidase family**

In order to further characterize the *Candida* Lap3p aminopeptidase, a series of experiments were conducted in which inhibitors to different proteases were incubated with the *Candida* LAP3-expressing *S. cerevisiae* strain. As evidenced in Table 1, use of cysteine protease inhibitors either eliminated (E-64) or greatly reduced (leupeptin) detectable Lap3p activity. Interestingly, inhibitors directed against other types of proteases

demonstrated little to no effect against *C. albicans* Lap3p. More specifically, incubation of whole cell extracts with bestatin (inhibitor of metalloproteases and leucine aminopeptidases), pepstatin (inhibitor of aspartyl proteases) and PMSF (inhibitor of serine proteases) had little effect on *C. albicans* Lap3p activity. Additionally, extracts incubated with EDTA or MgCl₂ (factors involved in metalloprotease activity) are not affected in their Lap3p activity. Taken together, the results in Table 1 further support the hypothesis that Lap3p functions as a cysteine aminopeptidase.

Increased bleomycin sensitivity in *Candida* is not solely attributed to Lap3p function

In studying the effects of DNA repair components in *C. albicans*, Garcia-Prieto, et al. (2010) demonstrated that *Candida* is more sensitive than *Saccharomyces* to the anticancer glycopeptide bleomycin. In that study it was hypothesized that there may be a previously uncharacterized bleomycin hydrolase activity present in *C. albicans*. The Lap3p aminopeptidase is thought to be solely responsible for bleomycin detoxification in *S. cerevisiae* (Pei, et al., 1995) and mammalian cells (Wang and Ramotar, 2002), which prompted a test of whether the *C. albicans* Lap3p was functionally distinct in such a way that it would impart increased bleomycin resistance in a Δlap3 *S. cerevisiae* strain. To address this possibility,

Table 1. Effects of protease inhibitors on *Candida albicans* Lap3p activity.

Inhibitor	Final Concentration	Activity (%)	
		H-Arg-AMC	H-Cit-AMC
None	-	100	100
E-64	10 μ M	0	0
Leupeptin	10 μ M	5	9
Bestatin	10 μ M	81	87
Pepstatin	1 μ M	73	72
PMSF	1 mM	78	72
EDTA	2 mM	103	120
DTT	2 mM	109	108
MgCl ₂	5 mM	101	107

Effects of protease inhibitors on *Candida albicans* Lap3p activity. Characterization of the *C. albicans* Lap3p was performed by mixing 50 μ g WCE per reaction with 4X assay buffer (200 mM KH₂PO₄, 200 mM NaHPO₄, pH 7.5, 8 mM EDTA, 40 mM DTT), and pre-warmed to 30°C. Sterile water was used to bring the final reaction volume to 100 μ l. Specific protease inhibitors were incubated with WCE for 15 min prior to the start of the reactions and the reactions were initiated by addition of H-Arg-7-amino-4-methylcoumarin hydrochloride (H-Arg-AMC) or H-citrulline-7-amino-4-methylcoumarin hydrobromic acid (H-Cit-AMC). Reactions progressed at 30°C for 2 h. Fluorescence of liberated AMC corresponded to aminopeptidase activity and was measured by excitation at 340 nm and emission at 465 nm. 100% activity corresponds to cleavage of 1 nM substrate/min.

the yeast strains described above were used in spot dilution test on rich medium (YEPD) plates containing 1, 2.5 or 5 μ g/ml bleomycin. As shown in Figure 4A (top left box), YEPD plates lacking bleomycin provided evidence that the strains do not exhibit noticeably different growth rates in this type of assay. Culture plates containing either 2.5 or 5 μ g/ml bleomycin (Figure 4A, bottom panels) showed the varied response to bleomycin by *S. cerevisiae* and *C. albicans*. In these conditions, *C. albicans* retained robust growth characteristics, but the growth of *S. cerevisiae* was inhibited by the presence of bleomycin to the extent that no detectable colonies were present. Each of the *S. cerevisiae* strains, whether *LAP3* was present or not, demonstrated the ability to grow in plates containing 1 μ g/ml bleomycin (Figure 4A, top right panel).

