Structure–function analysis of vaccinia virus mRNA cap (guanine-N7) methyltransferase

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ABSTRACT

The guanine-N7 methyltransferase domain of vaccinia virus mRNA capping enzyme is a heterodimer composed of a catalytic subunit and a stimulatory subunit. Structure–function analysis of the catalytic subunit by alanine scanning and conservative substitutions (49 mutations at 25 amino acids) identified 12 functional groups essential for methyltransferase activity in vivo, most of which were essential for cap methylation in vitro. Defects in cap binding were demonstrated for a subset of lethal mutants that displayed residual activity in vitro. We discuss our findings in light of a model of the Michaelis complex derived from crystal structures of AdoHcy-bound vaccinia cap methyltransferase and GTP-bound cellular cap methyltransferase. The structure–function data yield a coherent picture of the vaccinia cap methyltransferase active site and the determinants of substrate specificity and affinity.

Keywords: 7-methylguanosine; AdoMet; mRNA processing; poxvirus

INTRODUCTION

The m^7GpppN cap of eukaryal mRNA is formed by three enzymatic reactions: (1) the 5′ triphosphate end of the pre-mRNA is hydrolyzed to a diphosphate by RNA 5′ triphosphatase; (2) the diphosphate RNA end is capped with GMP by RNA guanylyltransferase; and (3) the GpppN cap is methylated by RNA (guanine-N7) methyltransferase. This pathway is conserved in all eukaryal organisms and many eukaryal viruses (Shuman 2002). Yet, differences in the structures and mechanisms of certain cap-forming enzymes in host versus pathogen have highlighted capping as a target for antiviral, antifungal, and antiprotozoal drug discovery (Shuman 2001).

RNA (guanine-N7) methyltransferase (cap methyltransferase) catalyzes transfer of a methyl group from S-adenosylmethionine (AdoMet) to GpppRNA to form m^7GpppRNA and S-adenosylhomocysteine (AdoHcy). Several lines of evidence point to viral and fungal cap methyltransferases as the targets of sinefungin (Pugh et al. 1978; Chrebet et al. 2005; Zheng et al. 2006, 2007; Li et al. 2007), a natural product analog of AdoMet with broad anti-infective activities. Poxvirus and yeast cap methyltransferases are exquisitely sensitive to inhibition by sinefungin in vitro, whereas the cap methyltransferase from the microsporidian parasite *Encephalitozoon cuniculi* is comparatively resistant (Pugh et al. 1978; Hausmann et al. 2005; Zheng et al. 2006; S. Hausmann and S. Shuman, unpubl.). Dissecting the mechanism of cap methylation and the principles of substrate recognition is a necessary step on the road to attacking cap methylation pharmacologically.

Vaccinia virus cap methyltransferase is a heterodimeric enzyme consisting of the carboxyl-terminal portion of the virus-encoded D1 polypeptide (amino acids 540–844; referred to herein as D1-C) and the 287-amino acid polypeptide encoded by the vaccinia D12 gene (Cong and Shuman 1992; Higman et al. 1992). The active site is located within D1-C, which has a weak intrinsic methyltransferase activity that is stimulated by D12 (Higman et al. 1994; Mao and Shuman 1994). Stimulation entails intersubunit allostery, whereby the D12 subunit enhances the catalytic subunit's affinity for AdoMet and the cap methyl acceptor and also increases k_{cat} (Schwer et al. 2006). Cellular cap-methylating enzymes are monomeric proteins with primary structure similarity to vaccinia D1-C (Mao et al. 1995). There are no known cellular homologs of vaccinia D12, nor is there evidence that cellular cap methyltransferase activity is regulated in an analogous fashion.

Initial mechanistic insights to cap methylation emerged from crystal structures of the *E. cuniculi* cap methyltransferase Ecm1 (Fabrega et al. 2004). Ecm1 contains two

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ligand-binding pockets, one for the methyl donor AdoMet, and one for the cap guanosine methyl acceptor and the 5′ triphosphate of the cap (Fig. 1A). A proposed catalytic strategy emphasizes the role of the enzyme in approximating the reactants and orienting the cap guanine-N7 nucleophile for in-line attack on the S–CH_3 of AdoMet (Fabrega et al. 2004). The essential functional groups in the methyl donor and methyl acceptor sites have been defined through extensive mutational analyses of Ecm1 and the homologous *Saccharomyces cerevisiae* cap methyltransferase Abd1 (Mao et al. 1996; Wang and Shuman 1997; Schwer et al. 2000; Fabrega et al. 2004; Hausmann et al. 2005; Zheng et al. 2006).

Many of the Ecm1 amino acids that contact either AdoMet or the GTP methyl acceptor are conserved in the vaccinia D1-C protein, consistent with a common ancestry for cellular and poxvirus cap methyltransferases (Mao et al. 1995; Bujnicki et al. 2001; Schwer and Shuman 2006). The recent crystal structure of the AdoHcy-bound D1-C/D12 heterodimer from Cusack and colleagues (De la Peña et al. 2007) nicely demonstrates the conserved fold in Ecm1 and D1-C (Fig. 1A). In the studies reported here, we exploited the Ecm1-ligand structures as a guide to mutational mapping of the essential functional groups of the poxvirus methyltransferase catalytic subunit, using both genetic and biochemical readouts of enzyme activity. Our results, which we interpret using the ensemble of Ecm1–GTP and D1-C/D12–AdoHcy structures (Fabrega et al. 2004; De la Peña et al. 2007), engender a coherent model of the poxvirus methyltransferase active site, the basis for substrate specificity, and the determinants of affinity for the cap.

