Mutational Analysis of *Encephalitozoon cuniculi* mRNA Cap (Guanine-N7) Methyltransferase, Structure of the Enzyme Bound to Sinefungin, and Evidence That Cap Methyltransferase Is the Target of Sinefungin’s Antifungal Activity*

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Cap (guanine-N7) methylation is an essential step in eukaryal mRNA synthesis and a potential target for antiviral, antifungal, and antiprotozoal drug discovery. Previous mutational and structural analyses of *Encephalitozoon cuniculi* Ecm1, a prototypical cellular cap methyltransferase, identified amino acids required for cap methylation *in vivo*, but also underscored the nonessentiality of many side chains that contact the cap and AdoMet substrates. Here we tested new mutations in residues that comprise the guanine-binding pocket, alone and in combination. The outcomes indicate that the shape of the guanine binding pocket is more crucial than particular base edge interactions, and they highlight the contributions of the aliphatic carbons of Phe-141 and Tyr-145 that engage in multiple van der Waals contacts with guanosine and S-adenosylmethionine (AdoMet), respectively. We purified 45 Ecm1 mutant proteins and assayed them for methylation of GpppA *in vitro*. Of the 21 mutations that resulted in unconditional lethality *in vivo*, 14 reduced activity *in vitro* to ≤2% of the wild-type level and 5 reduced methyltransferase activity to between 4 and 9% of wild-type Ecm1. The natural product antibiotic sinefungin is an AdoMet analog that inhibits Ecm1 with modest potency. The crystal structure of an Ecm1-sinefungin binary complex reveals sinefungin-specific polar contacts with main-chain and side-chain atoms that can explain the 3-fold higher affinity of Ecm1 for sinefungin *versus* AdoMet or S-adenosylhomocysteine (AdoHcy). In contrast, sinefungin is an extremely potent inhibitor of the yeast cap methyltransferase Abd1, to which sinefungin binds 900-fold more avidly than AdoHcy or AdoMet. We find that the sensitivity of *Saccharomyces cerevisiae* to growth inhibition by sinefungin is diminished when Abd1 is overexpressed. These results highlight cap methylation as a principal target of the antifungal activity of sinefungin.

The m7GpppN cap structure of eukaryotic messenger RNA is formed by three enzymes. RNA triphosphatase hydrolyzes the 5′-triphosphate end of the pre-mRNA to a diphosphate, which is capped with GMP by RNA guanylyltransferase. RNA (guanine-N7) methyltransferase then adds a methyl group from AdoMet to GpppRNA to form m7GpppRNA and AdoHcy. This pathway is conserved in all eukaryotic organisms and many eukaryotic viruses (1). The capping enzymes are considered attractive targets for antiviral, antifungal, and antiprotozoal drug discovery (2). Inhibition of cap methylation, in particular, has been touted as an anti-infective strategy based on two lines of evidence: (i) raising the cellular levels of AdoHcy by genetic or pharmacological inhibition of AdoHcy hydrolase blocks replication of many viruses (3, 4), and (ii) the AdoMet analog sinefungin (an inhibitor of cap methylation *in vitro*) inhibits the growth of diverse viruses, fungi, and protozoan parasites (5–11). Indeed, it was shown recently that sinefungin displays selectivity in inhibiting yeast cap methyltransferases *versus* the human enzyme *in vivo* (12).

The *Saccharomyces cerevisiae* cap methyltransferase Abd1 has been extensively characterized genetically, but biochemical and structural analyses of the yeast enzyme are not as far advanced (13–16). Cellular cap methyltransferases from humans, *Xenopus laevis*, *Candida albicans*, *Schizosaccharomyces pombe*, and *Trypanosoma brucei* have also been characterized (17–22). The cap methyltransferase Ecm1 from the microsporidian parasite *Encephalitozoon cuniculi* (23) is presently the best model for mechanistic studies of cap methylation. Ecm1 is the smallest cap methyltransferase known (298 amino acids), and it suffices for cap methylation *in vivo*, as gauged by complementation in yeast (23). Crystal structures of Ecm1 bound to its substrates have been determined (24, 25). Ecm1 contains two ligand-binding pockets, one for the methyl donor AdoMet, and one for the cap guanosine methyl acceptor and the 5′-triphosphate of the cap. Superposition of the structures of Ecm1-ligand complexes suggested a direct in-line mechanism of methyl transfer. It was remarkable that no Ecm1 residues were observed in contact with the guanine-N7 nucleo-

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§ The abbreviations used are: AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; DTT, dithiothreitol.
phile, the AdoMet methyl carbon, or the AdoHcy sulfur leaving group, implying that Ecm1 facilitates methyl transfer to cap guanine-N7 by optimizing proximity and geometry of the donor and acceptor. A similar catalytic strategy is used by glycine N-methyltransferase (26).

The availability of a crystal structure has spurred biochemical and structure-function analyses of Ecm1 (24, 25). Purified recombinant Ecm1 is a monomeric protein that catalyzes methyl transfer from AdoMet (K_m 25 μM) to GpppA (K_m 0.1 mM), GTP (K_m 1 mM), or GDP (K_m 2.4 mM), but not ATP, CTP, UTP, ITP, or m7GTP. A large collection of alanine and conservative mutants has been generated and tested for activity in yeast by complementation of an abd1Δ strain. This effort has pinpointed critical constituents of the active site that bind to AdoMet (Lys-54, Asp-70, Asp-78, and Asp-94), the cap triphosphate (Arg-106), or the cap guanosine (Phe-141). Tyr-145 is an essential residue that contacts both AdoMet and the cap guanine (Fig. 1). Purification and characterization of recombinant versions of a few Ecm1-Ala mutants verified that Lys-54, Asp-70, Asp-78, Asp-94, and Phe-141 were essential for methylation of GTP in vitro (25).