Interestingly, introduction of the *C. albicans* *LAP3* into the Δ *lap3* strain did not impart a significant difference in resistance to bleomycin by *S. cerevisiae*. Expression of *C. albicans* Lap3p derived from the episomal expression is “leaky” on a glucose containing culture medium, as evidenced by western blot analysis (Figure 2C), but may not have reached a critical amount to exert any effect on bleomycin detoxification.

To address the possibility that there is simply not a sufficient quantity of recombinant *C. albicans* Lap3p produced in *S. cerevisiae* to adequately detoxify bleomycin, culture plates lacking uracil (supplemented with galactose as the sole carbon source) as the sole carbon source were used in a spot dilution assay. Under these conditions, overexpression of the *C. albicans* Lap3p is observed (Figure 2C).

In Figure 4B (top left panel), no apparent difference in growth pattern was obvious when comparing the *S. cerevisiae* and *C. albicans* strains. It should be noted that the lack of growth in the *Sc001* and Δ *lap3* lanes was expected due to the lack of a *URA3* producing plasmid in each of those uracil deficient strains.

As in Figure 4A, *S. cerevisiae* strains were incapable of growth in 2.5 or 5 μ g/ml bleomycin, regardless of *C. albicans* *LAP3* presence. Additionally, overexpression of recombinant *C. albicans* *LAP3* did not increase resistance to 1 μ g/ml bleomycin in *S. cerevisiae*, suggesting that *C. albicans* Lap3p is not the sole factor involved in bleomycin detoxification in *Candida*.

Based on the collection of evidence suggesting that bleomycin resistance *in vivo* is significantly increased following introduction of recombinant Lap3p (Pei et al., 1995; Wang and Ramotar, 2002), a liquid culture bleomycin resistance assay was employed.

Following exposure to 1, 2.5, or 5 μ g/ml bleomycin in liquid culture, yeast cells were plated on bleomycin-free agar plates. In this experimental design, a measure of yeast colony forming units following bleomycin exposure was determined.

As shown in Figure 5, wild type *C. albicans* exhibited ability to grow in up to 5 μ g/ml bleomycin. In stark contrast, growth of each of the *S. cerevisiae* strains was severely impaired. Surprisingly, presence of either *S. cerevisiae* Lap3p (*Sc001*) or recombinant *C. albicans* Lap3p (Δ *lap3::CaLAP3*) resulted in no detectable increase in bleomycin resistance when compared to strains deleted for the *LAP3* open reading frame. Even at the lower bleomycin concentrations (1 μ g/ml), no signi-

