Requirement for cyclophilin A for the replication of vesicular stomatitis virus New Jersey serotype

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Several host proteins have been shown to play key roles in the life-cycle of vesicular stomatitis virus (VSV). We have identified an additional host protein, cyclophilin A (CypA), a chaperone protein possessing peptidyl cis-trans prolyl-isomerase activity, as one of the cellular factors required for VSV replication. Inhibition of the enzymatic activity of cellular CypA by cyclosporin A (CsA) or SDZ-211-811 resulted in a drastic inhibition of gene expression by VSV New Jersey (VSV-NJ) serotype, while these drugs had a significantly reduced effect on the genome expression of VSV Indiana (VSV-IND) serotype. Overexpression of a catalytically inactive mutant of CypA resulted in the reduction of VSV-NJ replication, suggesting a requirement for functional CypA for VSV-NJ infection. It was also shown that CypA interacted with the nucleocapsid (N) protein of VSV-NJ and VSV-IND in infected cells and was incorporated into the released virions of both serotypes. VSV-NJ utilized CypA for post-entry intracellular primary transcription, since inhibition of CypA with CsA reduced primary transcription of VSV-NJ by 85–90%, whereas reduction for VSV-IND was only 10%. Thus, it seems that cellular CypA binds to the N protein of both serotypes of VSV. However, it performs an obligatory function on the N protein activity of VSV-NJ, while its requirement is significantly less critical for VSV-IND N protein function. The different requirements for CypA by two serologically different viruses belonging to the same family has highlighted the utilization of specific host factors during their evolutionary lineages.

INTRODUCTION

Vesicular stomatitis virus (VSV), a non-segmented negative-strand RNA virus belonging to the Rhabdoviridae family, causes disease in economically important livestock (Hanson, 1952; Rose & Whitt, 2001). On infection, a virion-associated transcriptase transcribes the VSV genome of 11 kb into five mRNAs that code for the nucleocapsid (N) protein, phosphoprotein (P), the RNA polymerase L protein, matrix (M) and glycoprotein (G) (Banerjee, 1987). Of these five proteins, the N, P and L proteins comprise the viral ribonucleoprotein (RNP) complex, which is required for intracellular transcription/replication of the virus genome (Banerjee, 1987). The other two proteins, M and G, are involved in virus budding and entry, respectively (Chong & Rose, 1993; Lyles et al., 1992). In addition to virally encoded proteins, VSV utilizes several host proteins for its productive life-cycle (Gupta et al., 1998; Barik & Banerjee, 1992; Moyer et al., 1986; Das et al., 1998; Harty et al., 1999, 2001). We were initially interested in identifying and characterizing the cellular receptor(s) involved in the entry process of human parainfluenza virus type 3 (HPIV-3) and VSV (Bose et al., 2001; Bose & Banerjee, 2002). In that context, we were particularly interested in the observation from an earlier report that demonstrated the presence of cyclophilin A (CypA), a chaperone protein (Takahashi et al., 1989; Gothel & Marahiel, 1999), in the envelope of human immunodeficiency virus type 1 (HIV-1) virions and its requirement for HIV-1 cellular entry (Saphire et al., 1999). Interestingly, one of the results from this study showed that VSV-G pseudotyped virus containing the HIV-1 genome with a mutation in the CypA-binding domain of the HIV-1 Gag protein failed to bind to the cells, suggesting that CypA might also play a role in VSV entry. Thus, we evaluated a possible role for CypA in the life-cycle of two VSV serotypes, Indiana (VSV-IND) and New Jersey (VSV-NJ), which share moderate homology at the genomic level (Rose & Whitt, 2001).