It was surprising that many of the side chains that contact the substrates in the Ecm1 crystal structures were nonessential for activity in yeast, as surmised from the lack of a growth phenotype when the residue was replaced by alanine. Mutating other residues that contact the substrates resulted in temperature-sensitive growth. It was particularly noteworthy how few of the individual side-chain contacts to the edge of the guanine base were essential for Ecm1 function in vivo, given the stringent specificity for a guanine nucleotide as the methyl acceptor (25). This suggested that there might be functional redundancy among the substrate-binding residues. Here we sought to address this issue by analyzing the effects of new mutations in residues that comprise the guanine-binding pocket, alone and in combination. The outcomes indicate that neither of the polar contacts (from His-144 and Tyr-145) to the O6 atom of guanine are essential in vivo and suggest that the shape of the guanine binding pocket is more crucial than base edge interactions. The results also illuminate the contributions of the aliphatic carbons of Phe-141 and Tyr-145 that engage in multiple van der Waals contacts with guanosine and AdoMet, respectively.

To compare the growth phenotypes in yeast with mutational effects on enzyme activity, we purified 45 Ecm1 mutant proteins produced in bacteria and assayed them for methylation of GpppA in vitro. Whereas lethality in vivo generally correlated with ablation of methyltransferase activity, we noted many instances of mutations that were viable or conditional in yeast despite much reduced cap methyltransferase activity in vitro. We surmise that there is a threshold level of methyltransferase required for growth that is exceeded when wild-type Ecm1 is expressed in yeast. The mutational effects on activity in vitro provide a clearer view of the contributions of the active site functional groups.

To better understand the mechanism of sinefungin inhibition of cap methylation, we determined the crystal structure of an Ecm1-sinefungin binary complex, which revealed that sinefungin occupied a position similar to that observed for the AdoMet substrate and AdoHcy product in previous structures, although sinefungin makes additional contacts with Ecm1 main-chain and side-chain atoms. We report that S. cerevisiae Abd1 is inhibited potently by sinefungin and that the sensitivity of budding yeast to growth inhibition by the drug is diminished when Abd1 is overexpressed. These results suggest that the cap methyltransferase is a principal target of sinefungin action in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**—[^H-CH_3]AdoMet was purchased from PerkinElmer Life Sciences. GTP, m7GTP, AdoMet, and sinefungin were purchased from Sigma. GpppA and m7GpppA were purchased from New England Biolabs.

**Mutational Effects on Ecm1 Function in Vivo**—Missense mutations were introduced into the ECM1 gene by the PCR-based two-stage overlap extension method, and the mutated genes were inserted into the yeast CEN TRP1 plasmid p358-ECM1, where expression of ECM1 is under the control of the ABD1 promoter (25). The inserts were sequenced completely to exclude the acquisition of unwanted mutations during amplification and cloning. The in vivo activity of the mutated ABD1 alleles was tested by plasmid shuffle (24, 25). Yeast strain YBS40 (abd1::hisG p360-ABD1[CEN URA3 ABD1]) was transformed with CEN TRP1 plasmids containing the wild-type and mutant alleles of ECM1. Trp^- isolates were selected and then streaked on agar plates containing 0.75 mg/ml 5-fluoroorotic acid. Growth was scored after 7 days of incubation at 25°, 30°, and 37° C. Lethal mutants were those that failed to form colonies on 5-fluoroorotic acid at any temperature. Individual colonies of the viable ECM1 mutants were picked from the 5-fluoroorotic acid plate and transferred to YPD (yeast extract, peptone, dextrose) agar medium. Two isolates of each mutant were tested for growth on YPD agar at 25°, 30°, and 37° C. Growth was assessed as follows: +++ denotes colony size distinguishable from strains bearing wild-type ECM1; ++ denotes slightly reduced colony size; + indicates that only pinpoint colonies were formed; -- indicates no growth.

**Mutational Effects on Ecm1 Function in Vitro**—NdeI/BamHI fragments encoding Ecm1 mutants were excised from the respective p358-ECM1 plasmids and inserted into pET16b. The pET16-Ecm1 plasmids were introduced into Escherichia coli BL21(DE3). The recombinant Ecm1 mutant proteins were produced and purified from soluble bacterial lysates by nickelagarose chromatography as described previously for wild-type Ecm1 (25). The purification was performed at 4° C. Protein concentrations were determined using the Bio-Rad dye reagent with bovine serum albumin as the standard. Methyltransferase reaction mixtures (20 μl) containing 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 50 μM[^H-CH_3]AdoMet, 1 mM GpppA, and 1 μg of Ecm1 were incubated for 60 min at 25° C. Aliquots (5 μl) were spotted on polyethyleneimine cellulose TLC plates, which were developed with 0.2 M (NH_4)_2SO_4. The AdoMet- and m7GpppA-containing portions of the lanes were cut out, and the radioactivity in each was quantified by liquid scintillation counting. The activity of each protein was determined as the average of three separate experiments (Fig. 2B). The activity of each of the mutants was normalized to that of wild-type Ecm1 (defined as 100%) (Table 1).
Crystal Structure of Ecm1 Bound to Sinefungin—Ecm1 was produced for crystallographic analysis as reported previously (24). Ecm1 (5 mg/ml, 150 μM) was incubated at 4 °C in the presence of 300 μM sinefungin for 30 min prior to crystallization by vapor diffusion against a well solution containing 1.2 M sodium/potassium tartrate, 50 mM bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (pH 6.0 or 6.25), 20 mM DTT. Crystals appeared within 1–3 days at 18 °C and were cryo-protected with well solution containing 18% glycerol prior to freezing them in liquid nitrogen. Crystals diffracted x-rays to 2.6 Å (P321, a = b = 63.81 Å, c = 112.12 Å, α = β = 90°, γ = 120°). Data were collected using a Rigaku RU200 x-ray generator equipped with confocal Osicul multilayer optics and an R axis-IV imaging plate detector. Data were reduced with DENZ, SCALEPACK, and CCP4 (27, 28). The Ecm1-sinefungin data set was isomorphous to the previously determined crystal structures of Ecm1 (24, 25). Electron density maps were interpreted using O (29), and models were refined using CNS (30) to an R of 0.203 and R_free of 0.265. Waters were added manually into electron density derived from simulated annealing omit maps. The model has excellent geometry with no Ramachandran outliers. The coordinates have been deposited in PDB (accession code 2HV9).

