System for Expression of Microsporidian Methionine Amino Peptidase Type 2 (MetAP2) in the Yeast Saccharomyces cerevisiae

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Microsporidia are parasitic protists of all classes of vertebrates and most invertebrates. They recently emerged as important infections in various immunosuppressed and immunocompetent patient populations. They are also important veterinary and agricultural pathogens. Current therapies for microsporidiosis include benznidazoles, which bind tubulin-inhibiting microtubule assembly, and fumagillin and its derivatives, which bind and inhibit methionine amino peptidase type 2 (MetAP2). Benznidazoles are not active against Encephalitozoon bieneusi, the most common cause of human microsporidiosis. Fumagillin is active against most microsporidia, including E. bieneusi, but thrombocytopenia has been a problem in clinical trials. There is a pressing need for more-specific microsporidian MetAP2 inhibitors. To expedite and facilitate the discovery of safe and effective MetAP2 inhibitors, we have engineered Saccharomyces cerevisiae to be dependent on Encephalitozoon cuniculi MetAP2 (EcMetAP2) for its growth, where EcMetAP2 is harbored on an episomal uracil-selectable tetracycline-regulated plasmid. We have also constructed a leucine-selectable tetracycline-regulated expression plasmid into which any MetAP2 gene can be cloned. By utilizing a 5-fluoroorotic acid-mediated plasmid shuffle in the EcMetAP2 yeast strain, a yeast strain can be generated whose growth is dependent on MetAP2 from any organism. The level of heterologous MetAP2 gene expression can be controlled by the addition of tetracycline to the growth medium. These yeast strains should permit high-throughput screening for the identification of new inhibitors with high specificity and activity toward microsporidian MetAP2.

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Microsporidia are “emerging” human and veterinary pathogens. These protists belong to the phylum Microsporidia, which are obligate intracellular parasites. They are ubiquitous in nature, having been described as parasites of all classes of vertebrates and most invertebrates (8, 33). They have been reported to infect mainly their host’s digestive tract; however, infections have also been reported in reproductive, muscle, excretory, and nervous systems (4, 7, 10, 11, 30, 31). Microsporidia have been identified in water sources as well as in wild, domestic, and farm animals. This has raised concerns for waterborne, food-borne, and zoonotic transmissions of these infections. At least 10 species have been etiologic in several disease syndromes in humans (32). These pathogens have presented as opportunistic infections in immunodeficient hosts, such as patients with AIDS and/or organ transplantation, but also infect immunocompetent hosts (9). The two most commonly used drugs for treating microsporidiosis in animals and humans are albendazole and fumagillin (6). Albendazole is a benzimidazole that inhibits tubulin polymerization. Even though it is effective against many microsporidia, including Encephalitozoon spp., which infect mammals, including humans, it is only variably effective against Encephalitozoon bieneusi, the most common cause of human microsporidiosis.

Fumagillin is a sesquiterpene dipeoxide produced by the fungus Aspergillus fumigatus and is highly effective when administered systemically to humans but has caused neutropenia and thrombocytopenia in some patients (24). TNP-470 is a semisynthetic analogue of fumagillin that appears to be less toxic but has been reported to exhibit side effects in wound healing and female reproduction (15). Clinical trials with TNP-470 as an angiogenesis inhibitor suggest that resistance to TNP-470 can occur (2).

Ovalicin (5), fumagillin, and derivatives such as TNP-470 (23), cis-fumagillin (21), and fumagalone (35) specifically and covalently bind to a target enzyme identified as methionine amino peptidase type 2 (MetAP2 [Map2 in yeast]) and specifically inhibit its peptidase activity. MetAP2 catalyzes an essential posttranslational modification of nascent proteins by cleaving the initiator methionine and thus is a major component of the N-terminal methionine excision pathway (15). N-terminal methionine excision has been found to be an essential universal process conserved from eubacteria to higher eukaryotes. MetAP2 genes are essential genes in all prokaryotes since MetAP2 deletion is lethal. There are two major forms of MetAPs, designated type 1 and type 2 (1, 22). Although not an absolute rule, prokaryotes, which include eubacteria and archaeabacteria, will typically express the MetAP1 isoform, while eukaryotes express both type 1 and type 2 isoforms. Additionally, there are reports of other forms of MetAPs (16, 27). MetAP2 gained a lot of attention recently as an excellent therapeutic target not only in treatment of various parasite infections but also in anticancer therapy (26). Data suggest that the cellular target for fumagillin and its analogs in microsporidia is a microsporidian MetAP2 homologue and that this is an essential enzyme for these organisms (34).