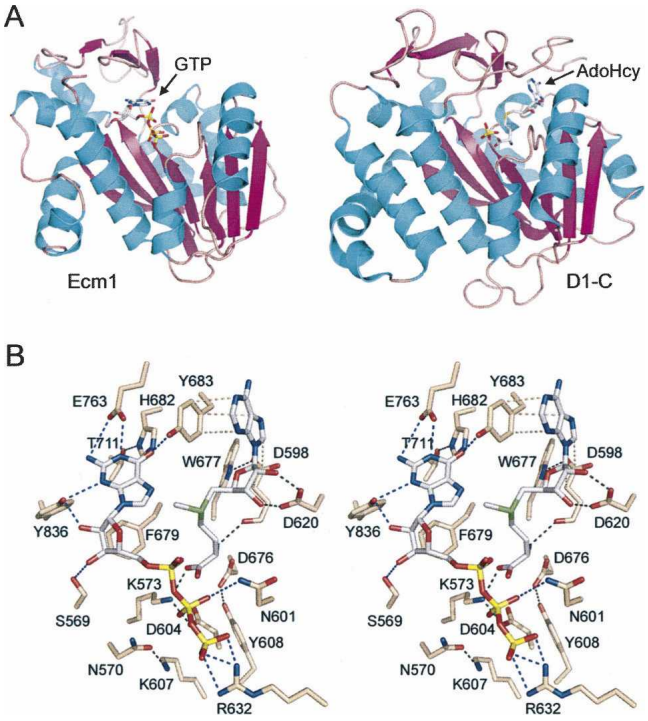

FIGURE 1. Tertiary structures of cellular and poxvirus cap methyltransferases and a model of the Michaelis complex of the vaccinia virus enzyme. (*A*) The structures of GTP-bound Ecm1 (PDB ID 1ri2) and AdoHcy-bound D1-C (PDB ID 2vdw) were superimposed and then offset horizontally. The folds are shown with β strands colored magenta and α helices in cyan. The GTP and AdoHcy ligands are depicted as stick models. A sulfate ion in the active site of vaccinia D1-C occupies the same position as the GTP γ-phosphate in the Ecm1 active site. (*B*) Stereo view of a model of the vaccinia D1-C active site with both substrates bound, generated by importing the GTP ligand from the aligned Ecm1 structure and adding a methyl group to the AdoHcy ligand in the otherwise unperturbed D1-C structure. The sulfur atom of the methyl donor is colored green. Actual polar atomic contacts of D1-C with the methyl donor and contacts between amino acid side chains are depicted as black dashed lines; Van der Waals contacts of Tyr683 and Asp598 with the adenosine moiety of the methyl donor are depicted as beige dashed lines. The modeled contacts between D1-C side chains and the GTP substrate are drawn as blue dashed lines.

RESULTS

Alanine scanning of D1-C and mutational effects on cap methylation activity in vivo

Our structure–function analysis of vaccinia cap methyltransferase relies on a yeast-based genetic assay in which cell growth depends on catalysis of cap synthesis by the viral enzyme acting in lieu of yeast Abd1 (Saha et al. 2003). Complementation of the *abd1*Δ yeast strain requires coexpression of the catalytic D1-C and stimulatory D12 subunits. The yeast assay was used previously to: (1) identify clusters of amino acids (and a few individual amino acids) that are important for cap methylation in vivo; (2) isolate temperature-sensitive mutants; and (3) select bypass mutants of the catalytic subunit that no longer require the stimulatory D12 subunit to sustain yeast growth (Saha et al. 2003; Schwer and Shuman 2006; Schwer et al. 2006).

The present study was directed at the following questions. What are the essential residues of the catalytic subunit? What are the contributions of the essential residues to substrate binding and catalysis? To answer these questions, we performed a new round of mutational analysis of the D1-C protein, targeting 21 positions for single-alanine scanning. The choice of residues to mutate was guided by a primary structure alignment of D1-C and Ecm1 and the available Ecm1 structures (Fabrega et al. 2004; Hausmann et al. 2005; Zheng et al. 2006). The targeted residues include: (1) the vaccinia equivalents of the Ecm1 amino acids that contact AdoMet or GTP in the Ecm1 cocrystals; (2) other conserved residues that are predicted to lie on the enzyme surface; and (3) residues unique to the poxvirus protein that are located within putative surface loops and, as such, were candidates to interact with the D12 subunit or mediate D12's activation of D1-C.

The new *D1-C-Ala* alleles on single-copy *CEN* plasmids were tested for function in vivo in the yeast *abd1*Δ strain by cotransformation with wild-type *CEN D12*. The recipient

yeast bears a *CEN URA3 ABD1* plasmid to sustain viability. If the vaccinia methyltransferase subunits associate in vivo to form a catalytically active heterodimer, the yeast cells can lose the *URA3 ABD1* plasmid and grow on medium containing 5-fluoroorotic acid (FOA), a drug that selects against *URA3*. The results of the plasmid shuffle assay are compiled in Table 1. Lethal mutations were those that failed to support the appearance of FOA-resistant colonies at 18, 25, 30, or 37°C (Table 1, scored as "–" at all temperatures). The viable FOA-resistant *D1-C-Ala/D12* strains

