



Short communication

The flaviviral methyltransferase is a substrate of Casein Kinase 1

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ABSTRACT

Serine/Threonine phosphorylation of the nonstructural protein 5 (NS5) is a conserved feature of flaviviruses, but the identity and function(s) of the responsible kinase(s) remain unknown. Serine 56 in the methyltransferase domain of NS5 can be phosphorylated intracellularly, is conserved in all flaviviruses, and is a critical residue in the catalytic mechanism. A negative charge at this residue inactivates the 2'-0 methyltransferase activity necessary to form a 5' cap structure of the viral RNA. Here we show pharmacologic inhibition of Casein Kinase 1 (CK1) suppresses yellow fever virus (YFV) production. We also demonstrate the alpha isoform of Casein Kinase 1 (CK1 α), a kinase previously identified as phosphorylating Hepatitis C Virus NS5A protein, also phosphorylates serine 56 of YFV methyltransferase. Overall these results suggest CK1 activity can influence flaviviral replication.

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The family *Flaviviridae* includes many diverse medical pathogens including Hepatitis C virus (HCV), arthropod-borne diseases such as Dengue virus (DV), YFV and west Nile virus (WNV), as well as veterinary pathogens. Serine/threonine phosphorylation of viral proteins has been demonstrated in at least one member of each of the three genera that constitute the family (Kapoor et al., 1995; Morozova et al., 1997; Reed et al., 1998) and appears to be a general feature of the family. In the genus *flavivirus*, which contain mostly arthropod borne viruses, NS5 has multiple serine/threonine phosphorylation sites (Bhattacharya et al., 2008; Kapoor et al., 1995).

NS5 has both carboxy-terminal RNA polymerase activity and an amino terminal methyltransferase domain, which methylates the cap structure found on the 5' end of viral RNA. This methyltransferase domain sequentially binds two molecules of S-adenosyl-L-methionine (SAM) at a single site and transfers a methyl group to the N7 as well as the 2'-0 positions of the viral RNA cap structure in two separate reactions. Methylation at the N7 site and to a lesser degree the 2'-0 site is critical for viral replication (Ray et al., 2006). The hydroxyl group of serine 56 is important for methyl transfer to the N7 of guanosine in the viral RNA cap and is critical for 2'-0 methylation (Bhattacharya et al., 2008; Dong et al., 2008; Kroschewski et al., 2008). Serine 56 is also phosphorylated intracellularly when NS5 is expressed in HEK293T cells, but the kinase responsible is unknown (Bhattacharya et al., 2008).

A neural net program designed to identify phosphorylation sites (NetphosK 1.0) (Blom et al., 2004) predicts that serine 56 is a CK1 recognition site. The CK1 family of kinases has several isoforms, including the CK1 α isoform which phosphorylates HCV NS5A, but no isoform of CK1 has previously been implicated in the viral life cycle of members of the flavivirus genus. We quantified viral production from HEK293T cells infected with the 17D YFV vaccine strain treated with varying concentrations of a CK1-specific cell permeable inhibitor, 4-(4-(2,3-dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol-2-yl)benzamide (D4776) (Calbiochem, CA) (Rena et al., 2004). Cells were infected at a multiplicity of infection of ~0.25. Media from infected cells was harvested at 48 h and a focus-forming assay (Payne et al., 2006) used to quantify viral yield. Virally infected cells treated with 5 μ M of D4776 produced 4–5-fold less YFV than untreated cells (p value = 0.00594, Student's t test) (Fig. 1). We also tested the effect of D4776 on a YFV replicon (Fig. 2A, Jones et al., 2005) which contains an in frame luciferase so that both luciferase and viral nonstructural proteins are translated from the ribosome binding to the same methylated 5' cap structure. Treatment of HEK293T cells bearing the YFV replicon with D4776 actually resulted in a slight increase in the luciferase marker (Fig. 2B). The increase in viral translation upon CK1 inhibition was confirmed by Western blotting of cell lysates for YFV NS3 (Fig. 2C). While the antibody was barely able to detect NS3 in the absence of D4776, detection was clearly present at the higher drug concentration even though this concentration of D4776 produced less infectious virus. This is consistent with D4776 blocking phosphorylation at S56, thereby allowing more cap methylation of the viral replicon RNA and subsequent protein translation. Concentrations of 10–100 μ M of D4776 are often used in other cell lines