Fumagillin and its analogs do not bind or inhibit the activity of MetAP1 or other amino peptidases. Saccharomyces cerevisiae cells deficient in MetAP1 (Δmap1) are killed by ovalicin,
but yeast cells deficient in MetAP2 (Δmap2) are not (17, 29). Deletion of both MetAP1 and MetAP2 is lethal in yeast (17, 34). These data confirm that fumagillin selectively targets MetAP2 and not MetAP1. The genome of the microsporidian Encephalitozoon cuniculi lacks MetAP1 (19, 25); therefore, in microsporidia, inhibition of MetAP2 by fumagillin most likely results in cell death analogous to the situation in Δmap1 yeast. This indicates that microsporidian MetAP2 is a physiologically relevant target for parasite drug development. Although the therapeutic potentials of MetAP2 have increasingly been recognized, there are only a few inhibitors against MetAPs reported in the literature.

Screening for potent novel inhibitors of the enzyme MetAP2 can conventionally be accomplished either by using the recombinant protein of the pathogen or by using in vitro cell culture of the parasite. The use of the recombinant enzyme is not ideal since it involves tedious heterologous expression, isolation, and purification procedures and an in vitro inhibitory assay may not reflect the true physiological conditions of the protein. In addition, since the enzyme requires metal ion for its activity, conversion of the recombinant apoenzyme to its corresponding active holoenzyme adds an additional complication. The use of an in vitro microsporidian culture for identifying the inhibitors of microsporidian MetAP2 is cumbersome and time consuming. In addition, this approach is impossible for those microsporidia which are noncultivable and for which there is no murine model of infection, such as E. bieneusi. As an alternative to the above-described methods, yeast-based screening strategies are attractive as they are simple and can be carried out in a high-throughput manner. They have successfully been used for studying drugs effective against protein targets of Apicomplexa (28) and for identifying the inhibitors of dihydrofolate reductase of Mycobacterium tuberculosis (13) and Cryptosporidium parvum (3). One of the main prerequisites required for conducting such studies is that parasite target protein needs to be able to complement its ortholog protein function in yeast.

We have reported the identification and characterization of the MetAP2 gene and its protein product from the human-pathogenic microsporidian E. cuniculi (34). In an attempt to design a simple and rapid approach to screen for novel E. cuniculi MetAP2 (EcMetAP2) inhibitors, we devised a complementary approach which is independent of recombinant protein or parasite culture and instead dependent on the expression of EcMetAP2 in the yeast S. cerevisiae.

MATERIALS AND METHODS

Strains, growth conditions, and DNA methods. All S. cerevisiae strains used and generated in the present study are derived from S288C (ATCC 201388), with the genotype MAPA his3Δ1 leu2Δ0 met15Δ0 ura3Δ0. Yeast was grown at 30°C on yeast-extract-peptone-dextrose (YPD) solid or liquid medium and synthetic complete (SC) liquid or solid medium. YPD-G418 plates contained YPD medium with 200 μg/ml of G418 (Life Technologies, Gaithersburg, MD). Escherichia coli strain Top10 and standard media and methods were used for plasmid manipulations. Yeast genomic DNA was isolated using a Yeast Star genomic DNA kit from Zymo Research (Zymo Research, Orange, CA). Plasmid DNA was isolated from E. coli and from agarose gels using QIAGEN kits (QIAGEN, Santa Clarita, CA). Oligonucleotides were purchased from Invitrogen (Carlsbad, CA). DNA for plasmid construction and yeast transformation was generated by PCR using the Expand system (Boehringer Mannheim, Indianapolis, IN) for 30 cycles.