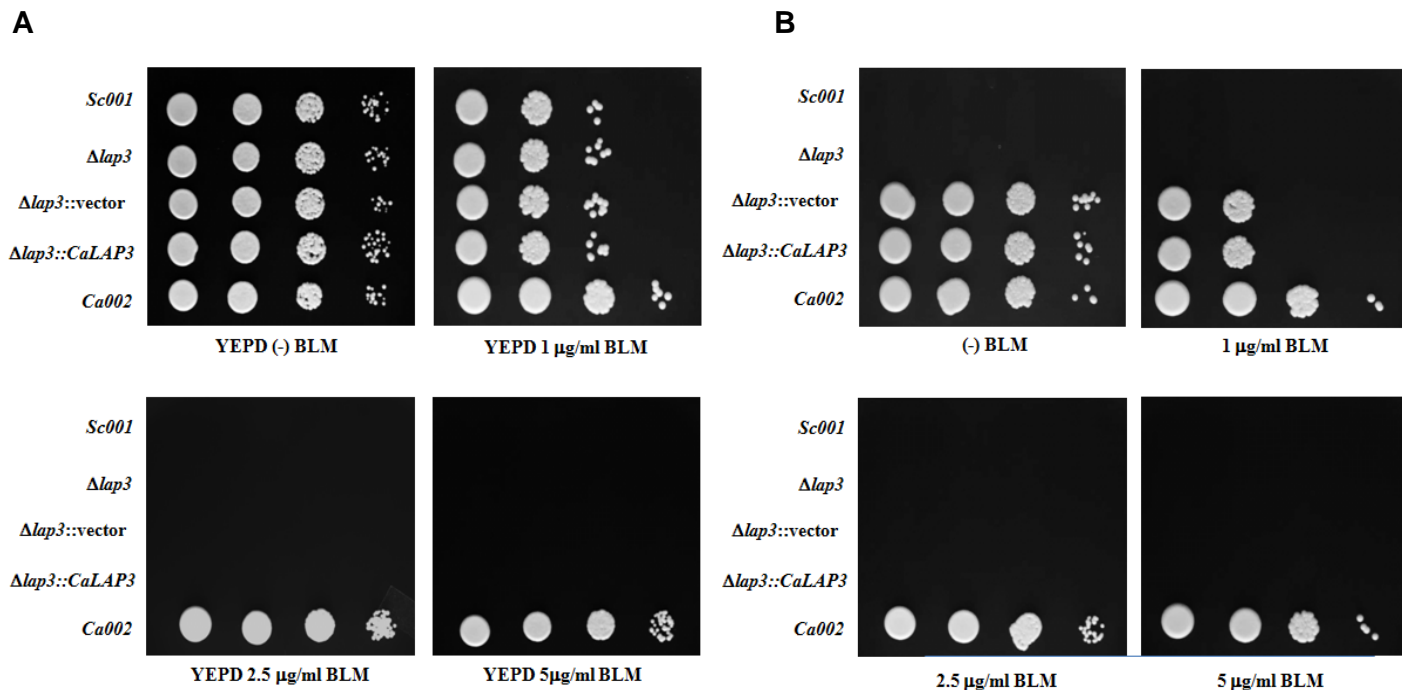


Figure 4. Sensitivity of *C. albicans* and mutant *S. cerevisiae* to bleomycin. Serial dilution assays were used to assess the relative sensitivity to bleomycin in *S. cerevisiae* cells expressing the *C. albicans* *LAP3* gene. Cells were spotted in 10-fold serial dilutions on rich medium (YEPD-Panel A) or synthetic medium lacking uracil (Panel B) supplemented with 2% galactose, 200 µg/ml Geneticin and either 1, 2.5 or 5 µg/ml of bleomycin in the molten agar. Untransformed $\Delta lap3$ cells, along with wild type *S. cerevisiae* (*Sc001*) and *C. albicans* (*Ca002*) were included on each plate. Plates were incubated at 30°C for 72 h. The data is representative of three independent assays.

ficant difference in growth patterns of the *S. cerevisiae* strains was observed. Taken together with the results shown in Figure 4, we conclude that the *C. albicans* *Lap3p* is not solely responsible for imparting bleomycin resistance in all fungal systems.

DISCUSSION

We have described the first experimental evidence regarding the cloning and functional analysis of the *Candida albicans* *Lap3p* aminopeptidase. The gene that encodes this protein (*LAP3*) is orthologous to the *S. cerevisiae* *LAP3* and mammalian *BLH1* genes, which encode a cysteine aminopeptidase that functions in detoxification of bleomycin *in vivo*. Utilizing a *S. cerevisiae* *lap3* deletion strain, we have provided evidence that *Candida* *Lap3p* aminopeptidase is capable of functionally replacing the proteolytic activity of the *Saccharomyces* *Lap3p*.

Enzyme inhibitor profile studies verified that *C. albicans* *Lap3p* does indeed function as a cysteine aminopeptidase. Introduction of the *Candida* *Lap3p* demonstrated no significant negative effects on growth of the organism. We have used *in vivo* cell survival assays to demonstrate the increased bleomycin resistance properties of *C.*

albicans when compared to *S. cerevisiae*. Furthermore, introduction of the *Candida* *Lap3p* did not significantly influence bleomycin resistance in *S. cerevisiae*.

