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Cyclophilin B stimulates RNA synthesis by the HCV RNA dependent RNA polymerase

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Abstract

Cyclophilins are cellular peptidyl isomerases that have been implicated in regulating hepatitis C virus (HCV) replication. Cyclophilin B (CypB) is a target of cyclosporin A (CsA), an immunosuppressive drug recently shown to suppress HCV replication in cell culture. Watashi et al. recently demonstrated that CypB is important for efficient HCV replication, and proposed that it mediates the anti-HCV effects of CsA through an interaction with NS5B (Mol. Cell 19:111). We examined the effects of purified CypB proteins on the enzymatic activity of NS5B. Recombinant CypB purified from insect cells directly stimulated NS5B-catalyzed RNA synthesis. CypB increased RNA synthesis by NS5B derived from genotype 1a, 1b, and 2a HCV strains. Stimulation appears to arise from an increase in productive RNA binding. NS5B residue Pro540, a previously proposed target of CypB peptidyl-prolyl isomerase activity, is not required for stimulation of RNA synthesis.

Keywords

RNA dependent RNA polymerase; peptidyl-prolyl isomerase; Hepatitis C virus; cyclosporin; cyclophilin

1. Introduction

The hepatitis C virus (HCV), a single-stranded RNA virus of the *Flaviviridae* family, chronically infects 170 million people worldwide [1,2]. The HCV genome directly encodes a single polyprotein precursor that is proteolytically processed into at least 10 proteins. Like other viruses, HCV also requires host cell proteins for replication. Recent work has implicated a class of cellular proteins known as cyclophilins in regulating HCV replication [3-6]. Cyclophilins are peptidyl prolyl isomerases that facilitate protein folding by catalyzing the cis-trans interconversion of peptide bonds at proline residues. Cyclophilins are also the intracellular ligands of cyclosporin A (CsA). CsA is an immunosuppressive drug, widely administered to transplant patients, that has also been shown to suppress HCV replication in a subgenomic replicon system [7-9]. A role for cyclophilins as viral cofactors was first uncovered when cyclophilin A (CypA) was found to have a critical role in HIV-1 infection [10,11]. The discovery that CsA has anti-HCV activity has led to investigations of cyclophilins as cofactors in HCV replication.

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CsA resistance studies have implicated the viral enzymes NS5A and NS5B, the RNA-dependent RNA polymerase responsible for replication of the HCV genome, as targets of CsA [12,13]. However, there is some controversy regarding which of the at least 16 human cyclophilins mediate the anti-HCV activity of CsA. CypB was the first to be implicated, in work by Watashi et al. showing that knockdown of CypB, but not of cyclophilins A, C, E or H, inhibited replication of HCV replicons in human hepatic cells [3]. In that study, CypB was found to interact with and stimulate the RNA binding activity of NS5B. Another study suggested that cyclophilins A, B and C all have roles [5]. More recently, a third group found that CypA, but not B, is important for replication of HCV replicons [4]. It is possible that various cyclophilins play multiple and/or overlapping roles in HCV replication. Encouraged by the compelling evidence that CypB is a likely cofactor for the NS5B polymerase [3,14], we have focused on characterizing the CypB-NS5B interaction using purified proteins. We report here that CypB expressed and purified using a eukaryotic protein expression system is capable of enhancing the RNA synthesis activity of NS5B.

2. Materials and methods

2.1. Proteins

NS5B proteins lacking the C-terminal 21 amino acids (NS5B Δ 21) and bearing C-terminal His tags were expressed in Rosetta (DE3) *E. coli* and purified as described previously [15].

For construction of the NS5B Δ 55_1b(Con1) expression plasmid, the coding sequence for NS5B lacking 55 C-terminal residues was PCR amplified from the cDNA of the genotype 1b_Con1 HCV isolate, pBDL429P⁺S⁺ [16]. The following primer pair was used: 5'-CTA GCT AGC ATG TCC TAC ACA TGG ACA-3' (NheI site underlined) and 5'-GCG CGC CTC GAG TTT GAG CTT GGT CCT TAC TGC CCA GTT-3' (XhoI site underlined). The purified PCR product and pET23a vector (Novagen, Madison, WI) were digested with NheI and XhoI and ligated to generate an expression plasmid for a C-terminally His-tagged protein. NS5B Δ 55_1b(Con1) protein was expressed and purified in the same manner as the NS5B Δ 21 proteins described previously [15].