Plasmids and vector construction. The EcMetAP2 gene was PCR amplified from a PCR1.1-TOPO vector containing the EcMetAP2 gene, (34) using primers EcMap2PmeFor (5’ CGTTTAAACAGATGAGGCTGTGAGGAGC3’ and EcMap2EagRev (5’ GAATCGGCCGTAACTTCCTCTGTCGAAC3’) (restriction sites are underlined). The 1.5-kb PCR product was ligated into the yeast expression vector pCM190 at the PmcI and EagI sites to generate the plasmid pCM190:EcMetAP2. Ligation of the PCR product was confirmed by restriction enzyme digestion and sequencing to confirm the absence of PCR-generated mutations in the vector construct. To construct plasmid pRS425Tet, plasmid pCM190 was cut with HindIII, followed by end blunting with Klenow. EcoRI treatment of this product yielded a 2,822-bp fragment containing the elements required for tetracycline-dependent transcription in yeast. This fragment was initially cloned into pRS423 at blunt-ended NotI and EcoRI sites to generate pRS423Tet. The 3.3-kb PvuII fragment from pRS423Tet was then excised and ligated into pRS425 at the PvuII site, resulting in the plasmid pRS425Tet.

Mutant EcMetAP2 was PCR amplified from the vector pVL1392:mutEcMetAP2 (34), using the same primers as described above for the amplification of the wild-type EcMetAP2 gene. A 1.1-kb PCR product was digested with PmeI and EagI and was ligated into PmeI/EagI-digested pRS425Tet. Positive clones (pRS425Tet:mutEcMetAP2) were confirmed by restriction digestion and sequencing.

Human MetAP2 was PCR amplified using a human cDNA library as the template, employing the primer set HuMap2PmeFor (CGTTTAAACAGATGAGGCTGTGAGGAGC) and HuMap2EagRev (GAATCGGCCGAATCTTCCTCTGTCGAAC) (restriction sites are underlined). The 1.5-kb PCR product was ligated at the PmcI and EagI sites of pRS425Tet, generating pRS425Tet:HuMetAP2.

PCR-mediated deletion of yeast MAP2 using a His3MX6 disruption cassette. A 1.6-kb PCR product of His3MX6 marker flanked by 60 nucleotides homologous to the region immediately upstream and downstream of the start and stop codons, respectively, of the yeast MAP2 gene locus was generated using the primers Map2HisFor (5’ GAAAGCTTAAATGCTGACTTATACCAACGGAACGTCAAACCGAAACGGGAGGAGCAGTACGTGAAGAAGCTCATTACGTAATGGAAAGGCGGAGGGCGCGGATGAGGAGGTACG) and Map2HisRev (5’ ATATAATCTACAAGATGCATTACATATACCTGAGTGAAGGCCCATTGTTAAAGGCCGCTTATTCTACATTGATCTGCTGACTG3’) and Map2HisRev (5’ ATATAATCTACAAGATGCATTACATATACCTGAGTGAAGGCCCATTGTTAAAGGCCGCTTATTCTACATTGATCTGCTGACTG3’) (in lower case are homologous to the plasmid sequence), using YPM2 plasmid (20) as the template. The PCR product was gel purified, and 5 μg of the product was used for transforming the BY4741 map1::KAN MATa yeast strain (ATCC 4005153) containing pCM190:EcMetAP2 plasmid by using the lithium acetate transformation procedure (14). Transformants were then selected on a SC-histidine (SC-His) plate.

Genomic DNA was prepared and used as the template for the diagnostic PCRs, using the primers Map2For (P1) (5’ CAAGGGACCATTTAATGACCTGGTCTC3’) and Map2Rev (P2) (5’ TGGACGACAATGGTTGAGAGAGAG5’), and Map2HisRev (P3) (5’ CTTACATCCTGCTGACTG3’) using a 1:5,000 dilution of anti-recombinant EcMetAP2 antibody overnight at 4°C. Immune complexes were visualized by chemiluminescence detection (GE Healthcare Biosciences, Piscataway, NJ).

Yeast drug assay. Yeast colonies were inoculated onto YPD liquid medium and grown at 30°C with shaking. At an A600 of 1.5, yeast cells were diluted to 0.5 absorbance units and were then 10-fold serially diluted. Using a multichannel pipettor, 3 μl of the yeast cell suspension was spotted onto solid YPD medium in the absence of any drugs or onto the plates containing either 10 μg/ml of doxycycline or different concentrations of ovalicin, fumagillin, or TNP-470. The plates were incubated at 30°C for 5 to 6 days and photographed.