TABLE 1. Mutational effects on methyltransferase activity in vivo and in vitro

	*abd1*Δ complementation					
D1-C	18°C	25°C	30°C	37°C	GpppA methylation (% of WT)	Contacts
WT	+++	+++	+++	+++	100	
S569A	+	++	++	+	39	Cap guanosine
N570A	–	–	–	–	37	Lys607
N570D	–	–	–	–	21	
N570Q	++	++	++	+	73	
K573A	–	–	–	–	<1	AdoMet carboxyl
K573R	–	–	–	–	1	
K573Q	–	–	–	–	<1	
D598N	–	–	–	–	<1	AdoMet adenosine, Trp677
D598E	–	–	–	–	1	
N601A	+	++	+	–	32	Cap triphosphate
D604N	–	–	–	–	<1	Lys573
D604E	+++	+++	+++	+++	112	
K607A	–	–	–	–	101	Asn570
K607R	–	–	–	–	13	
K607Q	–	–	–	–	74	
Y608A	–	–	–	–	3	Asp676
Y608F	+++	+++	+++	+++	74	
Y608L	+++	+++	+++	+++	62	
Y608S	–	–	–	–	2	
F609A	+++	+++	+++	+++	117	None
D620N	+++	++	+	+	11	AdoMet ribose hydroxyls
D620E	+++	+++	+++	+++	59	
R632K	+++	+++	+++	+++	72	Cap triphosphate
R632Q	–	–	–	–	58	
D657A	+++	+++	+++	+++	112	Thr692
F659A	+++	+++	+++	+++	96	None
D676A	–	–	–	–	<1	Tyr608
D676N	–	–	–	–	<1	
D676E	+++	+++	+++	+++	40	
W677A	++	++	+	–	4	Asp598
Q678A	+++	+++	+++	+++	55	AdoMet amine (via C=O)
F679A	–	–	–	–	5	Cap guaniine
F679H	+	+	+	+	13	
F679L	++	+++	+++	+++	55	
F679I	+	++	++	++	18	
F679V	+	++	++	++	20	
F679N	+	++	++	++	18	
H682A	–	–	–	–	<1	Cap guanine, Thr711
H682N	–	–	–	–	1	
H682Q	–	–	–	–	4	
V750A	+++	+++	++	++	59	None
E763A	–	–	–	–	16	Cap guanine
E763D	–	–	–	–	1	
E763Q	++	++	++	++	32	
Y764A	++	++	++	–	18	None
F814A	+++	+++	+++	++	99	(D12 Phe44)
F815A	++	++	++	+	92	(D12 Phe44)
E816A	+++	+++	+++	+++	76	None
Y836A	+	++	+	–	15	Cap guanosine

were tested for growth on rich medium at 18, 25, 30, and 37°C. Growth was scored as follows: "+++" indicates colony size indistinguishable from strains bearing wild-type *D1-C/D12*; "++" denotes reduced colony size; "+" indicates that only pinpoint colonies were formed; and "–" indicates no growth.

Alanine substitutions at eight positions were lethal, thereby defining the following amino acids as essential for methyltransferase activity in vivo: Asn570, Lys573, Lys607, Tyr608, Asp676, Phe679, His682, and Glu763 (Table 1). Six of these essential residues (Asn570, Lys573, Lys607, Tyr608, His682, and Glu763) are conserved in the D1 orthologs encoded by numerous diverse genera of vertebrate and invertebrate poxviruses. Glu replaces Asp676 in entomopoxvirus capping enzymes and Phe679 is occasionally substituted by Met or Leu.

Alanine changes at five positions had no overt effect on yeast cell growth at any temperature tested, thereby defining Phe609, Asp657, Phe659, Gln678, and Glu816 as unimportant for cap methylation in vivo (Table 1). Whereas Asp657 is typically replaced by dissimilar side chains in most other poxvirus D1 orthologs, and Glu816 is occasionally substituted (by Ala, Lys, or Asn), its is remarkable that the Phe609, Phe659, and Gln678 side chains are strongly conserved among poxvirus D1 orthologs, despite being nonessential for the methyltransferase. It is conceivable that such conserved residues play a role in other aspects of capping enzyme function, e.g., during transcription initiation and termination (Vos et al. 1991; Luo et al. 1995).

The remaining eight alanine mutations elicited constitutive or conditional growth defects that ranged from mild to severe. The most benign of these were *F814A* and *V750A*, which formed small colonies at either 37°C (*F814A*) or 30 and 37°C (*V750A*), but grew as well as wild-type *D1-C* cells at lower temperatures. The *Y764A* strain formed smaller (++) colonies at 18°C – 30°C and failed to grow at 37°C. The *F815A* strain formed smaller (++) colonies 18–30°C and pinpoint colonies at 37°C. *S569A* cells grew slowly at 25 and 30°C and formed pinpoint colonies at 18 and 37°C. *W677A* cells grew slowly at 18 and 25°C and failed to form colonies at 37°C. The *N601A* and *Y836A* strains displayed slow growth at 25°C, formed pinpoint colonies at 18 and 30°C, and failed to grow at 37°C. Six of the eight residues at which alanine mutations cause partial defects in vivo are highly conserved in other poxvirus D1 orthologs: Ser569, Trp677, Tyr764, Phe814, Phe815, and Tyr836.

Effects of alanine mutations on cap methyltransferase activity in vitro

Wild-type D1-C and all the D1-C-Ala mutants were produced as His_6-tagged proteins in *Escherichia coli* cells co-expressing untagged D12. Because some of the mutants of interest were temperature sensitive in yeast, we produced all of the proteins in IPTG-induced bacteria grown at 17°C. The vaccinia proteins were isolated from soluble bacterial extracts by nickel-affinity chromatography. Copurification of the untagged D12 protein with the tagged catalytic subunit provided an immediate test of whether a particular mutation disrupted subunit interaction. SDS-PAGE analysis showed that the wild-type and mutant protein preparations consisted of two predominant polypeptides with approximately equal staining intensity that corresponded to D1C and D12, respectively (Fig. 2A). We surmise that none of the mutated amino acids are crucial per se for heterodimerization.

Aliquots of each protein were assayed for methyl transfer from [^{3}H-CH$_3$]AdoMet to GpppA. The reaction products were separated by PEI-cellulose TLC and the transfer of the tritiated methyl group to generate labeled m^{7}GpppA was quantified (Fig. 3A). The activities of the mutants were normalized to that of wild-type D1-C/D12 (see results compiled in Table 1) and interpreted in light of the phenotypes elicited in yeast. As might be expected, all of the mutants that were fully functional in yeast (Table 1, F609A, D657A, F659A, Q678A, E816A) or conferred only a mild growth defect (Table 1, V750A, F814A, F815A)