Recombinant baculovirus expressing the human CypB gene was generated using the Bac-to-Bac system (Invitrogen, CA) using *Spodoptera frugiperda* (Sf9) cells that were maintained in HyQ-SFX –Insect medium (HyClone, UT) supplemented with 2.5% fetal bovine serum. Briefly, the coding sequence of CypB gene from pCMV-CypB cloning vector (Accession number NM_00942.4; OriGene, Rockville, MD) was sub-cloned into pFastBac HTB transfer vector using EcoR I and Not I sites, resulting in the full-length CypB fused to an N-terminal 6XHis tag. pFastBac-CypB was then transformed into *E. coli* DH10Bac to generate a bacmid containing the CypB gene. The recombinant bacmid was used to transfect Sf9 cells (single layer culture) to generate recombinant baculovirus. The first generation of recombinant virus was then amplified to generate a high titer virus stock (2×10^8). Sf9 cells (2×10^6 , in spinner flask) were infected with recombinant virus with MOI = 5-10, incubated at 27°C and harvested after 48 hours. The cell pellet was stored at -80°C until use. For purification of His-CypB, the cell pellet was resuspended in 50mM sodium phosphate, pH 7.8, 400mM NaCl, 10% glycerol, 0.5% Triton X-100, 5mM β -mercaptoethanol, and 20mM imidazole (buffer A) supplemented with protease inhibitor cocktail III from Calbiochem (Gibbstown, NJ). Cells were lysed by sonication and the extract was cleared by centrifugation at $13,000 \times g$ for 20 min. The cleared extract was filtered and loaded onto a Ni-nitrilotriacetic acid column (Novagen, Madison, WI) equilibrated with buffer A. The column was washed and eluted with buffer A containing a gradient of imidazole from 20 mM to 1 M. Fractions containing His-CypB were combined and dialyzed overnight against buffer B containing 50 mM Hepes (pH7.5), 200 mM NaCl, 30% glycerol, 0.2% Triton X-100, 0.1 mM DTT, 0.1 mM EDTA. The purified protein was concentrated in a Centricon-10 spin column (Amicon, Beverly, MA).

For expression of CypB in *E. coli*, the CypB coding sequence lacking the N-terminal 25 residues was amplified from pCMV-CypB using the following primers purchased from Integrated DNA Technologies (Coralville, IA): 5'- GCG CGC ACT AGT GAT GAG AAG AAG AAG GGG C -3' and 5'- GCG CGC CTC GAG CTA CTC CTT GGC GAT GGC-3' (restriction sites underlined). The purified PCR product was digested with SpeI and XhoI and ligated to the SpeI/XhoI digested pET41 vector (Invitrogen, CA) to generate an expression construct for N-terminally GST-tagged CypB. The GST-CypB expression plasmid was transformed into *E. coli* Rosetta (DE3) (EMD Chemicals, Inc., Gibbstown, NJ). Cells harboring the expression plasmid were grown overnight at 30°C. Cells from 20 ml of the starter culture were harvested and used to inoculate 2 liters of LB medium. Cells were grown at 37°C to an absorbance at 600 nm of 0.6 and induced with 1 mM isopropyl- β -D-thiogalactopyranoside. After growth at 25°C for 20 h, cells were harvested by centrifugation and resuspended in 50 mM sodium phosphate (pH 7.3), 150 mM NaCl (buffer A). Cells were lysed by sonication, and the extract was cleared by centrifugation at $18,500 \times g$ for 10 min. The cleared extract was filtered and loaded onto a GST-Bind column (Novagen, Madison, WI) equilibrated with buffer A. The column was washed with buffer A and eluted with buffer A containing 10 mM reduced glutathione. The fraction containing GST-CypB was dialyzed against buffer A containing 30% glycerol for storage.