The *Streptomyces verticillus*-derived glycopeptide bleomycin has long been used as an anticancer chemotherapeutic (Lazo, 1999). Shortly after the discovery of bleomycin-resistant cell lines (Lazo et al., 1989) the rabbit bleomycin hydrolase (*BLH1*) protein was identified as the responsible protein in this response (Sebti and Lazo, 1987). Identification of an orthologous protein in *S. cerevisiae* (*YCP1/BLH1/GAL6/LAP3*) prompted further genetic studies, but has yielded reports that differ in describing the response of *lap3*-deletion strains when compared to wild type strains. Upon deletion of the *BLH1* (Enekel and Wolf, 1993) or *GAL6* (Xu and Johnston, 1994) gene, cells exhibited hypersensitivity to bleomycin.

It has also been reported that strains deleted for *yBLH1* exhibited an equivalent level of bleomycin resistance as did wild type strains (Kambouris et al., 1992; Wang and Ramotar, 2002). Deletion of *LAP3* in *Saccharomyces* does not significantly influence the response of those cells to bleomycin. Our findings were consistent in both culture plate-based tests of resistance as well as in liquid culture, and suggest that a second protein (aside from *Lap3p*) may exist that functions in bleomycin

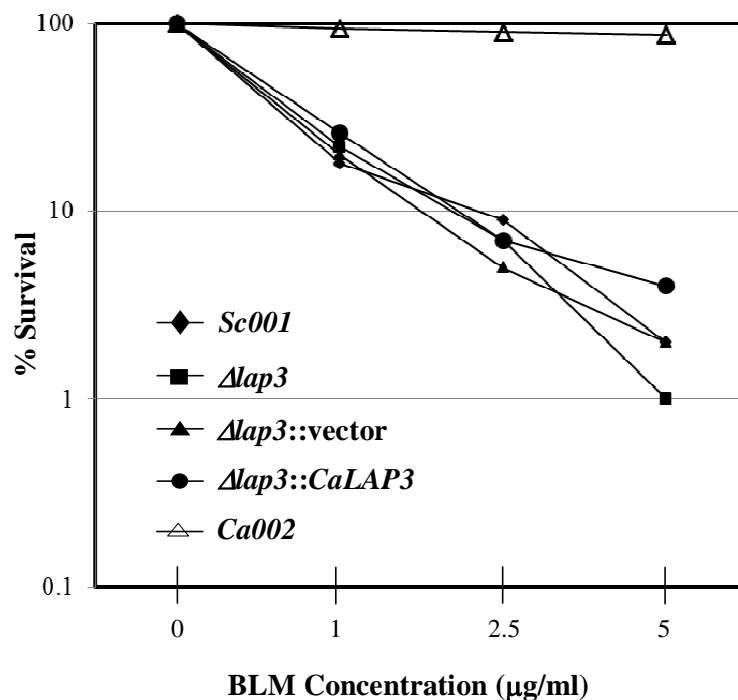


Figure 5. Cell survival of wild type and mutant yeast strains following exposure to bleomycin. Cells from the *Sc001*, *Ca002*, $\Delta lap3$, $\Delta lap3::vector$ and $\Delta lap3::CaLAP3$ strains were grown to mid log phase in synthetic medium lacking uracil and supplemented with 2% glucose. Cell pellets were washed in cold water and induced using 2% galactose for 2 h, and resuspended in with 1, 2.5 or 5 $\mu\text{g/ml}$ of bleomycin for 2 h. Two hundred μl from each condition were plated on culture medium lacking uracil and supplemented with 2% glucose. Plates were scored for survivors after 2 days of growth at 30°C.

detoxification in baker's yeast. Although a second bleomycin hydrolase has not been identified in yeast, the Lap aminopeptidases represent a family of 4 aminopeptidases that were isolated from the same mutant screen (Trumbly and Bradley, 1983). Analysis of the Lap aminopeptidase family revealed that activity of Lap1p, 2p and 4p greatly decreases upon introduction of EDTA to the assay system. Reactivation aminopeptidase activity was observed following addition of various cations, suggesting that these 3 Lap enzymes function as metallopeptidases. Interestingly, Lap3p is not classified as a metallopeptidase and possesses very little amino acid similarity to the 3 other members of the Lap family, suggesting that it is unlikely that members of the Lap family other than Lap3p function in detoxification of bleomycin. Through the use of active site Gal6p mutants, Zheng and Johnston (2008) demonstrated that hydrolysis of bleomycin was linked to the cysteine aminopeptidase activity of the protein both *in vivo* and *in vitro*. Evidence has been presented that describes changes in the Gal6p/Lap3p substrate specificity based on the carboxy-terminus of the enzyme (Zheng et al., 1998). It is possible

that functional redundancy with regard to bleomycin resistance can exist with another cysteine aminopeptidase.