Surprisingly, we found that CypA was not involved in either cellular entry or budding, but rather was required for post-entry primary transcription of VSV-NJ and to a significantly lesser extent, VSV-IND. In addition, both VSV-NJ and VSV-IND virions packaged CypA, although the role of CypA in VSV-NJ infection was more critical compared with its role in VSV-IND infection. These results demonstrate the specific utilization of a host factor by two serologically distinct viruses belonging to the same family, indicating their possible divergence during evolutionary lineages.
**METHODS**

**Virus, cells and viral RNP.** VSV-IND (Mudd–Summers strain) and VSV-NJ (Ogden strain) were propagated in baby hamster kidney (BHK) cells as described previously (Peluso & Moyer, 1983). BHK, HeLa, A549 and L929 cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco-BRL) supplemented with 10% foetal bovine serum, penicillin, streptomycin and glutamine. RNP was isolated from purified VSV-IND, VSV-NJ and HPIV-3 virions as described previously (De et al., 1991; Gupta & Banerjee, 1997). The purity of the isolated RNPs (2 μg protein) was confirmed by 10% SDS-PAGE and Coomassie blue staining of the gels. VSV-IND and VSV-NJ were biotinylated as described previously (Naunyck et al., 1999). [35S]Methionine-labelled VSV-IND and VSV-NJ were prepared by labelling VSV-infected cells with [35S]methionine and isolating radiolabelled virus from the medium supernatant (Bose et al., 2001).

**Cyclosporin A and SDZ-211-811 treatment.** To study the effect of cyclosporin A (CsA; Sigma) and SDZ-211-811 (gift from Barbara Willi, Novartis Pharma AG, Basel, Switzerland) treatment on VSV infection, BHK cells were inoculated with either VSV-IND or VSV-NJ (m.o.i.s of 0–1, 1, 2 or 5). Following adsorption for 1 h at 37 °C, the virus-containing medium was removed and fresh medium was added to the washed cells in the presence or absence of CsA (1–50 μM) or SDZ-211-811 (1–50 μM). At 6, 12 and 24 h post-infection (p.i.), the medium supernatant from these cells was subjected to plaque assay analysis on L929 cells, as described previously (Zhou et al., 1998). The cell lysates obtained from virus-infected cells (12 h p.i.) were subjected to Western blot analysis with VSV anti-P antibody.

**Transfection of mutant CypA cDNA.** Mutant CypA cDNA subcloned into pcDNA (Invitrogen) (a gift from Jim Patrick, Baylor College of Medicine, Houston, Texas, USA) and empty pcDNA vectors were used for the transfection experiment. HeLa cells grown to 70–80% confluency in 24-well plates were transfected with empty pcDNA vector or mutant CypA cDNA plasmid (700 ng per well) using Lipofectin (Gibco-BRL) as described previously (De et al., 2000). At 36 h post-transfection, the cells were infected with VSV-IND and VSV-NJ (m.o.i. of 0:1). Following adsorption for 1 h at 37 °C, the virus-containing medium was removed and fresh medium was added to the washed cells. At 12 h p.i., cells were lysed and the lysates were subjected to Western blot analysis with VSV anti-P antibody. In addition, the cell lysates obtained from the transfected cells (36 h post-transfection) were subjected to Western blot analysis with anti-CypA antibody to check the overexpression of CypA protein in transfected cells. The medium supernatants from VSV-infected cells (24 h p.i.) were also subjected to plaque assay analysis on L929 cells, as described previously (Zhou et al., 1998).

**Western blot analysis.** At 12 h p.i. (BHK cells) or 36 h post-transfection (HeLa cells), the cells were lysed as described previously (Choudhary et al., 2001). The protein concentration of the cell lysates was determined using a protein assay kit (Bio-Rad), and the cell lysates (10 μg protein) obtained from either BHK or HeLa cells were subjected to 10% SDS-PAGE followed by Western blotting on to a nitrocellulose membrane. Polyclonal VSV anti-P antibody (Gupta et al., 1998; Das et al., 1995) or polyclonal anti-CypA antibody (Saphire et al., 1999) was used to measure intracellular VSV P protein and CypA, respectively. Protein bands were visualized by staining with horseradish peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotech) followed by enhanced chemiluminescence according to the manufacturer’s protocol (Amersham-Pharmacia Biotech). Similar Western blot analysis with anti-CypA antibody was performed using RNP isolated from purified VSV-IND (6 μg protein), VSV-NJ (6 μg protein) and HPIV-3 (10 μg protein) virions.

Western blot analysis with VSV anti-P antibody was also performed using RNP (500 ng protein) isolated from purified VSV-IND and VSV-NJ virions.