2.2. RNA-dependent RNA polymerase assays

RdRp activity was assayed using polyC as a template for incorporation of [α - 32 P]GTP. Unless otherwise noted, reactions were performed in a total volume of 10 μ l containing 20 mM Hepes-KOH (pH 7.5), 10 mM NaCl, 10 mM MgCl₂, 4U Suprase-In™ (Ambion, Austin, TX), 3 mM DTT, 25 μ g/ml polyC (71.5 μ M C; Sigma, St. Louis, MO), 500 μ M [α - 32 P]GTP (MP Biomedicals, Solon, OH), and 0.2 μ M NS5B. Reactions were incubated at room temperature for 30 minutes and terminated with 5 μ l 0.5 M EDTA. The reactions were spotted onto DE81 filter papers which were washed three times for 15 minutes in 0.5 M sodium phosphate (pH 7) and washed twice in 70% ethanol before counting of the radioactive product in a liquid scintillation counter.

2.3. GST pull-down

100 μ l of GST-Bind resin (Novagen, Madison, WI) with $\sim$ 1 nmol GST-CypB protein was incubated with 50 pmol NS5B Δ 21_1b(Con1) protein in 1 ml of binding buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40) at 4°C for 17 hours. The resin was washed five times with the same buffer. Bound proteins were eluted with SDS-PAGE loading buffer and analyzed by 12% SDS-PAGE.

3. Results

3.1. *E. coli*-expressed GST-CypB does not stimulate NS5B-catalyzed RNA synthesis

Recent studies by the Shimotohno group have demonstrated that an *E. coli*-expressed GST-CypB fusion protein is capable of interacting with NS5B and enhancing its RNA binding activity [3,6]. To characterize the role of CypB in HCV replication further, we examined the effects of GST-CypB on RNA synthesis by NS5B. CypB with an N-terminal GST tag was expressed in *E. coli* and purified by GST affinity chromatography (Fig. 1A). GST-CypB was previously shown to coprecipitate full-length, in vitro translated NS5B [3,6,13]. Using a GST pull-down assay, we found that GST-CypB, but not GST, interacted with purified NS5B Δ 21_1b(Con1) (Fig. 1B). NS5B-catalyzed RNA dependent RNA synthesis was monitored by measuring the incorporation of [α - 32 P] GTP into new RNA using polyC RNA as a template. Reactions were carried out under optimized conditions as determined previously [15] and included 200 nM polymerase, 25 μ g/ml polyC (71.5 μ M C; average length 2500 nt), 0.5 mM [α - 32 P] GTP, and up to 600 nM GST-CypB. The amount of [α - 32 P] GTP incorporated

during a 30 minute reaction was used to calculate the rates of RNA synthesis at increasing concentrations of GST-CypB. GST-CypB had no effect on the amount of RNA synthesized and hence the specific activity (v/e) of NS5BΔ21_1b(Con1) (Fig. 1C) or NS5BΔ21_1b(J4) (not shown) at any concentration tested.

3.2. His-CypB expressed in insect cells stimulates RNA synthesis by HCV genotype 1a, 1b and 2a polymerases

We reasoned that for full activity, human CypB might require posttranslational modification that might be achieved in a eukaryotic protein expression system. We cloned CypB into a baculovirus vector and expressed it in insect cells as an N-terminally His-tagged protein. His-CypB was then partially purified by Ni²⁺ column chromatography (Fig. 2A). When this protein was added to RNA synthesis reactions containing 200 nM NS5BΔ21_1b(J4), we observed a concentration-dependent increase in RNA synthesis, up to approximately four-fold (Fig. 2B). The greatest increase in RNA synthesis was observed when polymerase and His-CypB were present in approximately equimolar concentrations. As discussed in detail below, His-CypB showed stimulation of NS5BΔ21 from the genotype 1b strains J4 and Con1, as well as from genotype 1a and 2a strains. A time course of RNA synthesis by 290 nM NS5BΔ21_1b(Con1) in the presence and absence of 90 nM His-CypB revealed a clear difference, and that the rate of RNA synthesis accelerated while CypB was incubated with NS5B (Fig. 2C).