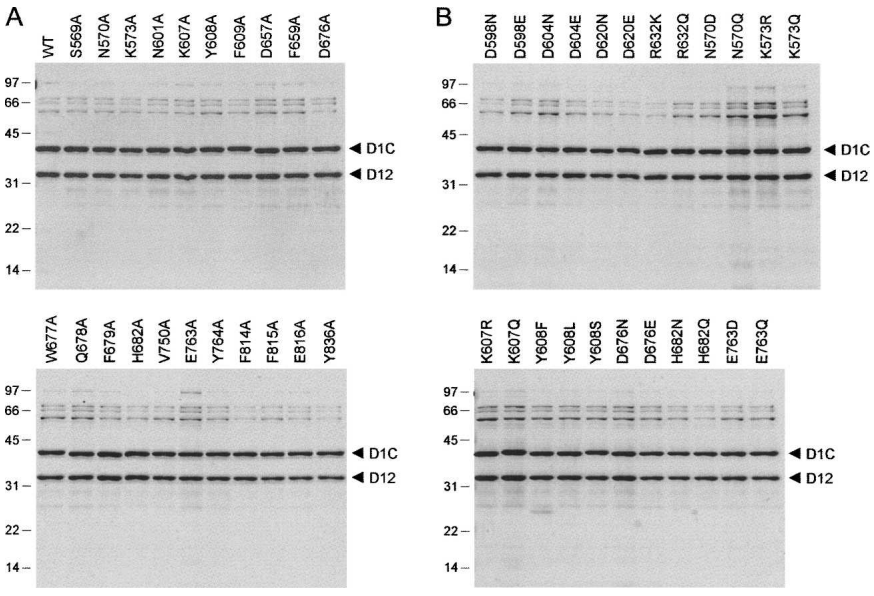

FIGURE 2. Recombinant wild-type and mutant D1-C/D12 heterodimers. Aliquots of the nickel-agarose preparations (containing 3 μg of the D1-C polypeptide) were analyzed by SDS-PAGE. The polypeptides were visualized by staining with Coomassie Blue dye. The positions and sizes (kDa) of marker polypeptides are indicated on the *left*. Alanine mutants are shown in *A*; conservative mutants are shown in *B*.

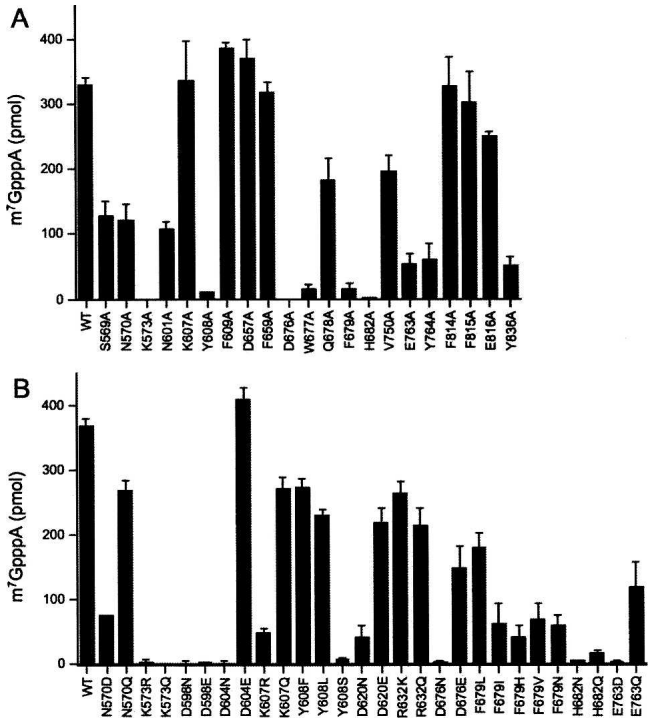

FIGURE 3. Mutational effects on vaccinia virus cap methyltransferase activity. The extents of ^{3}H-m^{7}GpppA formation by wild-type and mutant D1-C/D12 heterodimers are plotted with alanine mutants surveyed in *A* and conservative mutants in *B*. Each datum is the average of three experiments; standard error bars are shown.

displayed wild-type or near wild-type methyltransferase activity in vitro. Mutants that displayed more severe growth phenotypes with conditional lethality had progressively weaker methyltransferase activity in vitro, as follows: S569A (39%), N601A (32%), Y764A (18%), Y836A (15%), W677A (4%). The K573A, D676A, and H682A mutations that resulted in unconditional lethality in yeast effectively abolished methyltransferase activity in vitro (<1% of the wild-type level); the lethal Y608A and F679A mutants had 3% and 5% of wild-type methyltransferase activity, respectively. Thus, for the alanine mutants listed above, there was a reasonably good correlation between the in vivo growth phenotype and catalytic function in vitro.

We noted three exceptions whereby alanine mutants that were unconditionally lethal in vivo retained substantial methyltransferase activity in vitro: K607A (101% of wild-type), N570A (37%), and E763A (16%). Because the concentrations of AdoMet (40 μM) and GpppA (700 μM) included in the cap methylation reaction mixtures were in excess of the K_m values for the vaccinia methyltransferase (Schwer et al. 2006), it was possible that the catalytically active lethal mutants had defects in substrate binding that were not revealed by the initial screening assay. To address this issue, we determined kinetic parameters for the wild-type D1-C/D12 heterodimer and the K607A, N570A, and E763A mutants by titrating GpppA at a fixed concentration of AdoMet (40 μM). The results are summarized in Table 2. The wild-type enzyme had a K_m of 23 μM GpppA and a k_{cat} of 1.3 min^{-1}. The N570A and E763A mutants had threefold and 10-fold higher K_m values for GpppA (74 and 218 μM, respectively) and their k_{cat} values were also reduced by factors of 4 and 10 (to 0.34 and 0.13 min^{-1}, respectively). If one calculates catalytic efficiency as k_{cat}/K_m, then the N570A and E763A mutations elicited 12-fold and 95-fold reductions compared with the wild-type methyltransferase. These effects on in vitro catalysis could explain the lethality in vivo, especially in the case of E763A. It was most remarkable that the lethal K607A mutation had virtually no effect on GpppA affinity (K_m 31 μM) or k_{cat} (1.2 min^{-1}). We queried potential effects of the N570A and K607A changes on methyl donor binding by titrating AdoMet at a fixed concentration of GpppA (700 μM) (Table 3). The wild-type D1-C/D12 enzyme had a K_m of 7.5 μM AdoMet and a k_{cat} of 1.3 min^{-1}. The N570A and K607A changes had no significant effect on AdoMet binding, although they did have reduced turnover numbers.