**In vivo genome transcription of VSV.** To examine the efficiency of VSV genome transcription in vivo in the presence of CsA, BHK cells were infected with VSV-IND or VSV-NJ (m.o.i. of 0:5). Following adsorption for 1 h at 37 °C, fresh medium was added to the washed cells in the presence or absence of CsA (25 μM) and the incubation was continued for an additional 4 h. Actinomycin D (5 μg ml⁻¹; Sigma) was then added to the cells in the presence or absence of CsA and the cells were incubated for an additional 2 h. [3H]Uridine (100 μCi ml⁻¹; Perkin Elmer) was then added to the cellular medium containing actinomycin D in the presence or absence of CsA. After labelling for 4 h at 37 °C, cells were washed and lysed in a lystate buffer containing RNAase inhibitor (Roche). The cell lysates were then subjected to cold 10% trichloroacetic acid (TCA) precipitation, as described previously (Adam et al., 1986; Manders et al., 1972). The washed TCA pellet was counted on a gamma counter. The transcription efficiency of VSV genome expressed as percentage transcription, calculated as described in Fig. 4.

**Northern blotting.** BHK cells untreated or pretreated with cycloheximide (CHX, 10 μg ml⁻¹; Sigma) for 3 h were infected with VSV-IND or VSV-NJ (m.o.i. of 0:5) in the presence or absence of CHX. Following adsorption for 1 h at 37 °C, fresh medium was added to the washed cells in the presence or absence of CsA (25 μM) and CHX (10 μg ml⁻¹). At 6 h p.i., total RNA was isolated using Trizol reagent (Life Technologies) following the manufacturer’s protocol. RNA (10 μg) was separated on a 1:2% agarose gel containing formaldehyde and transferred to an Immobilon-N+ (Millipore) membrane. The membrane was hybridized with [32P]-labelled riboprobes corresponding to the VSV-NJ or VSV-IND N mRNA. The intensity of the N mRNA bands was quantified using a PhosphorImager.

**Co-immunoprecipitation of VSV N protein with CypA.** For the co-immunoprecipitation analysis, cells were inoculated with VSV-NJ or VSV-IND (m.o.i. of 0:5). Following adsorption for 1 h at 37 °C, fresh medium was added to the washed cells and the incubation was continued for an additional 8 h. Cells were then lysed and the lysates were immunoprecipitated with either a VSV-IND anti-N antibody (which cross-reacts with VSV-NJ N protein; Banerjee et al., 1984; Frazier & Shope, 1979) or control normal rabbit serum in the presence of washed protein A–Sepharose beads (Amersham-Pharmacia Biotech). Following incubation for 8 h at 4 °C in a rotor, the beads were washed extensively with PBS and the bound proteins were released from the beads by boiling in the presence of SDS-PAGE sample buffer. The released proteins were subjected to 15% SDS-PAGE and Western blot analysis with anti-CypA antibody. Similar co-immunoprecipitation analysis was also performed with VSV anti-P and anti-CypA antibodies.

**RESULTS**

**Effect of functional inhibition of CypA with CsA on VSV-IND and VSV-NJ multiplication**

Based on a previous study (Saphire et al., 1999), which showed that the pseudotyped VSV-G/HIV-1 virus failed to attach to the cells in a CypA-dependent manner, we investigated the role of CypA in VSV entry by using the same CypA-blocking antibody used for the HIV-1 studies in a similar cell surface blocking experiment with both VSV-NJ and VSV-IND. The failure of the blocking antibody to inhibit VSV infection (data not shown) suggested that
CypA is not involved in VSV entry. This finding has been further validated by various experimental means including: (i) unlike HIV-1 (Saphire et al., 1999), biotinylated CypA was not detected following Western blot analysis of avidin–agarose-bound biotinylated purified VSV-IND and VSV-NJ (Nauwynck et al., 1999) with anti-CypA antibody (data not shown); (ii) [35S]methionine-labelled VSV-IND (data not shown) and VSV-NJ (Bose et al., 2001) failed to bind protein–A Sepharose beads conjugated with anti-CypA antibody; (iii) VSV-IND (Bose & Banerjee, 2002) and VSV-NJ (data not shown) were capable of productively infecting cells that were devoid of heparan (following heparinase treatment; Bose & Banerjee, 2002), the cell surface receptor for CypA (Saphire et al., 1999); and (iv) there was no difference in the amount of CypA present in purified virus versus the amount detected in RNP (constituting the inner core of the virus envelope) isolated from purified VSV-IND and VSV-NJ (see below).