RESULTS

Microsporidian (E. cuniculi) methionine aminopeptidase type 2 complements the function of yeast methionine amino peptidase type 2. An amino acid sequence alignment of yeast Map2 and EcMetAP2 proteins demonstrates a 45% identity.
and 53% similarity. EcMetAP2 was PCR amplified and cloned into a 2-μm yeast expression plasmid, pCM 190, to generate pCM190:EcoMetAP2. A Δmap1 yeast strain (ATCC 4005153) was transformed with pCM190 and pCM190:EcoMetAP2 vectors. Transformants were selected on SC-uracil (SC-Ura) plates. Independent colonies from both of the plates were inoculated into SC-Ura medium for growth. Cell lysates were subjected to immunoblot analysis to investigate whether EcoMetAP2 protein was expressed in yeast, using mouse polyclonal antibodies raised against recombinant EcoMetAP2 (34). A minimum of four colonies was examined for each transformation, and a representative figure is shown in Fig. 1. A specific signal corresponding to the predicted size of the EcoMetAP2 protein is seen in the lysates of the yeast transformed with the pCM190:EcoMetAP2 vector (Fig. 1, lane 2), and this signal is absent in lysates from yeast transformed with an empty pCM190 vector (lane 1), indicating that EcoMetAP2 expresses in yeast from the TetO-CYC1 hybrid promoter in the vector pCM190.

In order to determine whether EcoMetAP2 complements the function of Map2 in yeast, we employed a one-step PCR-mediated gene disruption of the endogenous yeast MAP2 gene in the strains harboring pCM190:EcoMetAP2. A Δmap1 Δmap2 strain (ATCC 4005154) was transformed with pCM190 and pCM190:EcoMetAP2 vectors. Transformants were selected on SC-Ura plates. Independent colonies from both of the plates were inoculated into SC-Ura medium for growth. Cell lysates were subjected to immunoblot analysis to investigate whether EcoMetAP2 protein was expressed in yeast, using mouse polyclonal antibodies raised against recombinant EcoMetAP2 (34). A minimum of four colonies was examined for each transformation, and a representative figure is shown in Fig. 1. A specific signal corresponding to the predicted size of the EcoMetAP2 protein is seen in the lysates of the yeast transformed with the pCM190:EcoMetAP2 vector (Fig. 1, lane 2), and this signal is absent in lysates from yeast transformed with an empty pCM190 vector (lane 1), indicating that EcoMetAP2 expresses in yeast from the TetO-CYC1 hybrid promoter in the vector pCM190.
The pCM190:EcMetAP2 strain to replace pCM190:map2/H9004 HuMetAP2, a plasmid reshuffle was carried out in the HuMetAP2 in yeast. With vector alone (pRS425tet), confirming the expression of protein which is absent in the lysate from the cells transformed corresponding to the predicted size of the human MetAP2 HuMetAP2. As shown in Fig. 4 there is a specific signal in lane 4).

Evaluation of 5-FOA-mediated plasmid shuffle to convert EcMetAP2-dependent yeast to a strain dependent on HuMetAP2. Since the MetAP2 gene has been reported to be a therapeutic target for a wide range of infectious agents, we wanted to extend the present system to be useful for screening novel inhibitors for MetAP2 from other organisms. To achieve this, pRS425Tet, an episomal leucine (Leu) marker-containing plasmid, was constructed with the same basic elements required for the tetracycline-dependent expression of a foreign gene as pCM190. As human MetAP2 (HuMetAP2) has been reported to complement yeast Map2 function (12), this gene was selected to test this plasmid shuffle vector. The HuMetAP2 gene was cloned into the Pmel/EagI sites of pRS425Tet to generate pRS425Tet: HuMetAP2. This plasmid was initially transformed to a smap2 yeast strain, and the transformants were selected on SC-Leu plates. After the initial colony purification, individual colony lysates were used for the immunoblot analysis employing anti-human MetAP2 antibody. The specific signal corresponding to the HuMetAP2 protein is shown by an arrow.