Recombinant Abd1—Full-length Abd1 was produced as a His_C-Smt3 fusion protein (43) in E. coli BL21(DE3) CodonPlus RIL (Novagen) using a pET-based pSmt3-TOPO vector (Invitrogen). Cultures (10 liters) were grown at 37 °C in a Bioflo 3000 fermentor (New Brunswick Scientific) to an \( A_{600} \) of 3.0, then adjusted to 30 °C, supplemented with 0.75 mM isopropyl 1-thio-β-D-galactopyranoside, and incubated at 30 °C for 4 h. Cells were harvested by centrifugation and resuspended in 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20 mM imidazole, 0.1% IGEPAL, 20% sucrose, 1 mM β-mercaptoethanol. Cells were lysed by sonication, and insoluble material was removed by centrifugation. His_C-Smt3-Abd1 was purified from the soluble extract by nickel-nitrilotriacetic acid fast flow column chromatography (Qiagen). The His_C-Smt3 tag was removed by treatment with the Smt3-specific protease Ulp1 (43). Abd1 was purified free of the tag by gel filtration through a column of Superdex 200. Abd1 appeared homogeneous by SDS-PAGE and Coomassie Blue staining. The protein was concentrated to 10.5 mg/ml in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 3 mM DTT, and stored at −80 °C.

Inhibition of Yeast Growth by Sinefungin—The CEN URA3 ABD1 plasmid in the S. cerevisiae YBS40 strain was replaced by p358-ABD1 (CEN TRP1 ABD1) or p132-ABD1 (CEN TRP1 ABD1) by plasmid shuffling. In p358-ABD1, methyltransferase expression is directed by the ABD1 promoter, whereas in p132-ABD1, methyltransferase expression is driven by the strong constitutive TPI1 promoter. Wild-type Abd1-expressing and “High-ABD1” yeast cells were grown in YPD medium to mid-log phase (\( A_{600} \) of 0.7 to 0.9) and \( 10^6 \) cells were spread on YPD agar plates (15-cm diameter). After incubation of the plates for 1 h at 30 °C to allow the cell suspension to dry, 2-μl aliquots of aqueous solutions of sinefungin (125, 250, 500, or 1000 μM) were spotted on the agar plates. Water alone was spotted as a control and resulted in no zone of growth inhibition. The plates were incubated for 2 days at 30 °C, and then photographed.

**RESULTS AND DISCUSSION**

New Mutations in the Active Site of Ecm1—Amino acids that comprise the GTP binding pocket include Phe-141, His-144, Tyr-145, Pro-175, Phe-214, Glu-225, and Tyr-284 (Fig. 1A). We showed previously that Phe-141, which makes multiple van der Waals contacts with the cap guanine and ribose, is essential for Ecm1 function in yeast, i.e. the F141A mutation was unconditionally lethal (24). Here we tested the effects of replacing Phe-141 conservatively with leucine, isoleucine, valine, and histidine. The mutant ECM1 alleles were placed under control of the ABD1 promoter on a centromeric plasmid and assayed by methyltransferase Ulp1 (43). Abd1 was purified free of the tag by gel filtration through a column of Superdex 200. Abd1 appeared homogeneous by SDS-PAGE and Coomassie Blue staining. The protein was concentrated to 10.5 mg/ml in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 3 mM DTT, and stored at −80 °C.
TABLE 1
Mutational effects on Ecm1 activity in vivo and in vitro
The indicated mutant alleles were tested for cap methyltransferase activity in vivo by complementation of the S. cerevisiae abd1Δ strain as described under “Experimental Procedures.” The new alleles constructed for the present study are indicated in boldface type. The complementation data for the other mutants was reported previously (25). Cap methyltransferase activity of all of the purified recombinant proteins was determined as described in Fig. 2. The activity of each of the mutants (in boldface type) was normalized to that of wild-type Ecm1 (defined as 100%). The atomic contacts of each mutated side chain are indicated in the rightmost column, on the same line as the alanine mutant.