In the absence of a role for CypA in VSV cellular entry, we next investigated whether CypA might have an alternative role in the life-cycle of VSV, such as uncoating, transcription or budding, as observed for HIV-1 (Braaten et al., 1996a, b; Luban et al., 1993; Streblow et al., 1998). For these studies, we infected BHK cells with VSV in the presence of CsA, an immunosuppressant drug that interacts with CypA to alter its conformation and inhibit the prolyl-isomerase activity (Lodish & Kong, 1991; Steinmann et al., 1991). A dose response curve with various concentrations of CsA (1–50 μM) revealed that, at 24 h p.i., VSV-IND replication (m.o.i. of 0·1; Fig. 1A) was less affected by CsA compared with the significant inhibition of VSV-NJ replication (m.o.i. of 0·1; Fig. 1B) when CsA was added to the cells following virus adsorption. Since both 25 μM and 50 μM CsA rendered maximal inhibition, we used 25 μM CsA in subsequent studies. To examine the replication capability of VSV following infection in the presence of CsA, a growth kinetics analysis of VSV infectivity (m.o.i. of 0·1) was performed at 6, 12 and 24 h p.i. by plaque assay. As shown in Fig. 1(C, D), CsA had a significantly reduced effect (inhibition by approximately 1 log) on VSV-IND infection compared with that observed with VSV-NJ (inhibition by 3 logs). Similar results were obtained when cells were infected at higher m.o.i.s of 1, 2 or 5 (data not shown). Western blot analysis (Fig. 1E, F) of cell lysates obtained at 12 h p.i. from cells infected with VSV in the absence or presence of CsA using VSV anti-P antibody were consistent with the results obtained by plaque assay, i.e. the synthesis of P protein decreased minimally for VSV-IND in the presence of CsA (Fig. 1E, lanes 3 and 4), whereas the extent of P protein synthesis of VSV-NJ was drastically reduced in the presence of CsA (Fig. 1F, lanes 3 and 4). Note that there were lower molecular mass bands beneath the VSV P protein in Western blots, which probably represent partially degraded products of the P protein. Western blot analysis of untreated and CsA-treated cell lysates with VSV N protein antibody yielded similar results to those observed with VSV P antibody (data not shown). These data suggested that VSV-NJ, and to a much lesser extent VSV-IND, utilize CypA during infection and that a functional intracellular pool of CypA is required for virus multiplication.

**Effect of inhibition of the peptidyl cis-trans prolyl-isomerase activity of CypA by SDZ-211-811 on VSV-NJ and VSV-IND multiplication**