Conflicting reports also exist regarding the effect of Lap3p expression on bleomycin detoxification. Cells expressing high levels of Ycp1p demonstrate increased resistance to bleomycin than do those expressing normal levels of the protein (Kambouris et al., 1992). Additionally, introduction of yeast Ycp1p resulted in an increase in bleomycin resistance of mammalian cells (Pei et al., 1995).

Wang and Ramotar (2002), however presented data to show that addition of Bih1p imparts no additional resistance to bleomycin *in vivo*. This outcome was obtained when bleomycin hydrolase was either expressed at normal levels or over-expressed. Data shown in Figures 4 and 5 demonstrate that over-expression of *C. albicans* Lap3p in a *S. cerevisiae* *lap3* deletion strain does not significantly increase the bleomycin resistance properties of *Saccharomyces*. The *C. albicans* protein may require a secondary factor not present in *Saccharomyces* in order to exert its bleomycin

detoxification effects. Alternatively, a possibility may arise in which *C. albicans* cells exhibit resistance to killing by bleomycin by physically excluding the drug from cells.

Jayaguru and Raghunathan (2007) used bleomycin-treated cells to investigate the possibility of targeting the splicing machinery of *C. albicans* as a method by which to design new antifungals. Additionally, in studies of *Candida* DNA repair processes; it was found that bleomycin was capable of killing *C. albicans* cells at concentrations greater than 5 µg/ml (Garcia-Prieto et al., 2010). We have also found that concentrations at or over 10 µg/ml bleomycin serve to induce cell death of *C. albicans* (data not shown). Taken together, it would seem unlikely that *C. albicans* exhibits greater resistance to bleomycin simply by excluding it from the cell.

Finally, the ability of the *C. albicans* Lap3p to bind DNA may be a key determinant in its activity against bleomycin. The Gal6p was originally identified bound to the *GAL1/10* promoter region (Xu and Johnston, 1994), and Zheng and Johnston (1998) demonstrated that *S. cerevisiae* strains housing a DNA binding mutant Gal6p were decreased in their capacity to neutralize bleomycin. The DNA binding properties of the *C. albicans* protein have yet to be determined.

Although the *Candida* Lap3p is predicted to have an overall more negative charge than the *Saccharomyces* enzyme (CGD), this does not appear to be the sole determinant to nucleic acid binding by Lap3p-orthologous proteins. As an example, although the rat bleomycin hydrolase enzyme is able to bind single stranded Gal4 binding site (Takeda et al., 1996), this enzyme does not associate with DNA cellulose.

In this study it was proposed by the authors that there may be an accessory factor required for localization onto DNA. In *Escherichia coli*, the LexA protein is also known to be a DNA binding protease, but its association with DNA demonstrates an absolute requirement for the co-factor RecA (Kim and Little, 1993). In either case, we believe it is imperative to fully characterize the nucleic acid binding properties of *Candida* Lap3p to further understand the implications of this activity in fungal cellular processes.

Numerous cellular functions other than bleomycin detoxification have been described for yeast Lap3p and its orthologs (Koldamova et al., 1998; Montoya et al., 1998; Zimny et al., 2006; Montoya et al., 2007; Kamata et al., 2011) Furthermore, cysteine proteases throughout nature have demonstrated a role in a large variety of processes, ranging from signaling pathways in many organisms to programmed cell death (Degterev et al., 2003). Our results presented here raise further questions regarding the nature of the function of the *Candida* protein. Our evidence presented here provides the first evidence of cloning and functional characterization of the *C. albicans* Lap3p, and opens the door for dissecting its function(s) in *Candida* physiology.

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