| Ecm1        | 25 °C | 30 °C | 37 °C | GpppA methyltransferase |
|-------------|-------|-------|-------|-------------------------|
| WT          | +++   | +++   | +++   | 100                     |
| N51A        | +++   | +++   | −     | 15                      |
| N51D        | −     | −     | −     | 4                       |
| K54A        | −     | −     | −     | 1                       |
| K54R        | −     | −     | −     | 1                       |
| K54Q        | −     | −     | −     | <1                      |
| R59A        | +++   | +++   | +++   | 88                      |
| D70A        | −     | −     | −     | 6                       |
| D70N        | −     | −     | −     | 8                       |
| D70E        | +++   | +++   | +++   | 40                      |
| K75A        | +++   | +++   | −     | 8                       |
| D78A        | −     | −     | −     | 24                      |
| D78N        | −     | −     | −     | 19                      |
| D78E        | +++   | +++   | −     | 12                      |
| K81A        | −     | −     | −     | 110                     |
| K81R        | −     | −     | −     | 9                       |
| K81Q        | −     | −     | −     | 23                      |
| R84A        | +++   | +++   | +++   | 2                       |
| D94A        | −     | −     | −     | 4                       |
| D94N        | −     | −     | −     | 1                       |
| D94E        | +++   | +++   | +++   | 4                       |
| Y124A       | +++   | +++   | −     | 17                      |
| F141A       | −     | −     | −     | <1                      |
| F141L       | +++   | +++   | +++   | 45                      |
| F141I       | +++   | +++   | +++   | 47                      |
| F141H       | +++   | +++   | +++   | 16                      |
| F141V       | −     | −     | −     | 1                       |
| H144A       | +++   | +++   | +++   | 2                       |
| Y145A       | −     | −     | −     | 2                       |
| Y145F       | +++   | +++   | +++   | 34                      |
| Y145L       | +++   | +++   | +++   | 7                       |
| Y145S       | −     | −     | −     | 2                       |
| Y145H       | +++   | +++   | +++   | 11                      |
| Y145I       | +++   | +++   | +++   | 2                       |
| Y145V       | −     | −     | −     | 2                       |
| H144A/Y145F | +++   | +++   | +++   | 12                      |
| H144A/Y145L | +++   | +++   | +++   | 1                       |
| P175A       | +++   | +++   | +++   | 18                      |
| Y212A       | +++   | +++   | +++   | 30                      |
| F214A       | +++   | +++   | +++   | 10                      |
| E225A       | −     | −     | −     | 13                      |
| K267A       | +++   | +++   | +++   | 120                     |

Pro-175 makes a van der Waals contact from Cγ to the exocyclic N2 atom of the guanine base (Fig. 1A) and might thereby contribute to methyl acceptor specificity. However, we found presently that changing Pro-175 to Ala had no apparent effect on Ecm1 activity in yeast (Table 1). Given that guanine N2 makes multiple additional contacts to the enzyme (via Tyr-284 Cε2 and OH, Glu-225 Oε1, and Phe-214 C82 and Cε2), it is likely that loss of any one contact will not abolish activity. Indeed, we found previously that the F214A, E225A, and Y284A yeast mutants are viable, albeit unable to grow at 37 °C (Table 1) (24).

His-144 donates a hydrogen bond from Ne to guanine O6 (Fig. 1A) and was initially thought to be a methyl acceptor specificity determinant. The findings that the H144A mutation had no effect on yeast growth at 25 or 30 °C, although it did slow growth at 37 °C (Table 1), cast doubt on this idea. It was suggested that the guanine O6 contact to His-144 and the water-

C2’) observed in the crystal structure are not the critical interactions of this side chain. The isoleucine change resulted in a temperature-sensitive growth defect, whereby the F141I strain grew well at 25 and 30 °C but formed pinpoint colonies at 37 °C. The F141V and F141H strains also formed pinpoint colonies at 37 °C and displayed a slow growth phenotype at 30 °C as well. Thus, we surmise that an aliphatic branched amino acid at position 141 is the minimal requirement for Ecm1 activity and that the van der Waals contacts of Phe-141 with asparagine, which is nearly isosteric with leucine, resulted in unconditional lethality (Table 1). We infer from this result that the Oδ atom of asparagine was the deleterious factor, insofar as a histidine (to which Asn is partially isosteric, and which, like Asn, contains an Nδ atom) was able to support cell growth.
mediated contact to the Tyr-145 hydroxyl might be functionally redundant. To address this issue presently, we constructed and tested a H144A/Y145F double mutant, which we found to be fully functional in yeast (Table 1). Thus, it seems that neither of the hydrogen-bonding interactions with the guanine O6 seen in the crystal structure are essential for cap methyltransferase activity in vivo. This is not to say that there is no structural basis for the guanine O6 specificity; rather, we suppose that it is the shape of the guanine-binding pocket into which the O6 atom fits that is the defining determinant. Indeed, the O6 atom is sandwiched between the Phe-141 main-chain carbonyl oxygen and the Glu-225 Oε2, which are located 3.1 Å from guanine O6 at positions roughly orthogonal to, and on opposite faces of, the plane of the purine base (Fig. 1A). Also, guanine O6 makes a van der Waals contact (4.1 Å) to the Tyr-145 Cβ1 atom, which is located apical to the guanine 6-carbonyl. The Tyr-145 Cβ1 contact was not essential per se, insofar as introducing leucine in place of Tyr-145 had no effect on yeast growth (Table 1). However, we found presently that an H144A/Y145L double mutant was defective in vivo, failing to grow at 37 °C, forming microcolonies at 30 °C, and growing slowly at 25 °C (Table 1). The pairwise comparison of the benign H144A/Y145F and severe H144A/Y145L alleles implicates the contour of the guanine O6 binding pocket as the functionally critical feature. Note that the combinatorial effects of mutating residues surrounding guanine O6 on Ecm1 function are not the consequence of steric hindrance (all changes made involve removal of atoms lining the pocket rather than introduction of bulkier groups) but, rather, of opening up ever more space surrounding the ligand.