Structure–function relationships gleaned from effects of conservative mutations

Conservative amino acid substitutions were introduced in lieu of the eight residues defined as essential in vivo in the present alanine scan and four essential residues identified previously: Asp598, Asp604, Asp620, and Arg632 (Saha et al. 2003). The 28 conservative mutants were tested by plasmid shuffle for methyltransferase activity in vivo (Table 1). The mutant His$_6$-D1-C and wild-type D12 subunit were coproduced in bacteria and affinity purified. SDS-PAGE analysis showed that the tagged D1-C and untagged D12 proteins copurified in each case (Fig. 2B; data

TABLE 2. Mutational effects on affinity for the cap methyl acceptor

	GpppA	
	K_m (μM)	k_{cat} (min^{-1})
WT	23 ± 5	1.3 ± 0.1
N570A	74 ± 1	0.34 ± 0.05
N570D	446 ± 11	0.18 ± 0.02
K607A	31 ± 5	1.2 ± 0.1
K607R	314 ± 28	0.11 ± 0.01
K607Q	200 ± 22	1.1 ± 0.1
E763A	218 ± 16	0.13 ± 0.02
R632Q	183 ± 18	0.44 ± 0.09
WT	30 ± 7	1.3 ± 0.1
F679H	255 ± 25	0.18 ± 0.03
F679L	50 ± 14	0.57 ± 0.10
F679I	176 ± 15	0.24 ± 0.01
F679V	293 ± 28	0.24 ± 0.02
F679N	143 ± 7	0.21 ± 0.01

TABLE 3. Mutational effects on affinity for AdoMet

	AdoMet	
	K_m (μM)	k_{cat} (min^{-1})
WT	7.5 ± 0.5	1.3 ± 0.1
N570A	9.5 ± 0.5	0.43 ± 0.02
K607A	8.0 ± 1.0	0.98 ± 0.06

not shown for the Phe679 mutants). Aliquots of each mutant were assayed for methyl transfer from [^{3}H-CH_3] AdoMet to GpppA (Fig. 3B), and the activities of the mutants were normalized to wild-type D1-C/D12 activity (Table 1). Useful structure–activity relationships were thereby delineated for the essential residues and there was a good correlation in most cases between in vitro and in vivo mutational effects. The findings are summarized as follows.

No restoration of function in vivo was achieved by replacing Lys573 with arginine or glutamine, signifying that lysine is strictly essential at this position. The recombinant K573R and K573Q proteins were catalytically defective in vitro (≤1% of wild-type activity). Neither asparagine nor glutamine could sustain cell growth in place of the essential His682 side chain. The H682N and H682Q proteins were severely impaired for cap methylation in vitro (1% and 4% as active as wild-type, respectively). Because Asn and Gln can potentially mimic hydrogen-bonding interactions of the His Nδ and Nε atoms, respectively, the findings suggest either that both hydrogen-bonding atoms of His682 are required or that positive charge or proton transfer could be the relevant properties of the histidine.

Distinctive structure–activity relations were observed at the essential acidic residues. Changing Asp598 to asparagine or glutamate was lethal in vivo and abolished cap methylation in vitro (≤1% activity), indicating that the carboxylate is essential and suggesting a steric constraint that precludes the longer glutamate side chain from functioning. Asparagine mutations at Asp604 and Asp676 were lethal in vivo and catastrophic in vitro (<1% activity), but glutamate substitutions restored normal growth at all temperatures and revived the catalytic activity in vitro (to 112% for D604E and 40% for D676E); thus, the carboxylate groups are essential and there is little or no steric hindrance by the extra methylene group of glutamate. Glutamate in lieu of Asp620 supported normal growth at all temperatures, while asparagine conferred partial restoration of activity, evinced by slow growth at higher temperatures. The phenotypes correlated with the methyltransferase activities of D620E and D620N in vitro, which were 59% and 11% of wild type, respectively. We surmise that whereas hydrogen bonding by a carboxylate or amide functional group is the minimally relevant property at position 620, the carboxylate promotes optimal activity. The vaccinia methyltransferase was nonfunctional in vivo when Glu763 was changed to aspartate, which suppressed activity in vitro (1%) to an even greater degree than E763A (16%). However, glutamine restored ++ growth at all temperatures and raised the level of methyltransferase to 32% of wild type. It would appear that the hydrogen-bonding capacity of residue 763 is the key feature and can be satisfied by Gln; retraction of the functional group closer to the main chain (in the case of Asp) precludes the essential contacts and imposes new deleterious effects not seen when the side chain was deleted to the β carbon in the alanine mutant.

Full activity in vivo was restored when the essential Tyr608 side chain was substituted with phenylalanine or leucine, which correlated with a significant gain of catalytic function, to 74% and 62% for Y608F and Y608L, respectively. In contrast, the Y608S change was lethal and afforded no gain of activity in vitro (2%). Thus, neither the hydroxyl group nor the aromatic quality is crucial; a bulky hydrophobic group sufficed for function.

The lethal effects of the N570A mutation were reversed by glutamine, which conferred ++ growth and increased catalytic activity to 73% of wild type, compared with 37% for N570A. In contrast, the N570D change was lethal in vivo and even less active in vitro (21%) than N570A. These results highlight the importance of the amide nitrogen as a likely hydrogen-bond donor.

No restoration of function in vivo was achieved by replacing Lys607 with arginine or glutamine, signifying that lysine is strictly essential for activity in yeast. Whereas K607Q was 74% as active as wild-type D1-C/D12 in vitro in the screening assay, the K607R protein was impaired (13% activity). GpppA titration experiments revealed that the Gln and Arg changes elicited ninefold and 14-fold increases in K_m for the cap (to 200 and 314 μM, respectively). Whereas K607Q had little effect on k_{cat} (1.1 min^{-1}), the K607R change reduced turnover by an order of magnitude (0.11 min^{-1}) (Table 2). The summary effects of K607Q and K607R were to reduce catalytic efficiency by factors of 10 and 160, respectively. This is a remarkable instance in which side-chain removal had no discernible effect on activity in vitro, but conservative substitutions conferred significant defects. We surmise that arginine imposes steric effects that account for the drastic decline in activity and cap binding, and likely accounts for its lethality. It remains unclear why K607A is lethal despite its vigorous activity in vitro. We will speculate below on the structural role of Lys607.