A point mutation in EcMetAP2 abolished its ability to complement yeast MetAP2. Site-directed mutagenesis of EcMetAP2 was performed using a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA), changing the residue Ala to Thr at position 241 in EcMetAP2 (this Ala corresponds to the Ala at position 362 in HuMetAP2) (34). Independent in vitro enzymatic assays were done using methionine-para-nitroanilide and Met-Pro-para-nitroanilide as substrates and confirmed that both of these enzyme preparations exhibited comparable in vitro enzyme activities (data not shown). The EcMetAP2-Thr 241 mutant gene was cloned into

[FIG. 4. Immunoblots demonstrating the expression of human MetAP2 from the TetO-CYC1 hybrid promoter in the vector pRS425Tet:HuMetAP2. Whole-cell lysates from Δmap1 cells which were transformed with either pRS425Tet:HuMetAP2 (lane 1) or pRS425Tet (lane 2) are shown. Fifty micrograms of total protein was loaded in each lane, and the immunoblot was probed with mouse anti-human MetAP2 antibody. The specific signal corresponding to the HuMetAP2 protein is shown by an arrow.]

[FIG. 5. Expression of mutant EcMetAP2 in yeast. Yeast cell lysates from Δmap1 cells containing pCM190:EcMetAP2 (lane 1), pRS425Tet (lane 2), and pRS425Tet:mutEcMetAP2 (lane 3) are shown. Immunoblots were probed with mouse antibody against rEcMetAP2. map2 pCM190:EcMetAP2 strain was transformed with pRS425Tet (control) or pRS425Tet:HuMetAP2 (test) plasmid, and the transformants were selected on SC-Leu plates. Leu-positive colonies were streaked after colony purification on SC-5-FOA-containing Ura plates for facilitating the ejection of Ura-containing plasmid causing the loss of EcMetAP2. There was a significant number of colonies in the plate streaked with the cells transformed with test plasmid, indicating that HuMetAP2 is the only copy of the MetAP2 gene in these cells and that it complements yeast Map2 function. There were no colonies in the plate spread with the cells transformed with control plasmid, confirming our earlier observation that in the absence of a copy of HuMetAP2, cells require EcMetAP2 activity for survival. When pRS425Tet:HuMetAP2-containing cells were spotted after a 10-fold serial dilution on YPD plates containing doxycycline, they also exhibited doxycycline sensitivity (Fig. 2), consistent with the phenotype observed earlier for EC METAP2-containing cells. This experiment demonstrates the generation of Δmap1 map2 pRS425Tet:HuMetAP2 yeast cells starting from Δmap1 map2 pCM190:EcMetAP2 and suggests that the MetAP2 gene from any organism can be used for plasmid reshuffle to generate a new yeast strain dependent on that organism’s MetAP2 (provided that the organism’s MetAP2 can complement the function of endogenous Map2 in yeast).]
pRS425Tet to generate pRS425Tet:mutEcMetAP2. A Δmap1 strain was transformed with this plasmid, the transformants were selected on SC-Leu plates, and the positive transformants were used for the immunoblot analysis with anti-rEcMetAP2. As demonstrated in Fig. 5 there was expression of both EcMetAP2 (lane 1) and mutEcMetAP2 (lane 3) in transformed cells and no expression in cells transformed with empty pRS425Tet vector (lane 2). Despite multiple attempts, we were unable to replace the pCM190:EcMetAP2 plasmid with pRS425Tet:mutEcMetAP2 following 5-FOA-mediated selection. In all of the experiments, no colonies grew in the presence of 5-FOA, suggesting that mutEcMetAP2 does not complement yeast Map2.

Valuation of a drug screening strategy for EcMetAP2 and HuMetAP2 by using the newly generated yeast strains. For validation of the use of these newly generated yeast strains as a screen for potential MetAP2 inhibitors, we used fumagillin, ovalicin, and TNP-470, which are known inhibitors of MetAP2. After pilot experiments to determine the optimum concentrations of each drug, each yeast strain was spotted after 10-fold serial dilutions on YPD agar plates containing the appropriate amount of drug and the plates were incubated at 30°C. As shown in Fig. 6 fumagillin at 1 and 5 nM inhibited MetAP2 from all three sources, i.e., yeast, *E. cuniculi*, and human; however, yeast Map2 was more resistant to fumagillin than both *E. cuniculi* MetAP2 and HuMetAP2. We have observed that higher concentrations of the drugs were required to inhibit yeast Map2, resulting in growth arrest (data not shown). Ovalicin was more effective than fumagillin. For ovalicin, 0.5 to 1 nM of the drug was inhibitory for all three MetAP2s. For TNP-470, 0.25 μM of the drug was used and all of the MetAP2s were less sensitive than to fumagillin or ovalicin.