To confirm further the inhibitory effect of CsA on VSV-NJ replication, we used a non-immunosuppressive inhibitor of CypA called SDZ-211-811 (Billlich et al., 1995; Dorfman & Gottlinger, 1996). This analogue binds to the active site of CypA to inhibit completely its isomerase activity. A dose response curve with various concentrations of SDZ-211-811 (1–50 μM) revealed that at 24 h p.i., VSV-IND replication (Fig. 2A) was less affected compared with VSV-NJ (Fig. 2B), whose replication was severely compromised when SDZ-211-811 was added to the BHK cells following virus adsorption. Interestingly, in contrast to CsA (Fig. 1B), 50 μM SDZ-211-811 conferred approximately fivefold greater inhibition compared with 25 μM SDZ-211-811. However, since the extent of inhibition exerted by 25 μM and 50 μM SDZ-211-811 was not significantly different, we used 25 μM SDZ-211-811 in subsequent studies. To assess further the inhibitory effect of SDZ-211-811 on virus replication in BHK cells, a growth kinetics analysis of VSV infectivity performed at 6, 12 and 24 h p.i. by plaque assay demonstrated that 25 μM SDZ-211-811 had a significantly reduced effect (inhibition by less than 1 log) on VSV-IND infection (Fig. 2C) compared with VSV-NJ infection, which was inhibited by almost 3 logs (Fig. 2D). Similar results were obtained when cells were infected with VSV-IND and VSV-NJ in the presence of SDZ-211-811 at higher m.o.i.s of 1, 2 or 5 (data not shown). Consistent with these results, Western blot analysis of VSV-IND-infected (12 h p.i.) cell lysates with VSV-IND anti-P antibody showed a minimal effect of SDZ-211-811 on P protein accumulation during infection (Fig. 2E, lanes 3 and 4). In contrast, P protein synthesis during VSV-NJ infection (12 h p.i.) was significantly inhibited in the presence of 50 μM or 25 μM SDZ-211-811 (Fig. 2F, lanes 3 and 4). Western blot analysis of untreated and SDZ-211-811-treated cell lysates with VSV anti-N protein antibody yielded similar results to those observed with VSV anti-P antibody (data not shown). Moreover, pretreatment of cells with CsA or SDZ-211-811 prior to virus adsorption followed by virus infection in the presence of CsA or SDZ-211-811 had similar inhibitory kinetics compared with those observed when the drugs were added to the cells following virus adsorption (data not shown). These results indicate an important role for functional CypA in VSV-NJ replication at a step following VSV cellular entry.

**Effect of overexpressing a catalytically inactive prolyl-isomerase mutant of CypA on VSV-NJ and VSV-IND infection**

To test further the role of the prolyl-isomerase function of CypA in VSV infectivity, overexpression of a catalytically
deficient CypA harbouring a mutation in its active site was carried out by transient transfection and VSV infectivity was evaluated in the cells. The CypA mutant used in our studies contained an alanine residue in place of an arginine at position 55, which resulted in 1000-fold reduction in its catalytic efficiency, as reported previously (Helekar & Patrick, 1997; Zydowsky et al., 1992). For the transfection studies, we utilized HeLa cells (Kopecky et al., 2001), in which both CsA and SDZ-211-811 drastically inhibited VSV-NJ replication compared with VSV-IND replication (data not shown) similar to that observed with BHK cells.

Initially, we examined the extent of overexpression of the mutant CypA compared with control cells transfected with an empty vector. As shown in Fig. 3(A), Western blot analysis of cell lysates (36 h post-transfection) obtained from control cells (lane 1) and mutant CypA-transfected cells (lane 2) using an anti-CypA antibody revealed a 10–12-fold increase in CypA protein levels in mutant CypA-transfected cells, confirming that significant levels of transfected CypA are expressed at 36 h post-transfection. We next examined the infection efficiency of VSV in control and mutant CypA-expressing cells. For these studies, the CypA

Fig. 1. Effect of cyclosporin A (CsA) on VSV-IND and VSV-NJ infection. (A, B) BHK cells infected with VSV-IND (A) or VSV-NJ (B) at an m.o.i. of 0.1 in the presence or absence of 1–50 μM CsA were subjected to plaque assay analysis to calculate virus titre at 24 h p.i. (C, D) BHK cells infected with VSV-IND (C) or VSV-NJ (D) at an m.o.i. of 0.1 in the presence (■) or absence (□) of 25 μM CsA were subjected to plaque assay analysis to calculate virus titre at 6, 12 and 24 h p.i. Data represent the mean of three independent experiments. (E, F) BHK cell lysates obtained from mock-infected cells (lane 1) or from cells infected with VSV-IND (E) or VSV-NJ (F) at 12 h p.i. in the absence (lane 2) or presence of 25 μM (lane 3) or 50 μM (lane 4) CsA were subjected to Western blot analysis with VSV-IND or VSV-NJ anti-P antibody.
mutant-transfected and control cells (transfected with an empty vector) were inoculated with VSV-NJ and VSV-IND at 36 h post-transfection. At 24 h p.i., the medium supernatants from the mutant CypA-transfected and control cells were analysed by plaque assay. As shown in Fig. 3(B), there was no significant change in VSV-IND virus titre between control and mutant CypA-expressing cells. In contrast, there was a significant decrease in VSV-NJ virus titre (inhibition by approximately 2 logs) from mutant CypA-expressing cells compared with control cells. Consistent with the plaque assay results, Western blot analysis revealed that expression of mutant CypA had no effect on VSV-IND infection, while its expression reduced VSV-NJ P and N protein levels significantly compared with the control cells (data not shown).