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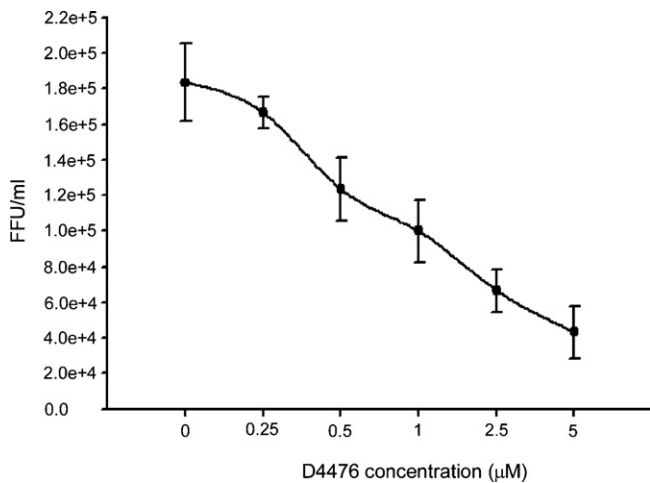


Fig. 1. CK1 inhibitor decreases YFV replication. HEK293T cells were infected with YFV vaccine 17D strain at a multiplicity of infection of ~0.25 and treated with the indicated concentration of D4476. After 48 h the media was harvested and the viral yield was quantitated in a focus-forming assay in triplicate. The viral yield at 5 μM D4476 was statistically different than at 0 μM D4476 (p value = 0.00594, Student's t test).

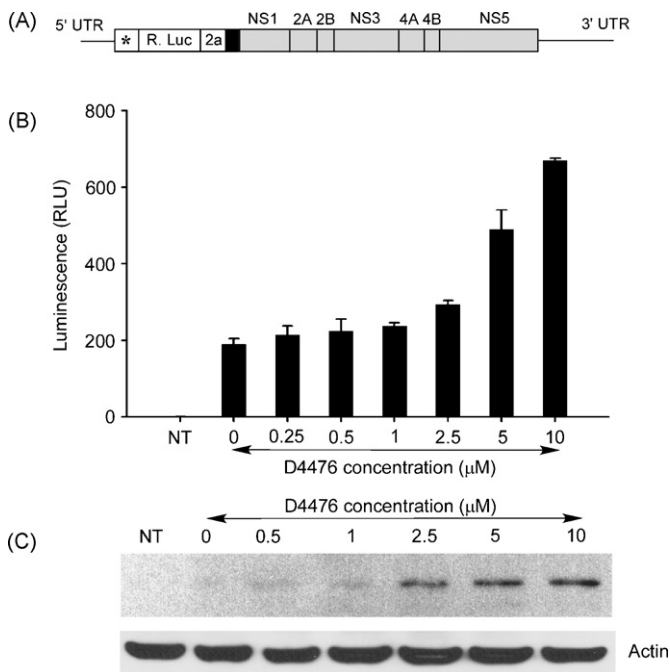


Fig. 2. CK1 inhibitor increases the YFV replicon growth. (A) The schematic representation of YFV replicon construct (Jones et al., 2005) was used to measure the luciferase activity. * indicates first 22 amino acids of capsid protein for RNA cyclization sequences; R. Luc, *Renilla* luciferase; 2a, 17 amino acid residue auto proteolytic peptide from foot and mouth disease virus; black box, NS1 signal sequence; gray box, nonstructural protein sequences. (B) YFV replicon RNA was transfected into HEK293T cells in presence of indicated concentration of D4476 and incubated for 48 hours. The cells were then lysed and the luciferase activity of viral RNA was measured. Non-transfected (NT) cells were also measured parallel for background control. The luciferase activity was increased ~4-fold in presence of 10 μM D4476. (C) Western blot of 4 μg of total protein from whole cell lysate of same preparation as in B by anti YFV NS3 antibody indicates the protein level of YFV replicon was increased in presence of higher concentration of D4476. The lower panel shows a reprobing of this blot with an anti-actin antibody to demonstrate equal protein loading.

to inhibit CK1 (MacLaine et al., 2008; Tillement et al., 2008), but we observed cytotoxicity via a soluble tetrazolium salt cytotoxicity assay at concentrations over 30 μM (data not shown).