**FIG. 6.** Validation of drug screening against *E. cuniculi* and human MetAP2s, using yeast as the surrogate system. Tenfold serial dilutions of the wild type and isogenic yeast strains containing vector alone or vectors containing either *E. cuniculi* or human MetAP2 were spotted onto YPD medium containing fumagillin (1 and 5 nM), ovalicin (0.5 and 1 nM), or TNP-470 (250 nM), the known inhibitors of MetAP2. Photographs were taken after the plates were incubated at 30°C for 4 to 5 days.

DISCUSSION

A main goal of the present study was to develop a new strategy to identify potent inhibitors of microsporidian MetAP2. To accomplish this, using yeast as a surrogate system, the primary criterion was to determine whether microsporidian MetAP2 complements Map2 function in yeast. It had already been shown that human MetAP2 complements yeast Map2 function only when overexpressed from a 2-μm episomal plasmid. When human MetAP2 was cloned into a centromere-containing yeast expression plasmid, it failed to support the viability of Δmap1 map2 yeast cells, suggesting that the amount of HuMetAP2 protein expressed from a single-copy centromere plasmid may not be sufficient to sustain yeast growth (12). Overexpression of a heterologous protein may, however, force nonspecific protein-protein interactions titrating out a specific protein from its biologically relevant association with another protein, resulting in a lethal phenotype. Systematic characterization of proteins associated with either yeast Map1 or Map2 have not been reported; however, genome-wide affinity purification-based protein interaction network studies of yeast have identified a physical interaction of Map2 with an essential DNA binding protein, Rfc3p (a subunit of heteropen-tameric replication factor C) (18). Overexpression of a heterologous MetAP2 protein may result in its increased association with Rfc3p, making this protein unavailable for DNA replication and leading to cell death and the conclusion that the heterologous protein is nonfunctional in yeast. Therefore, pCM190, a 2-μm-based yeast expression plasmid in which the expression of the foreign gene can be modulated using tetracycline derivatives, was utilized for these studies.

A Δmap1 yeast strain was obtained from the ATCC in which
genomic MAP1 is replaced by a KanMX4 cassette, rendering the cells G418 positive. The absence of the MAP1 gene in this strain was reconfirmed by diagnostic PCR using gene-specific primers. Transformation of this strain with pCM190:EcMetAP2 vector and subsequent immunoblot analysis clearly established that EcMetAP2 can be expressed in yeast. In the next step, three independent approaches were employed to ascertain the complementation of yeast Map2 function by EcMetAP2 protein. We hypothesized that if EcMetAP2 complements the function, we should be able to delete the endogenous chromosomal copy of the yeast MAP2 gene. A histidine-selectable marker cassette was used for the disruption of the yeast MAP2 gene by a one-step PCR-mediated gene deletion strategy.

Transformation of yeast cells containing either pCM190 (control plate) or pCM190:EcMetAP2 (test plate) vector resulted in the appearance of a significant number of colonies in SC-His plates, which represent cells in which the transformation module is recombined either at the target MAP2 gene locus or elsewhere in the genome. To facilitate screening for those cells in which homologous recombination has occurred at the MAP2 locus, we employed 5-FOA selection. When His- and Ura-positive transformants from the test plate are selected on plates containing 5-FOA, only those cells which possess the intact endogenous copy of yeast MAP2 will grow. Those cells in which the recombination of the histidine marker module has occurred at the genomic MAP2 locus will not be able to survive because in the presence of 5-FOA, yeast cells will lose Ura-containing plasmid which harbors the EcMetAP2 gene. Consistent with this, all of the colonies from the control plate survived in the presence of 5-FOA and a significant number of colonies from the test plate failed to grow in the presence of 5-FOA.

In the vector pCM190:EcMetAP2, the expression of EcMetAP2 is under the regulation of doxycycline. In the Δmap1 map2 yeast transformants, the only source of MAP2 activity is EcMetAP2. In these cells, if EcMetAP2 complements the function of yeast MAP2, then their growth should be modulated by doxycycline. Accordingly, we found that 5-FOA-sensitive colonies from the test plate had a clear sensitivity toward doxycycline, confirming that in these cells, the genomic yeast MAP2 locus has been replaced by a His-selectable marker cassette (Fig. 2). The final demonstration of the genomic yeast MAP2 deletion in the presence of EcMetAP2 was provided by a diagnostic PCR of yeast genomic DNA. All of these independent experimental results conclusively suggest that we have generated a Δmap1 map2 yeast strain which is dependent on EcMetAP2 for its viability. These yeast strains exhibited growth rates similar to that of their parental Δmap1 yeast strain.