The essential Tyr-145 residue is situated uniquely at the interface between the methyl donor and acceptor sites. It makes extensive van der Waals contacts to the adenine base and ribose sugar of AdoMet (Fig. 1B). Previous findings that the Y145F and Y145L mutants were viable, whereas Y145S was lethal (25), suggested that the van der Waals interactions with AdoMet (via the Cβ, Cγ, and Cε1 atoms of Tyr-145) are the decisive contribution of this side chain to Ecm1 function. To examine in greater detail the structure-activity relationships at this position, we replaced Tyr-145 with histidine, isoleucine, and valine. Whereas the Y145H allele supported yeast growth at all temperatures, the Y145V change was unconditionally lethal (Table 1). Y145I elicited an intermediate phenotype of slow growth at 25 and 30 °C and microcolony formation at 37 °C (Table 1). We surmise that the γ-branched leucine and histidine side chains, which are partially isosteric with tyrosine, can engage in the essential AdoMet contacts of Tyr-145, whereas the β-branched valine and isoleucine side chains cannot. Tyr-145 Cβ makes a 3.8-Å van der Waals contact to the adenine N7 of AdoMet (Fig. 1B). Tyr-145 Cγ is 3.9 Å from the adenine C8 atom of AdoMet; Tyr-145 Cε1 is 3.6 Å from adenine C8 and 4.1 Å from the ribose O4 atom of AdoMet (Fig. 1B). It is expected that adding a branched methyl group at Cβ would sterically hinder AdoMet occupancy of the methyl donor pocket. The deleterious isoleucine substitution creates a steric clash from Cβ while presumably allowing the contacts from Cγ and Cε. The lethality of the valine mutant likely reflects the combination of creating steric clash at Cβ and eliminating the salutary interactions from Cγ.

In summary, the new mutational analysis highlights the theme that nonpolar contacts with the atoms that line the methyl donor and methyl acceptor sites are collectively critical for cap methyltransferase activity in vivo. This conclusion resonates with our previous inferences (24) that proper orientation of the AdoMet and guanosine substrates within their binding pockets is the principal driving force for the transmethylation reaction.

Mutational Effects on Ecm1 Activity in Vitro—Forty-five Ecm1 mutants were produced in E. coli as His145-Ecm1 fusions. Because many of the mutations of interest were temperature-sensitive in yeast, we produced all of the proteins in isopropyl 1-thio-β-D-galactopyranoside-induced bacteria grown overnight at 17 °C. The proteins were purified from soluble extracts by nickel-affinity chromatography in parallel with wild-type Ecm1 (Fig. 2A). Aliquots (1 μg) of each protein were assayed for methyl transfer from 50 μM [3H-CH3]AdoMet to 1 μM GpppA during a 60-min reaction at 25 °C (Fig. 2B). The activities of the mutants were normalized to that of wild-type Ecm1 (results compiled in Table 1) and interpreted in light of the phenotypes elicited in yeast. We included as prospective positive controls alanine mutations at Arg-59, Arg-84, and Lys-267, which are located on the surface of Ecm1 and do not directly contact the methyl donor or acceptor. We had shown previously that the R59A, R84A, and K267A alleles were functional in vivo in yeast (25). Here we find that the recombinant R59A, R84A, and K267A protein were as active as wild-type Ecm1 in GpppA methylation in vitro (Fig. 2B and Table 1).

Of the twenty-one mutations that resulted in unconditional lethality in yeast, fourteen reduced methyltransferase activity in vitro to ≤2% of the wild-type level (these being K54A, K54R, K54Q, D78A, D78N, D94A, D94N, R106A, R106Q, F141A, F141N, Y145A, Y145S, and Y145V). Five lethal mutations reduced methyltransferase activity to between 4 and 9% (N51D, D70A, D70N, K81R, and R106K) and two lethal changes resulted in between 12 and 19% of wild-type activity (K81A and K81Q). Thus, there was a good correlation between in vivo lethality and a severe decrement in enzymatic function in vitro (except perhaps at Lys-81, where the catalytic impairment was not so dramatic). Structure-activity relationships were delineated clearly for several essential residues implicated in binding the cap triphosphate or AdoMet. Arg-106, which is located near the cap triphosphate bridge, was strictly essential, insofar as the lethal alanine, lysine, and glutamine substitutions eliminated GpppA methylation in vitro. Lys-54, which contacts the AdoMet carboxylate, was also strictly essential; alanine, arginine, and glutamine substitutions abolished GpppA methylation in vitro. At Asp-70 and Asp-78, which together make a water-mediated contact to the AdoMet amine, the lethal alanine, arginine, and glutamine changes suppressed activity in vitro (to 6–8% at Asp-70 and ≤1% at Asp-78), but the glutamate substitutions restored function in yeast and also resulted in recovery of methyltransferase activity in vitro (to 40% for D70E and 24% for D70Q). Also at Asp-94, which coordinates both ribose hydroxyls of AdoMet, we found that the lethal asparagine change abolished GpppA methylation in vitro, whereas the glutamate replacement elicited gains of function in vivo and in vitro (to 23% of the wild-type level). We surmise that the car-
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Asn-51 (which is proposed to interact with the cap triphosphate) and Tyr-124 (which stacks on the adenine of AdoMet), both of which were conditionally lethal at 37 °C in yeast, were active in vitro to a level of 15 and 17% of wild-type Ecm1, respectively. An alanine change at Lys-75, which is also near the triphosphate bridge and results in a more severe conditional growth defect, retains 8% of wild-type activity in vitro (Table 1). However, there is not a strict correlation between residual activity in vitro and yeast growth when mutations in different amino acids are compared, e.g. mutations at Lys-81 (which interacts via Nz with both Asp-78 Oδ and Asn-51 Oδ, but not directly with the substrates) are uniformly lethal despite having in vitro activity comparable to the “viable” N51A, Y144A, and K75A mutants.