Several control RNA synthesis reactions were carried out to address the specificity of the effect of His-CypB on NS5BΔ21_1b(Con1)-catalyzed RNA synthesis (Fig. 2D). It was possible that the increase in RNA synthesis was due to a contaminant that either possessed RNA synthesis activity or enhanced RNA synthesis by NS5B (e.g., RNA or metal cations). No RNA synthesis was observed when His-CypB was added to reactions in the absence of polymerase, indicating that the effect was not due to an RNA synthesis activity co-purifying with His-CypB (not shown). 80 nM His-CypB that was boiled prior to addition to reactions had no effect on RNA synthesis by 200 nM NS5BΔ21_1b(Con1), indicating that a protein component of the preparation was responsible for the stimulatory activity and that there were no non-heat labile contaminants affecting RNA synthesis (column 3). To examine further whether a contaminant from the insect cells in which His-CypB was expressed could be influencing our results, an extract from uninfected Sf9 cells was prepared in the same manner as His-CypB. A slight increase in RNA synthesis was observed when 0.1, 0.25 or 0.5 μl of this extract was added to the 10 μl reactions containing 200 nM NS5BΔ21_1b(Con1); however, the effect was minor compared to that achieved with His-CypB (~1.5- vs. 3.4-fold), and did not correlate with the amount of extract added (columns 4, 5, 6).

3.3. NS5B Pro540 is not required for stimulation by His-CypB

Cyclophilins isomerize peptide bonds amino-terminal to proline residues. The study by Watashi et al. implicated Pro540 of NS5B in activation by CypB, with a P540A mutation reducing the interaction of NS5B with CypB in GST pull-down and immunoprecipitation assays [3]. The P540A mutation also decreased RNA binding by NS5B in replicon cells, as demonstrated in an RNA-protein binding precipitation assay [3]. To examine the role of P540 we took advantage of HCV strains that naturally vary at this residue, and also tested the interaction of CypB with a truncated NS5B lacking the P540 region.

We examined the effect of His-CypB on purified NS5BΔ21 derived from the genotype 1a (H77), 1b(Con1), 1b(J4) and 2a(J6) strains. We previously characterized these four NS5BΔ21 variants and found that, while the specific activities vary substantially, optimal conditions for RNA synthesis are similar for all four [15]. Alignment of the protein sequences reveals differences in two regions separately identified as important for CypB interaction [3,13]. These

differences include P/A 540 and two residues implicated in CsA resistance (V/I 432 [12] and S/G 556 [13]) (Fig. 3A).

In contrast to most HCV isolates including the 1b and 2a strains studied here, NS5B_1a(H77) naturally bears an alanine at residue 540. RNA synthesis by NS5B Δ 21_1a(H77) increased ~1.6-fold in the presence of His-CypB, while that of the 1b(J4), 1b(Con1) and 2a (J6) NS5B Δ 21 enzymes increased 1.8-, 3.0- and 3.5-fold, respectively (Fig. 3B).

To examine the role of P540 further, we also tested the effect of His-CypB on RNA synthesis catalyzed by NS5B Δ 55_1b(Con1), whose C-terminal truncation encompasses residue P540. Again increased RNA synthesis was observed in the presence of His-CypB, although less than for NS5B Δ 21 from the same strain (~2.3- vs. ~3.8-fold) (Fig. 3C). These results confirm that Pro540 is not necessary for stimulation by CypB.

3.4. His-CypB increases the productive RNA binding capacity of NS5B Δ 21

To examine the molecular basis for the stimulatory effect of His-CypB, we tested its effects on the interaction of NS5B Δ 21 with template and substrates in the RNA synthesis assay. First we examined if His-CypB affects the K_m of GTP. We previously determined the K_m of NS5B Δ 21_1b(Con1) for GTP to be 57 μ M [15]. 200 nM His-CypB had no effect on the K_m for GTP, as determined by titrating GTP into RNA synthesis reactions containing 200nM NS5B Δ 21_1b(Con1) (data not shown).

We next examined how CypB affects the interaction of NS5B with the template that it uses to synthesize new RNA. Watashi et al. previously showed that in the presence of CypB, an increased proportion of in vitro translated NS5B binds to an RNA-sepharose column [3]. Such a result could be explained if either CypB increases the affinity of NS5B for RNA or if CypB increases the binding capacity of NS5B. To distinguish between these two possibilities, we examined NS5B catalyzed RNA synthesis at various NS5B Δ 21, polyC RNA, and His-CypB concentrations. When NS5B Δ 21_1b(Con1) was titrated with polyC, its ability to synthesize new RNA increased until the protein was saturated, after which point excess polyC behaved as a classic “substrate inhibitor” (Fig. 4A). At low RNA concentrations, binding was stoichiometric, meaning that all RNA was bound to the protein, making an estimation of the affinity impossible to accurately estimate. In other words, binding was tight, and the dissociation constant must be below the lowest protein concentration used, *i.e.* less than 25 nM. Other groups have also observed such tight binding and noted the fact that excess template RNA inhibits steady-state rates of RNA synthesis by NS5B [15,17,18]. Tight binding is shown more clearly when the same titration is repeated at several different protein concentrations, and when this is done, it is immediately apparent that optimal activity depends on the template/enzyme ratio (Fig. 4A). The RNA binding capacity of each protein concentration was estimated by fitting a bell-shaped curve to the data. The peak of the curve was used to determine the RNA concentration that leads to peak activity, and this RNA concentration was linearly related to the amount of enzyme in the reaction (Fig. 4C).