It was reported previously that the R632A mutant was lethal in yeast and reduced cap methylation in vitro to <1% of wild type (Saha et al. 2003). Here, we found that replacing Arg632 with glutamine was lethal, but a lysine substitution restored wild-type growth at all temperatures. We surmise that a positive charge at position 632 is crucial for cap methylation in vivo. In contrast, the screening assay

for cap methylation in vitro revealed that R632K and R632Q were 72% and 58% as active as wild type, respectively (Table 1), raising the issue of why the seemingly benign glutamine mutant failed to sustain cell growth. A substrate titration experiment showed that R632Q caused an eightfold increase in the K_m for GpppA (183 μM) and a threefold decrement in k_{cat} (0.44 min^{-1}) (Table 2). The net 24-fold decrease in catalytic efficiency could plausibly account for the in vivo phenotype.

Finally, At Phe679, we found that partial function was restored by a variety of γ-branched side chains (Leu, His, Asn) or β-branched side chains (Ile, Val), each of which partially restored cap methylation activity in vitro—to 13%–55% of wild type, compared with 5% for F679A. Previous studies of Ecm1 had shown that the Phe141 side chain (equivalent to vaccinia Phe679) is an essential component of the cap guanosine binding pocket (Fabrega et al. 2004; Zheng et al. 2006). To gain a better understanding of the contributions of Phe679 to cap binding by the vaccinia enzyme, we performed a series of GpppA titration experiments and determined kinetic parameters for the five conservative Phe679 mutants (Table 2). The F679L change had little impact on GpppA affinity (K_m of 50 μM, compared with 30 μM for wild type) and only a twofold decrement in k_{cat} (0.57 min^{-1}). Thus, we surmise that the aromatic quality of this residue is not crucial, nor are potential contacts made by the Cε and Cζ atoms of the phenyl ring. The other substitutions for Phe679 resulted in five- to 10-fold increases in K_m for GpppA and suppressed k_{cat} by five- to sevenfold (Table 2). Thus, the hydrophobic character and γ-branched configuration of this essential residue combine to promote optimal cap methylation.

DISCUSSION

The 2.8 Å structure of the D1-C/D12 heterodimer bound to AdoHcy (De la Peña et al. 2007) provides an invaluable guide to interpret the mutational data for vaccinia cap methyltransferase reported here. To gain maximum hermeneutic value, we have superimposed the AdoHcy-bound D1-C structure on the structure of GTP-bound Ecm1 (Fabrega et al. 2004) and used that superposition to infer the position of the GTP methyl acceptor in the active site of D1-C. This maneuver places the cap acceptor near the AdoHcy in an orientation that is compatible with in-line attack of guanine N7 on a modeled $-CH_3$ group of AdoMet (Fig. 1B). Moreover, the γ phosphate of GTP in our model occupies the same position as a sulfate ion in the AdoHcy complex of D1-C (Fig. 1A). The model provides a plausible picture of the Michaelis complex. The likely contacts between the enzyme and the GTP methyl acceptor are depicted as blue dashed lines in Figure 1B.

Structural basis of cap guanosine recognition

Vaccinia cap methyltransferase isolated from virus particles can use a variety of guanine nucleotides as methyl acceptors, including GpppG, GpppA, GTP, dGTP, and GTPγS, (Martin and Moss 1976; Shuman et al. 1980; S. Hausmann and S. Shuman, unpubl.). Recombinant vaccinia virus capping enzyme is equally adept at methylating GTP, dGTP, and 3′-OMeGTP substrates (S. Hausmann and S. Shuman, unpubl.). ATP, UTP, and CTP are inert as acceptors (Martin and Moss 1976; S. Hausmann and S. Shuman, unpubl.). Our structural model depicts a constellation of D1-C side chains engaging the cap guanosine via hydrogen bonds to the ribose hydroxyls (Ser569 and Tyr836), the guanine ring nitrogens (Tyr836 and Glu763), and the guanine carbonyl oxygen (His682 and Tyr683) (Fig. 1B). The finding that Ser569, which we model as contacting the ribose O3′ atom, is nonessential for methyltransferase activity in vivo and contributes only a modest (2.5-fold) enhancement of activity in vitro, is consistent with the biochemical evidence that dideoxy-GTP is utilized ∼45% as well as dGTP as a substrate for the vaccinia methyltransferase (S. Hausmann and S. Shuman, unpubl.). Tyr836A makes a predicted bifurcated hydrogen bond to the ribose O2′ and guanine N3 atoms (Fig. 1B). Elimination of the tyrosine compromises activity in vivo and reduces cap methylation in vitro by a factor of 6. Given that the ribose O2′ atom is not important for activity in vitro, we surmise that the proposed guanine base contact is the relevant contribution of Tyr836.

Glu763 is essential for cap methylation in vivo and in vitro and our model indicates that it makes a bidentate interaction (as a hydrogen-bond acceptor) with the guanine N1 and exocyclic N2 atoms. The loss of these contacts could explain the deleterious effects of the E763A mutation on cap binding and catalysis (Table 2). Our finding that the E763Q change restored function compared with alanine suggests that one of the hydrogen-bond acceptor interactions is more critical than the other. We speculate that the contact with the protonated guanine N1 atom is most relevant as a determinant of guanine specificity, in so far as Glu763 would not interact with the unprotonated N1 atom present in adenine. We regard the Glu763 contact to the exocyclic N2 atom as having lesser importance, in light of the fact that ITP (which lacks the C2-amine) is utilized 11%–25% as well as GTP by the vaccinia methyltransferase (Martin and Moss 1976; S. Hausmann and S. Shuman, unpubl.).