D4476 is considered specific for CK1, but the observed decrease in viral yield could either be mediated by a CK1-like kinase or could be an indirect effect of CK1 inhibition on YFV. Therefore we directly tested whether the CK1α or CK1γ2 isoform could phosphorylate YFV methyltransferase. We used a bacterial expression system and metal affinity chromatography to purify histidine tagged YFV NS5 methyltransferase domain (amino acids 1–296) from wild type YFV as well as from a YFV mutant with a serine to alanine change at residue 56 (S56A) (Bhattacharya et al., 2008). We then incubated each NS5 substrate with γ - 32 P-ATP and one of the CK1 isoforms (Upstate, CA) for 15 min at 30 °C. Samples were separated using SDS-PAGE and labeled proteins visualized using autoradiography. CK1α was able to label the methyltransferase protein when the phosphoacceptor site is present (serine 56), but to a much lower degree with the alanine mutant. CK1γ2 was only able to autophosphorylate itself (Fig. 3).

This reaction was also performed with wild type methyltransferase protein, nonradioactive ATP and CK1α, and the products subjected to SDS-PAGE. The band was excised and trypsinized. Reaction products were analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. This analysis found a peak 80 Da larger (m/z 983.54815) than the predicted mass of the peptide containing serine 56 (peptide VDTGVAVSR) and is consistent with a single phosphorylated residue (Fig. 4A). The same phosphorylation event was detected in YFV NS5 expressed *in vivo* (Bhattacharya et al., 2008). Of the six sites found after intracellular expression, only serine 56 is predicted to be a CK1α phosphorylation site. The tryptic digest of the *in vitro* phosphorylation reaction was also analyzed using Electrospray ionization liquid chromatography mass spectrometry (LC-MS/MS). The observed fragment ionization from both directions also supports serine 56 phosphorylation in this peptide (Fig. 4B).

Several groups including ours have shown that the hydroxyl side chain of S56 is critical for methyltransferase activity and viral growth in at least three flaviviruses including YFV (Bhattacharya et al., 2008; Dong et al., 2008; Kroschewski et al., 2008). This is the first report that CK1α can phosphorylate S56, although we previously showed that S56 is phosphorylated in the cell when NS5 is expressed by itself. A phosphomimetic aspartic acid mutant replacing serine 56 is defective in the replicon (Bhattacharya et al., 2008). This severe defect from mutant S56 is consistent with the requirement for the hydroxyl group in the methylation reaction, and does not occur from inhibiting CK1α activity.

The CK1 protein kinase family has at least seven mammalian isoforms (α, β, γ1, γ2, γ3, δ, and ε) and multiple splice variants (Knippschild et al., 2005). CK1 isoforms phosphorylate a wide variety of host cell substrates involved in cell differentiation, proliferation, and circadian rhythms. While CK1α has been implicated in HCV NS5A hyperphosphorylation (Quintavalle et al., 2007), it remains unclear why this event has been conserved throughout the divergent evolution of HCV. CK1α is also known to phosphorylate Rotavirus NS5 (Eichwald et al., 2004). The serine 56 phosphoacceptor site, as well as the surrounding residues that constitute the recognition site, are highly conserved in all flaviviral genomes including the mosquito borne lineage (YFV, DV and WNV), as well as the more divergent tick born lineage (Bhattacharya et al., 2008). While CK1α has been implicated in viral infections, we are unaware of other CK1 isoforms being implicated.

While CK1 isoforms are highly conserved in the kinase domain, they vary widely in substrates. Typically CK1 recognition sites are "primed" by a previous phosphorylation event N terminal to the CK1 recognition site. Primed sites are 15–25-fold more efficient compared to unprimed site (Flotow et al., 1990; Marin et al.,

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