To examine how CypB influences RNA binding, an RNA titration was repeated with 25 nM His-CypB added to RNA synthesis reactions containing 50 nM of NS5B Δ 21_1b(Con1). The presence of His-CypB shifted peak RNA synthesis from 12 μ M to 40 μ M polyC, an RNA concentration that was inhibitory in the absence of His-CypB (Fig. 4B). Our data suggest that His-CypB-activated NS5B Δ 21 productively binds 3 times as much RNA as the untreated polymerase (Fig. 4C). A plausible explanation for these data is that His-CypB simply increases the RNA binding capacity of NS5B by increasing the proportion of purified protein that is competent for RNA synthesis. Such an explanation is consistent with evidence that preparations of purified NS5B Δ 21 contain only a small portion of active protein [19]. It should

be noted, however, that these data do not exclude the possibility that His-CypB also increases the affinity of NS5B for RNA.

3.5. Mn^{2+} masks the CypB stimulatory effect on NS5B Δ 21

We next examined the effect of His-CypB on metal cation usage by the polymerase. NS5B Δ 21 proteins require divalent metal cations to synthesize RNA. We previously determined 10 mM $MgCl_2$ and 1 mM $MnCl_2$ to be the optimal concentrations for the four NS5B Δ 21 enzymes [15]. However, we noted that the ability of His-CypB to stimulate RNA synthesis was reduced in the presence of $MnCl_2$. We examined the impact of varying metal ion concentrations on stimulation of NS5B Δ 21_1b(J4) RNA synthesis activity by His-CypB (Fig. 5). Standard RNA polymerase assays containing 200 nM NS5B Δ 21_1b(J4) were titrated with $MgCl_2$ only (Fig. 5A), $MnCl_2$ only (Fig. 5B), or $MnCl_2$ in the presence of 10 mM $MgCl_2$ (Fig. 5C) in the presence or absence of 200 nM His-CypB. For all concentrations of $MgCl_2$ tested, approximately twice as much RNA synthesis was observed when His-CypB was present (Fig. 5A). In contrast, increasing concentrations of $MnCl_2$, either alone or in the presence of 10 mM $MgCl_2$, resulted in a decrease in the stimulatory activity of His-CypB (Fig. 5B & C). A similar effect of $MnCl_2$ was observed with all four NS5B Δ 21 enzymes (data not shown).

4. Discussion

Understanding the mechanism(s) by which CsA inhibits HCV could provide new therapeutic targets and allow improvements of drugs currently in development. Indeed, non-immunosuppressive cyclosporine derivatives, including NIM811 and Debio-025, have been found to be potent inhibitors of HCV replication. NIM811 suppressed replication of subgenomic and full-length genomic HCV replicons in cell culture [20,21]. Debio-025 likewise showed potent inhibition of HCV replication in vitro, and also significantly reduced viral load in HCV patients [8,14,20,21]. Along with CsA, these compounds are thought to function as HCV inhibitors by sequestering cyclophilins, blocking their ability to stimulate viral replication. Consistent with this model, in patients treated with Debio-025 reductions in viral load coincided with decreased CypB levels [14]. However, details about the mechanism of these inhibitors remain unclear, as several studies have produced conflicting results regarding which cyclophilins are involved, as well as which viral proteins are the principal targets [3,4,6,12,13]. The results of enzyme assays presented here support a role for CypB as a cofactor in HCV replication through direct stimulation of RNA synthesis by the viral polymerase. However, one must not over-interpret these results, as there is still direct no evidence that CypB alters the NS5B protein structure. On a similar note, we have not yet observed any effect of CsA in our in vitro NS5B assays, either in the presence or absence of CypB (data not shown). This inability of CsA to disrupt the interaction observed here is inconsistent with the idea that CsA inhibits HCV replication by sequestering CypB. Different reaction conditions may be needed to observe an effect of CsA in our system, but further work is needed to address this issue.