Since several species of microsporidia have caused human infections, we wanted to extend our study to facilitate the generation of yeast strains whose growth is dependent on the functional expression of MetAP2 from any organism of choice. To this end, we generated the pRS425Tet vector. Using the two vectors pCM190 and pRS425Tet with Ura and Leu markers, respectively, one can generate a yeast strain containing the MetAP2 of any organism by employing a 5-FOA-mediated plasmid shuffle. Using these vectors, we demonstrated the conversion of a yeast strain dependent on EcMetAP2 to one dependent on human MetAP2 for its growth. This validates our strategy and is consistent with earlier reports of yeast MAP2 complementation by HuMetAP2.

In the case of HuMetAP2, it has been reported that a point mutation resulting in the change of a single amino acid at position 362 from Ala to Thr abolishes its sensitivity to ovalicin. This study was carried out to screen for ovalicin-resistant HuMetAP2 after random mutagenesis using a yeast system (2), and the exact mechanism of A362T-mediated resistance of HuMetAP2 to ovalicin is not clear. We wondered whether EcMetAP2 would exhibit properties similar to those of ovalicin and thus would be useful for validating ovalicin drug assays. We, therefore, mutated the Ala at 241 of EcMetAP2 to threonine and successfully expressed this mutant protein in a baculovirus system. The in vitro enzyme activity of the mutant protein was comparable to that of the wild-type protein. Despite this, mutEcMetAP2 failed to complement the function of MAP2 in yeast. This may be because this mutation interferes with biologically relevant potential protein-protein interactions in yeast. This suggests that in addition to the peptidase activity, yeast MAP2 may also participate in other important functions required for yeast growth and mutEcMetAP2 may not be able to complement such functions. This may not be surprising given the fact that in yeast, MAP1 point mutations in the zinc finger region exhibited a more detrimental effect on the enzyme function in vivo than did truncation of the entire zinc finger domain (12).

We validated our strategy of using the yeast strains generated in the present study for drug screening by using known inhibitors of MetAP2. Serial dilution and spotting of yeast strains dependent on MetAP2 of yeast, E. cuniculi, and human on plates containing fumagillin, ovalicin, and TNP-470 are clearly consistent with intravitro data for E. cuniculi showing that these drugs are inhibitory to this organism. In our assays for all of the drugs tested, human MetAP2 was more sensitive to these agents than either EcMetAP2 or yeast Map2. This may be due to our observation that EcMetAP2 complements yeast Map2 function more efficiently than human MetAP2. The doubling time of a yeast Δmap1 map2 strain dependent on EcMetAP2 was 3.5 to 4 h compared to 7.5 to 8 h for a yeast Δmap1 map2 strain dependent on HuMetAP2. We have observed that the yeast strains exhibited a higher sensitivity toward all of the drug concentrations tested than toward the drug concentrations found optimal by Brdlík and Crews (2). This may be because of a difference in the parental yeast strains used by Brdlík and Crews (2) as well as a difference in the compositions of media used for our respective drug assays. The higher concentration of TNP-470 required in the present study to inhibit MetAP2 activities and thereby the growth of yeast cells is consistent with the reported observation that TNP-470 is less potent in arresting the growth of S. cerevisiae cells (2).

The strategy of generating new yeast strains dependent on MetAP2 of an organism of choice and the demonstration of their use for high-throughput drug screening should prove to be very useful for developing new inhibitors for MetAP2. Once novel inhibitors are identified in screens using these yeast strains dependent on heterologous MetAP2, yeast cells can be grown in liquid culture to determine their respective 50% inhibitory concentration values against a parasite’s MetAP2. In addition, random mutagenesis studies can be carried out with various MetAP2s from different sources with yeast to identify
critical amino acid residues essential for the binding of the drug molecule, thus helping to understand protein-drug interactions and the possible development of resistance to any newly identified compounds.

Overall, the technique we have described should prove to be very useful for the development of new therapeutic agents against microsporidia as well as other pathogenic organisms.

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