The hierarchy of mutational effects at Phe-141 of the guanosine binding site on methylation in vitro was instructive, and was generally in agreement with the severity of the growth phenotypes in yeast (Table 1). F141A and F141N were catalytically inert, which agreed with their unconditional lethality, whereas F141L, which performed as well as wild-type in yeast, displayed the highest level of activity in vitro (45% of wild-type). The decrement in relative methyltransferase activities of F141I (25% of wild-type) and F141H (8%) recapitulated their relative conditional growth defects (Table 1). Similar trends were seen for other components of the cap guanosine binding pocket. Alanine substitution at Pro-175, which contacts the guanine N2, reduced activity to 18% of wild-type, which sufficed for yeast growth. Phe-214 makes van der Waals contacts via its C2 atoms to guanine N2 and N3 (Fig. 1A). The F214A mutant was 10% as active in vitro as wild-type Ecm1 and was conditionally lethal at 37 °C in yeast (Table 1). Glu-225 makes multiple base-edge contacts, to guanine O6, N1, and the exocyclic N2. The E225A mutant retained 13% methyltransferase activity in vitro and had a strong conditional growth phenotype in yeast (Table 1). Tyr-212 lines the binding pocket but does not contact the guanosine directly; rather, it contributes a hydrogen bond to Glu-225. The Y212A mutant had 30% activity in vitro and the Y212A yeast strain grew well at 30 °C but not at 37 °C (Table 1). His-144 donates a hydrogen bond to guanine O6; the H144A mutant retained 47% of wild-type enzyme activity and was functional in yeast (Table 1).

The stratification of mutational effects at Tyr-145 was also instructive and generally supportive of the importance of specific van der Waals contacts discussed above. The lethal Y145A, Y145S, and Y145V mutants retained scant enzymatic activity (2% of wild type), whereas Y145F, which was fully functional in yeast (Table 1). Tyr-212 lines the binding pocket but does not contact the guanosine directly; rather, it contributes a hydrogen bond to Glu-225. The Y212A mutant had 30% activity in vitro and the Y212A yeast strain grew well at 30 °C but not at 37 °C (Table 1). His-144 donates a hydrogen bond to guanine O6; the H144A mutant retained 47% of wild-type enzyme activity and was functional in yeast (Table 1).
mRNA Cap Methyltransferase

TABLE 2

| Crystallographic data and refinement statistics | Ecm1-Sinefungin |
|-----------------------------------------------|-----------------|
| PDB ID                                       | 2HV9            |
| Source                                       | Rigaku RU2000   |
| Wavelength (Å)                               | 1.5418          |
| Resolution limits (Å)                        | 39.2–6.29 (2.69–2.69) |
| Space group                                  | P3,21           |
| Unit cell (Å) a, b, c, α, β, γ               | 63.81, 63.81, 112.12, 90, 90, 120 |
| Number of observations                       | 33,416          |
| Number of reflections                        | 8,444           |
| Completeness (%)                             | 98.1 (96.8)     |
| Redundancy                                    | 4.0 (3.2)       |
| Mean I/σ                                     | 12.4 (2.6)      |
| Rmerge on I*                                  | 9.7 (42.6)      |
| Cut-off criteria I/σ                          | -0.5            |

Refinement statistics

| Resolution limits (Å)                        | 39.2–6.29 (2.69–2.69) |
| Number of reflections                       | 8,357               |
| Completeness (%)                            | 97.4 (95.4)         |
| Cut-off criteria I/σ                         | 0                   |
| Protein/ligand/water atoms                  | 2033/27/62          |
| Rmerge (%)                                   | 0.203 (0.351)       |
| Rfree (%) (5% of data)                       | 0.265 (0.399)       |
| Bonds (Å)                                    | 1.007               |
| Angles (°)                                   | 1.2                 |

Average B-factor (mc/sc/ligand/water) 42.6/46.3/34.1/37.9

H144A/Y145F double mutant (which was more active in yeast) had 12% activity in vitro (Table 1).

Structure of Ecm1 in Complex with Sinefungin—Sinefungin is an analog of AdoMet that has been shown to have antifungal, antiprotozoal, and antiviral activities (5–12). Sinefungin differs from AdoMet in that the S-CH₃ sulfonium moiety is replaced by a C-NH₂ amine. We showed previously that Ecm1 was inhibited by sinefungin in a concentration-dependent manner and that Ecm1 has a 3-fold higher affinity for sinefungin than it does for either AdoMet or AdoHcy (25). Here we determined a structure of Ecm1 in complex with sinefungin (Table 2 and Fig. 3A). The 2.6-Å simulated annealing omit electron density map covering the ligand is shown in Fig. 3B contoured at 1.1 σ.

Sinefungin is bound within the Ecm1 methyl donor site in a conformation similar to that observed previously for AdoMet, AdoHcy, and aza-AdoMet (24, 25) (Fig. 3). The ensemble of Ecm1 side chains that contacts sinefungin (Fig. 3A) is the same that engages AdoMet (Fig. 3C). However, the present structure reveals new protein contacts specific to sinefungin, including: (i) a hydrogen bond between the Phe-141 backbone carbonyl and the sinefungin C-NH₂ amine (Ne), in lieu of the van der Waals interaction between Phe-141 C=O and the S-CH₃ methyl group in AdoMet and (ii) a hydrogen bond between the Ser-142 Oγ and sinefungin ribose O4’ atom, instead of the van der Waals contact of Ser-142 CB with the ribose O4’ of AdoMet (Fig. 3). The Ecm1/sinefungin structure reveals other differences compared with the AdoMet complex, including: (i) closer hydrogen bonding contacts between the Asp-94 carboxylate and the ribose O2’ and O3’ atoms and (ii) closer contact of the Asn-140 and Gly-72 backbone carbonyls to the α-amino nitrogen of sinefungin. Whereas these interatomic distance differences are within the respective coordinate errors derived from Luzatti plots for either the AdoMet (0.29 Å) or sinefungin (0.31 Å) structures, the trend toward multiple closer contacts between Ecm1 and sinefungin, and the additional hydrogen bonding interactions specific to sinefungin, might well account for the 3-fold higher affinity of Ecm1 for sinefungin versus AdoMet or AdoHcy (25).