His682 is poised to donate a hydrogen bond from Nε to the O6 carbonyl atom of the cap guanine in our model. His682 Nδ forms a hydrogen bond to Thr711 Oγ atom in the D1-C crystal structure (Fig. 1A). Both contacts are apparently relevant in light of the findings that neither Asn nor Gln in lieu of His682 restored activity in vivo or in vitro. Tyr683 is an essential residue defined previously

(Mao and Shuman 1996; Saha et al. 2003) that donates another hydrogen bond to the guanine O6 atom in the modeled Michaelis complex; in addition, Tyr683 stacks on the adenine base of the methyl donor (Fig. 1B). In light of the previous report that replacing Tyr683 with phenylalanine restored cap methylation activity in vitro to 60% of wild type (Mao and Shuman 1996), we surmise that the hydrogen bond from the tyrosine hydroxyl to guanine O2 is either minimally contributory or else functionally redundant to the O6 contact of His682. It appears that the π-stacking of Tyr683 on the AdoMet adenine is the key role of this side chain. Phe679 lines the cap guanosine-binding pocket beneath the ribose sugar (Fig. 1B). Our results show that Phe679 provides as essential hydrophobic surface that contributes to the affinity of the enzyme for the cap substrate.

Recognition of the cap triphosphate bridge

The substrate-binding cleft in the D1–D12 crystal structure includes a sulfate anion coordinated by several amino acid side chains (De la Peña et al. 2007), including some of those mutated in the present study. Our model of the Michaelis complex superimposes the γ-phosphate of the GTP methyl acceptor on this sulfate. The model implicates the essential Arg632 residue as a ligand for the γ-phosphate, to which it makes a bidentate contact from the terminal guanidinium nitrogens. The ability of lysine to functionally replace Arg632 in vitro and in vivo suggests that the electrostatic interactions with the cap triphosphate bridge are most relevant. Perhaps lysine Nζ can make a bifurcated contact with more than one phosphate oxygen, as is seen for one of the arginine nitrogens in Figure 1B. Note that replacing Arg632 with glutamine, a neutral amino acid, was lethal in vivo and diminished affinity for the GpppA substrate. The equivalent arginines in the cellular cap methyltransferases Ecm1 (Arg106), yeast Abd1 (Arg206), and human Hcm1 (Arg239) are also essential for activity (Wang and Shuman 1997; Saha et al. 1999; Hausmann et al. 2005; Zheng et al. 2006).

The D1-C structural model suggests that Asn601 is poised to coordinate the β phosphate of the cap triphosphate bridge (Fig. 1B). Removal of Asn601 elicits a tight temperature-sensitive growth defect in yeast and causes a threefold decrement in methyltransferase activity in vitro. Asn601 is strictly conserved in chordopoxvirus capping enzymes, but is replaced by arginine in entomopoxvirus capping enzymes and lysine in cellular cap methyltransferases. The Lys81 equivalent in Ecm1 is located on the floor of the cap triphosphate-binding groove and is essential for Ecm1 function (Hausmann et al. 2005; Zheng et al. 2006).

Essential contacts to the methyl donor

Sequence comparisons of the AdoMet-binding motifs of poxvirus cap methyltransferases (VLAIDFG^{600}) versus cellular cap methyltransferases (VLDLGCG) had prompted suggestions that their AdoMet interactions might differ in functionally interesting ways (Wang and Shuman 1997; Bujnicki et al. 2001). Comparison of the structures of AdoHcy-bound D1-C and Ecm1 revealed that this was indeed the case (De la Peña et al. 2007). Specifically, the adenosine nucleoside of the methyl donor in the poxvirus structure adopts a unique *syn* conformation (Fig. 1B), as opposed to the *anti* conformation observed in Ecm1 and other AdoMet-dependent methyltransferases (De la Peña et al. 2007). The *syn* conformation is enforced by the stacking of the adenine base on Tyr683 and the Van der Waals contacts between the ribose C1 and adenine C8 atoms and Asp598, the defining residue of the poxvirus-style AdoMet motif (Fig. 1B). Asp598 also accepts a hydrogen bond to its side-chain carboxylate from Trp677 and to its main-chain carbonyl from the AdoHcy(AdoMet) amine (Fig. 1B). Replacing Trp677 with alanine or Asp598 with asparagine or glutamate suppresses vaccinia methyltransferase activity (Table 2). It was noted previously that a D598A mutation in vaccinia D1-C precluded growth of yeast at 30° and 37°C and supported only + growth at 19°C (Saha et al. 2003).

The other components of the D1-C methyl donor site that we subjected to mutational analysis are conserved in Ecm1 and other cellular cap methyltransferases. The essential Asp620 side chain accepts hydrogen bonds to its carboxylate oxygens from the AdoHcy(AdoMet) ribose hydroxyls. The essential Lys573 side chain forms a bifurcated ion pair with the carboxyl group of AdoHcy(AdoMet) and the essential Asp604 carboxylate (Fig. 1B). We suggest that Asp620, Asp604, and Lys573 are crucial to bind and orient AdoMet so that the sulfonium center is aligned properly for attack by the cap guanine-N7.

Essential residues with imputed substrate-binding or structural roles

Four of the residues identified presently as essential for vaccinia cap methyltransferase function in vivo are located near the substrate-binding sites, but not close enough to make direct contacts with the methyl donor or the modeled GTP methyl acceptor. These are Asp676, Tyr608, Asn570, and Lys607. It is remarkable that these side chains engage in pairwise contacts to each other. Asn570 accepts a hydrogen bond from Lys607 at a site on the enzyme surface ~6 Å from the GTP γ-phosphate. To explain the conundrum that eliminating Lys607 by alanine substitution has little or no effect on GpppA methylation in vitro, while severely affecting cap methylation in vivo, we speculate that Lys607 (with its partner Asn570) is involved in coordinating one of the 5′-terminal phosphodiesters of the RNA chain of the mRNA substrate (GpppN**p**N**p**N–) and docking the RNA 5′ end in place to serve as a methyl acceptor. The assays of cap methylation in vitro using a GpppA substrate would, per

force, not be sensitive to alterations of a putative RNA-docking site.