We observed that His-CypB purified from insect cells, but not GST-CypB purified from *E. coli*, was capable of elevating levels of RNA synthesis by NS5B derived from HCV strains of the 1a, 1b and 2a genotypes. Studies using HCV replicons have suggested that the effect of CypB on HCV replication varies with genotype, with the 2a(JFH1) replicon showing less dependence on CypB than genotype 1b replicons [6]. While we did observe some variation with genotype, NS5B from the genotype 2a(J6) strain studied here showed significant enhancement of RNA synthesis in the presence of CypB. Our future plans include an analysis of the 2a(JFH1) polymerase to compare it to the 2a(J6) polymerase and to determine whether enzyme assays will confirm the observations made with replicons.

The observed stimulation of the NS5B Δ 21_1a(H77) and NS5B Δ 55_1b(Con1) polymerases by CypB was unexpected, given that they lack the Pro540 residue previously suggested to be a target of CypB peptidyl isomerase activity [3]. However, it is consistent with other work indicating that the CypB interaction domain of NS5B does not encompass residue 540 [13]. NS5B Δ 21_1a(H77) and NS5B Δ 55_1b(Con1) showed slightly less of an increase in RNA synthesis in the presence of CypB than polymerases from the other strains and NS5B Δ 21_1b(Con1), respectively. CypB may target multiple peptide bonds within NS5B; it is also possible that isomerase activity is not required for its effect on NS5B.

Surprisingly, *E. coli*-expressed CypB was recently reported to inhibit RNA synthesis by HCV NS5B Δ 21 [22], in contrast to its previously proposed role as an activator [3] and to the results presented here. In that study, CypA also inhibited the polymerase. In our hands, bacterially-expressed GST-CypB interacted with NS5B Δ 21_1b(Con1) (Fig. 1B), but neither inhibited nor stimulated NS5B RNA synthesis. It may be that post-translational modifications of CypB are necessary for its ability to interact productively with NS5B, and/or that the relatively bulky GST tag interferes with CypB activity. Work is under way in our lab to test additional CypB constructs, including CypB co-expressed with NS5B, for NS5B stimulatory activity. Differences in assay conditions may also have contributed to the different results. We found that Mn^{2+} and RNA template concentrations influenced the effects of His-CypB on RNA synthesis. The effects of CypB could also differ depending on the template used; whereas we observed stimulation with a polyC template, the inhibition by CypB reported by the Kao group was observed with an oligo template [22].

CypB appears to alter the interaction of NS5B with RNA, increasing the RNA binding capacity of an enzyme preparation, which suggests an increase in productive RNA binding (Fig. 4). Wang *et al.* have proposed that template inhibition of NS5B is due to binding of template to a secondary site on the polymerase, excluding productive binding at the primary site [17]. CypB might act by blocking binding at the secondary site, directly or by inducing a conformational change in NS5B. Our results are consistent with previous work showing that CypB enhances RNA binding by the polymerase [3].

The ability of CypB to increase NS5B-catalyzed RNA synthesis depended in part on the metal cation used. A greater effect of CypB on RNA synthesis was observed when Mg^{2+} was the sole metal cation, whereas increasing concentrations of Mn^{2+} resulted in decreasing stimulation by CypB (Fig. 5). Mn^{2+} is thought to aid formation of the initiation complex [23]. One explanation for our observations is that CypB likewise enhances initiation complex formation, and that excess Mn^{2+} therefore masks its stimulatory effect. As Mg^{2+} is likely the physiological metal of choice, aiding the initiation of RNA synthesis could be an important function of CypB. This hypothesis is presently being tested in our lab.

Our finding that CypB stimulates NS5B-catalyzed RNA synthesis supports a model in which the CypB-NS5B interaction contributes to regulation of HCV replication. A recent report indicates that CypA likewise interacts with NS5B and may have a greater role than CypB in mediating the effects of CsA [4], an idea that can now be tested using the system reported here. Further insight into the various contributions of cyclophilins to HCV replication will be valuable in allowing these interactions to be exploited for therapeutic purposes.