The present crystal structure of the Ecm1/sinefungin complex revealed mostly subtle differences compared with the Ecm1-AdoMet structure. This is in contrast to the case of the Thermus aquaticus DNA adenine-N6 methyltransferase M.TaqI, for which crystal structures of the AdoMet and sinefungin complexes revealed major differences in the conformation and protein contacts of the methionine and ornithine amino acid components of AdoMet versus sinefungin, although the conformations and contacts of the adenosine moieties of the ligands were identical (38). Sinefungin binds 6-fold more avidly to M.TaqI than does AdoMet, which was explained by the presence of two additional hydrogen bonds to the sinefungin ligand that were not seen in the enzyme-AdoMet structure (38). The DNA adenine-N6 methyltransferase M.RsrI has also been cocrystallized with AdoMet and sinefungin; here, too, there were significant differences in the conformations and contacts of the methionine and ornithine components of AdoMet and sinefungin, although the adenine bases of the ligands occupied identical positions (39). The crystal structures of the AdoMet and sinefungin complexes of the rRNA adenine-N6 methyltransferase ErmC’ highlighted small differences in the conformation of the sinefungin ornithine versus the AdoMet methionine, but significant differences in the protein contacts to the two ligands, including several novel hydrogen bonds to the sinefungin Ne atom (40).

These cases differ from the structure of a ternary complexes of T4 Dam adenine-N6 methyltransferase with target DNA and sinefungin, which shows that sinefungin binds in a mode that would be productive for catalysis, i.e. in a conformation that places the sinefungin Ne less than 3 Å away from the N6 of the adenine methyl acceptor (41). Thus, sinefungin binds to T4Dam (and to Ecm1) in a way that mimics the binding of the natural substrate AdoMet. However, the sinefungin Ne engages in a hydrogen bond with a tyrosine side chain of T4Dam, which presumably does not apply when AdoMet is bound (41). We find here that Ecm1 acquires a new main-chain hydrogen bond to the sinefungin Ne.

Inhibition of Yeast Cap Methyltransferase by Sinefungin—Sinefungin inhibits the growth of S. cerevisiae with an IC₅₀ of 22 ng/ml (12). An isogenic strain, in which the Saccharomyces capping enzymes were replaced by Candida albicans enzymes (31), was equally sensitive, but a yeast strain containing the mammalian capping apparatus (17) was 5-fold more resistant to sinefungin (IC₅₀ 112 ng/ml) (12). The same trends were seen when comparing isogenic yeast strains that differed only in the source of the cap (guanine-N7 methyltransferase component (12)). It was suggested that the selective inhibition of growth of yeast cells expressing fungal cap methyltransferases might reflect the intrinsic sensitivity of the fungal enzymes to sinefungin (12), but there was no direct test of the sinefungin sensitivity of a fungal cap meth-
yltransferase in vitro. Here we purified the yeast cap methyltransferase Abd1 and tested its response to sinefungin.

Initial experiments showed that Abd1 was capable of utilizing the cap dinucleotide GpppA as a methyl acceptor. Methyl transfer from 50 μM AdoMet to 2.5 mM GpppA was optimal at pH 8.0–9.5 in Tris-HCl buffer (data not shown). We found that 2.5 mM GpppA was a much more effective methyl acceptor than 2.5 mM GTP (Fig. 4A). From the slope of the Abd1 titration curve we estimated a turnover number of 5.4 min⁻¹ with GpppA. Methyl transfer from 50 μM AdoMet to GpppA increased with increasing concentration of the methyl acceptor (Fig. 4B); from a double-reciprocal plot of the data, we calculated a $K_m$ of 240 μM GpppA and a $k_{cat}$ of 5.9 min⁻¹. Methylation of 1 mM GpppA by Abd1 also increased with [3H-CH₃]AdoMet concentration (not shown); from a double-reciprocal plot of the data, we calculated a $K_m$ of 6 μM AdoMet. For comparison, the $K_m$ and $k_{cat}$ values for vaccinia virus cap (guanine-N7) methyltransferase are 3.2 μM AdoMet, 62 μM GpppA, and 2.1 min⁻¹ (44). The $K_m$ values for E. cuniculi Ecm1 are 25 μM AdoMet and 100 μM GpppA (25).

Abd1 activity in the presence of 25 μM [3H-CH₃]AdoMet was inhibited in a concentration-dependent fashion by AdoHcy; the apparent IC₅₀ for AdoHcy was 21 μM (Fig. 4C). We surmise that Abd1 has similar affinities for the substrate AdoMet and the product AdoHcy. Abd1 was inhibited by sinefungin in a concentration-dependent manner, with an apparent IC₅₀ of 24 nM (Fig. 4D). We conclude Abd1 has ~900-fold higher affinity for sinefungin than it does for AdoHcy.

Overexpression of Abd1 Confers Resistance to Sinefungin in Vivo—Sinefungin can inhibit a wide variety of AdoMet-dependent methyltransferases in addition to the mRNA cap methyltransferase. The classic genetic approach to identifying the target of a drug is screen for a drug-resistant mutant and then clone the gene responsible for the resistance phenotype. There have been several reports of the isolation of sinefungin-resistant mutants or resistant strain variants of protozoan or-

FIGURE 3. Structure of Ecm1 in complex with sinefungin. A, stereo view of sinefungin (SFG) in the Ecm1 methyl donor site. B, a simulated annealing omit map calculated at 2.6 Å is shown contoured at 1.1 σ covering the sinefungin ligand. C, stereo view of AdoMet (SAM) in the Ecm1 methyl donor site. Potential hydrogen-bonding and van der Waals interactions are shown by dashed lines with interatomic distances (Å). The figure was generated with PyMOL (DeLano, W. L. (2002)).
organisms that are normally sensitive to the drug (32–37). A sinefungin-resistant isolate of Leishmania displayed impaired uptake of the drug compared with the wild-type strain (37). To our knowledge, sinefungin resistance-conferring genetic changes have not been assigned to single genes or been correlated with a mutation leading to biochemical resistance of a specific AdoMet-dependent methyltransferase. Sinefungin’s catholic action in vitro raises the caveat that there may be multiple intracellular methyltransferase targets for sinefungin that contribute to its inhibition of fungal growth, in which case mutation of any one AdoMet-dependent methyltransferase to achieve “biochemical resistance” would not impact on drug inhibition of cell growth.