Tyr608 and Asp676 are buried in the protein behind the AdoMet-binding motif; Asp676 accepts a hydrogen bond from the side-chain hydroxyl of Tyr608. We suggest that these residues play a structural role. Although conservative mutational effects at Asp676 highlight the importance of the carboxylate, we infer that it must be making contributions beyond the hydrogen bond to Tyr608, in so far as changing Tyr608 with phenylalanine or leucine (which precludes the polar interaction) has little effect on methyltransferase activity. The structure indicates that Tyr608 makes Van der Waals contacts with nearby residues (Ile577 and Cys581) that could be relevant for proper folding.

MATERIALS AND METHODS

Mutations of the D1-C subunit of vaccinia cap methyltransferase

Missense mutations were introduced into the *D1(540–844)* gene by the PCR-based two-stage overlap extension method and the mutated genes were inserted into yeast expression vector pYN132 (*CEN TRP1*), where expression of *D1-C* is under the control of the yeast *TPI1* promoter (Saha et al. 2003). The inserts were sequenced completely to ensure that no unwanted mutations were introduced during amplification and cloning. The mutated D1-C genes were excised from the yeast vectors by digestion with EcoRI/XhoI and then inserted into the bacterial expression plasmid pET28a, where they are fused to a 5′ leader sequence encoding a His_{10} tag.

Yeast-based assay of vaccinia cap methyltransferase function in vivo

Saccharomyces cerevisiae strain YBS40 is deleted at the chromosomal *ABD1* locus encoding the yeast cap methyltransferase. Growth of YBS40 depends on the maintenance of plasmid p360A-ABD1(*CEN URA3 ADE2 ABD1*). *abd1Δ* cells were cotransformed with *CEN TRP1 D1-C* and *CEN HIS3 D12* plasmids encoding wild-type or mutated versions of the D1-C subunit and the wild-type D12 subunit, respectively. Individual Trp^+ His^+ transformants were streaked on agar medium containing 0.75 mg/mL 5-fluoroorotic acid (FOA). Growth was scored after 7 d of incubation at 18, 25, 30, and 37°C. Lethal mutants were those that failed to form colonies on FOA at any temperature. Individual FOA-resistant colonies with viable *D1-C* mutants were transferred to YPD agar medium. Two isolates of each mutant were tested for growth on YPD agar at 18, 25, 30, and 37°C. Growth was assessed as follows: (+++) colony size indistinguishable from strains bearing wild-type *D1-C*; (++) slightly reduced colony size; (+) only pinpoint colonies were formed; (–) no growth.

Wild-type and mutant methyltransferase heterodimers

pET-His_6-D1-C plasmids encoding tagged wild-type and mutant catalytic subunits were transformed into *E. coli* BL21-CodonPlus(DE3) together with plasmid pET-D12 encoding the nontagged stimulatory subunit. Cultures (500 mL) derived from single transformants were grown at 37°C in LB medium containing 50 μg/mL kanamycin, 100 μg/mL ampicillin, and 50 μg/mL chloramphenicol until the A_{600} reached ~0.6. The cultures were placed on ice for 30 min and then adjusted to 0.2 mM IPTG and 2% (v/v) ethanol, and incubation was continued for 20 h at 17°C with constant shaking. Cells were harvested by centrifugation and stored at −80°C. All subsequent procedures were performed at 4°C. Thawed bacteria were resuspended in 25 mL of buffer A (50 mM Tris-Hcl at pH 8.0, 150 mM NaCl, 10% glycerol). Phenylmethylsulfonyl fluoride and lysozyme were added to final concentrations of 500 μM and 100 μg/mL, respectively. After incubation on ice for 30 min, Triton X-100 was added to a final concentration of 0.1%, and the lysates were sonicated to reduce viscosity. Insoluble material was removed by centrifugation. The soluble extracts were mixed for 30 min with 2 mL of Ni^{2+}-NTA-agarose (Qiagen) that had been equilibrated with buffer A containing 0.01% Triton X-100. The resins were recovered by centrifugation, resuspended in buffer A, and poured into columns. The columns were washed with 10 mL of 20 mM imidazole in buffer A and then eluted stepwise with 2.5 mL aliquots of buffer A containing 50, 100, 250, and 500 mM imidazole. The 250-mM imidazole eluates containing the recombinant vaccinia proteins were dialyzed against buffer B (50 mM Tris-Hcl at pH 8.0, 100 mM NaCl, 10% glycerol, 2 mM DTT, 2 mM EDTA, 0.01% Triton X-100) and then stored at −80°C. The concentrations of D1-C protein were determined by SDS-PAGE analysis of serial dilutions of the preparations in parallel with serial dilutions of a BSA standard. The gels were stained with Coomassie Blue, and the staining intensities of the D1-C and BSA polypeptides were quantified using a BIO-RAD Molecular Imager ChemiDoc gel densitometry analysis system. D1-C concentrations were calculated by interpolation to the BSA standard curve.

Methyltransferase assay

Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 700 μM GpppA (New England Biolabs), 40 μM [^{3}H-CH_3]-AdoMet (Perkin Elmer Life Sciences), and aliquots of the Ni-agarose preparations of wild-type or mutant recombinant D1-C/D12 (containing 0.3 μg of the D1-C polypeptide) were incubated for 30 min at 30°C. Aliquots (4 μL) were spotted on PEI-cellulose TLC plates, which were developed with 0.05 M ammonium sulfate. The AdoMet- and m^7GpppA-containing portions of the lanes were cut out and the radioactivity in each was quantified by liquid scintillation counting.

Kinetic parameters

GpppA titrations

Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 40 μM [^{3}H-CH_3]-AdoMet, increasing concentrations of GpppA, and wild-type or mutant D1-C/D12 proteins were incubated for 30 min at 30°C.

AdoMet titrations

Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 700 μM GpppA, increasing concentrations of

[^{3}H-CH$_3$]-AdoMet, and wild-type or mutant D1-C/D12 proteins were incubated for 30 min at 30°C. The extents of methyl transfer were plotted as a function of the variable substrate concentration. K_m and k_{cat} values were calculated from double-reciprocal plots of the data. The results are summarized in Tables 2 and 3. Each datum is the average of three independent substrate titration experiments (± mean error).

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