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Fig. 1.

Effect of *E. coli* expressed CypB on NS5B catalyzed RNA synthesis. **(A)** Analysis of purified recombinant *E. coli* expressed GST-CypB protein by SDS-PAGE. Protein bands were separated on a 12% polyacrylamide-SDS gel and visualized by Coomassie blue staining. Lane 1, marker; lane 2, crude extract; lane 3, fraction obtained by GST affinity chromatography (7.4 μg GST-CypB). The sizes of the protein markers in kDa are indicated. **(B)** Interaction of GST-CypB with NS5B Δ 21_1b(Con1) was analyzed by GST pull-down. 50 pmol NS5B Δ 21_1b(Con1) was incubated with resin alone, ~ 1 nmol GST or ~ 1 nmol GST-CypB. After extensive washing, the samples were analyzed by 12% SDS-PAGE. Protein bands were visualized by Coomassie blue staining. Input represents one fifth of the amount used in the pull-down assay.

(C) RNA dependent RNA synthesis catalyzed by 200 nM NS5B Δ 21_1b(Con1) in the presence of increasing concentrations of GST-CypB was monitored by measuring the incorporation of [α - 32 P] GTP with a polyC RNA template, as described in Materials and Methods. Reactions were carried out in duplicate, and error bars represent standard deviations.

Fig. 2.

Effect of insect cell expressed CypB on NS5B catalyzed RNA synthesis. **(A)** Analysis of His-CypB expressed and purified from Sf9 insect cells by SDS-PAGE. Protein bands were separated on a 15% polyacrylamide-SDS gels and visualized by Coomassie blue staining. Lane 1, marker; lane 2, fraction obtained by Ni²⁺ column chromatography (0.4 μg His-CypB). The sizes of the protein markers in kDa are indicated. **(B)** RNA dependent RNA synthesis catalyzed by 200 nM NS5BΔ21_1b(J4) in the presence of increasing concentrations of His-CypB was monitored as described in Materials and Methods, except that reactions were terminated after 60 min. Mean values were obtained from three independent replicates, and error bars represent standard deviations. **(C)** Time course of RNA synthesis in reactions with 290 nM NS5BΔ21_1b (Con1) +/- 90nM His-CypB. All reactions in panel B included 25 mM KCl, but were otherwise as described in Materials and Methods. **(D)** RNA synthesis catalyzed by NS5BΔ21_1b(Con1) was monitored as described in Materials and Methods, in the presence of storage buffer (NS5B only), 80 nM His-CypB, 80 nM boiled His-CypB, or three different volumes of insect cell extract (0.1, 0.25 and 0.5 μl). The insect cell extract ("mock extract") was derived from uninfected Sf9 cells and was prepared in the same manner as His-CypB. Relative rates of RNA synthesis were normalized to reactions containing 200 nM NS5BΔ21_1b(Con1), i.e., "NS5B only," which was set to 1. Mean values were obtained from two to four independent replicates, and error bars represent standard deviations.

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Fig. 4.

Effect of His-CypB on RNA binding by NS5BΔ21. A) RNA synthesis reactions containing various amounts of NS5BΔ21_1b(Con1) were titrated with polyC RNA. Initial rates of RNA synthesis (nmol/min) and the amount of protein in each reaction (nmol) were used to calculate protein specific activity (v/e) under each condition. B) RNA synthesis reactions containing 50 nM NS5BΔ21_1b(Con1) with or without 25 nM His-CypB were titrated with PolyC RNA. In both *panel A* and *panel B* data are fit to a steady state rate equation describing substrate inhibition ($v/e = k_{\text{cat}} * [\text{RNA}] / (K_m + X * (1 + [\text{RNA}] / K_i))$) using non-linear regression. C) PolyC template concentration conferring peak specific activity observed in each titration plotted versus enzyme concentration used in each series of reactions. Data are fit by linear regression.

Fig. 5.

Effects of metal cations on the stimulatory activity of His-CypB. NS5B Δ 21_1b(J4)-catalyzed RNA synthesis reactions with or without 200nM His-CypB were titrated with (A) MgCl_2 , (B) MnCl_2 , and (C) MnCl_2 in the presence of 10mM MgCl_2 . Reactions were performed in duplicate; error bars represent standard deviations.