We reasoned that, if there is a dominant methyltransferase target in yeast for sinefungin, then overexpression of that methyltransferase ought to confer resistance to sinefungin. This is the classic mechanism of acquisition of resistance to an enzyme inhibitor, e.g. methotrexate resistance by amplification of dihydrofolate reductase.) However, if a particular methyltransferase is not the principal target, then its overexpression should have no impact on sinefungin sensitivity. We used a simple spot test to gauge sinefungin’s effect on the growth of S. cerevisiae bearing a single copy of the ABD1 gene under the control of its native promoter. Aliquots (2 μl) of an aqueous solution of sinefungin (either 125, 250, 500, or 1000 μM) were spotted on a freshly plated lawn of yeast cells on YPD agar medium. Diffusion of the drug from the site of application resulted in a circular zone of growth inhibition with a sharp demarcation at the circumference. The diameter of the zone of inhibition increased with the increasing sinefungin concentration, as expected (Fig. 5). The instructive finding was that overexpression of ABD1 (by placing the wild-type ABD1 gene under the control of the strong constitutive TPI1 promoter) resulted in sinefungin resistance, as evinced by the smaller zones of inhibition at equivalent concentrations and the indiscernible demarcation of the zone boundaries. By extrapolation of zone size to drug concentration in Fig. 5 (and confirmed by measurements of zone diameter as a function of drug concentration in four independent experiments) we conclude that ABD1 overexpression confers 4-fold resistance to sinefungin. These results highlight the cap methyltransferase ABD1 as a principal target of sinefungin in S. cerevisiae.

Prospects for Cap Methylation as an Anti-infective Target—The present study bolsters the case for cap methylation as a target for anti-infective drug discovery by showing that the yeast cap methyltransferase ABD1 is exquisitely sensitive to inhibition by sinefungin. We operationally define a potent methyltransferase inhibitor as one that is significantly more active than the product AdoHcy. We can conveniently quantify potency as the ratio of the IC50 values for AdoHcy versus the inhibitor of interest. By this criterion, sinefungin is a superb inhibitor of ABD1 (IC50[AdoHcy/sinefungin] = 875) but a comparatively weak antagonist of Ecm1 (IC50[AdoHcy/sinefungin] = 3). As discussed above, the finding that overexpression of ABD1 confers resistance to sinefungin is a good indicator that cap methyltransferase is indeed the proximal target of the drug in S. cerevisiae. This is not to say that changes in ABD1 are the only means to confer resistance of an organism to sinefungin, e.g. overexpression of AdoMet synthase in Leishmania confers 10-fold resistance to sinefungin (42). The latter maneuver is non-target-specific in that it raises the intracellular level of the AdoMet substrate and thereby generally decreases the ability of sinefungin to inhibit AdoMet-dependent enzymes.

**FIGURE 4.** Cap methylation by Abd1 and inhibition by sinefungin. A, methyl acceptor preference. Reaction mixtures (10 μl) containing 50 mM Tris-HCl, pH 8.5, 5 mM DTT, 30 mM NaCl, 50 μM [3H-CH3]AdoMet, 2.5 mM GpppA or 2.5 mM GTP, and Abd1 as specified were incubated for 20 min at 30 °C. The extents of methyl transfer are plotted as a function of input enzyme. B, methyl acceptor dependence. Reaction mixtures (10 μl) containing 50 mM Tris-HCl, pH 8.5, 5 mM DTT, 30 mM NaCl, 50 μM [3H-CH3]AdoMet, 63 ng of Abd1, and GpppA as specified were incubated for 20 min at 30 °C. The extent of methyl transfer is plotted as a function of GpppA concentration. C, AdoHcy inhibition. Reaction mixtures (10 μl) containing 50 mM Tris-HCl, pH 8.5, 5 mM DTT, 30 mM NaCl, [3H-CH3]AdoMet, 2 mM GpppA, 63 ng of Abd1, and AdoHcy as specified were incubated for 20 min at 30 °C. The extents of methyl transfer are plotted as a function of AdoHcy (C) or sinefungin (D) concentration.

**FIGURE 5.** Abd1 overexpression confers resistance to sinefungin inhibition of yeast growth. Wild-type Abd1-expressing and “High-ABD1” yeast cells were spread on YPD agar plates. Aliquots (2 μl) of aqueous solutions of sinefungin (125, 250, 500, or 1000 μM) were spotted on the agar in the four quadrants as indicated. The plates were incubated for 2 days at 30 °C, and then photographed. Water spotted as a control resulted in no zone of growth inhibition (not shown).
We infer that sinefungin interacts differently with the yeast cap methyltransferase Abd1 than it does with Ecml, e.g. that there are unique contacts of Abd1 to the sinefungin amine that enhance its potency and/or that the amino acid conformation differs in the Abd1-sinefungin complex. The strong and selective inhibition of Abd1 by sinefungin is similar to that seen with vaccinia virus cap guanine-N7 methyltransferase ($K_i = 12$ nM sinefungin versus 1.3 $\mu M$ AdoHcy) (7). Obtaining crystal structures of the fungal and viral cap methyltransferases with bound AdoMet and sinefungin will be an important step in understanding the selectivity of sinefungin against some members of the conserved cap methyltransferase family and in guiding the design of additional inhibitors of cap methylation.

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