Fig. 2. Effect of SDZ-211-811 on VSV-IND and VSV-NJ infection. (A, B) BHK cells infected with VSV-IND (A) or VSV-NJ (B) at an m.o.i. of 0.1 in the presence or absence of 1–50 μM SDZ-211-811 (SDZ) were subjected to plaque assay analysis to calculate virus titre at 24 h p.i. (C, D) BHK cells infected with VSV-IND (C) or VSV-NJ (D) at an m.o.i. of 0.1 in the presence (■) or absence (□) of 25 μM SDZ-211-811 were subjected to plaque assay analysis to calculate virus titre at 6, 12 and 24 h p.i. Data represent the mean of three independent experiments. (E, F) BHK cell lysates obtained from mock-infected cells (lane 1) or from cells infected with VSV-IND (E) or VSV-NJ (F) (12 h p.i.) in the absence (lane 2) or presence of 50 μM (lane 3) or 25 μM (lane 4) SDZ-211-811 were subjected to Western blot analysis with VSV-IND or VSV-NJ anti-P antibody.
Role of CypA in VSV-IND and VSV-NJ intracellular genome transcription

To investigate the stage of the VSV infection cycle at which the CypA activity is required, we first investigated the role of CypA in VSV transcription and specifically in the post-entry primary transcription of the VSV genome. We chose to examine primary transcription of VSV in CypA-inhibited cells, since CypA did not play any role in VSV entry (see above) or budding (data not shown), as measured by a previously described budding assay (Bose et al., 2001; Ono et al., 1987). For these experiments, the extent of VSV genome transcription in vivo was investigated in the presence of the CypA inhibitor CsA. Initially, VSV RNA synthesis was monitored by labelling BHK cells with [3H]uridine in the presence of actinomycin D (which inhibits cellular transcription, but not VSV genome transcription) and CsA, followed by the measurement of cold 10% TCA-precipitable [3H]radioactivity, as described in Methods. This method for examining the in vivo transcription rate of VSV has been utilized in several studies (Adam et al., 1986; Manders et al., 1972). CsA was found to inhibit transcription of VSV-NJ significantly (90% inhibition; Fig. 4B) compared with its effect on VSV-IND transcription (15–20% inhibition; Fig. 4A).

To confirm further the results shown in Fig. 4(A, B) and investigate whether CypA is required during primary transcription of the VSV genome following viral entry, Northern blot analysis was performed with VSV N mRNA. Cells were pretreated with CHX (10 μg ml⁻¹) for 3 h, followed by the addition of VSV in the presence of CHX and in the absence or presence of CsA (25 μM). At 6 h p.i., total RNA was extracted from cells for Northern blot analysis with a VSV N mRNA riboprobe. This method has been utilized previously to monitor intracellular primary transcription of viruses (Choudhary et al., 2001). As shown in Fig. 4(C), the presence of CsA had minimal effect on both steady state VSV-IND mRNA levels (in the absence of CHX) (Fig. 4C, compare lanes 3 and 6) and primary transcription (in the presence of CHX) (Fig. 4C, compare lanes 2 and 5) of the VSV-IND genome. Quantification of the VSV-IND N mRNA bands from Fig. 4(C) revealed less than 15% inhibition of VSV-IND transcription by CsA (Fig. 4D). In contrast, both VSV-NJ steady-state mRNA levels (Fig. 4E, compare lanes 2 and 5) and primary transcription (Fig. 4E, compare lanes 3 and 6) were drastically inhibited by CsA. Quantification of the VSV-NJ N mRNA bands from Fig. 4(E) revealed 85–90% inhibition of VSV-NJ transcription by CsA (Fig. 4F). Similar inhibition in VSV-NJ, but not VSV-IND, RNA synthesis was observed following SDZ-211-88 treatment (data not shown). These results demonstrated that active CypA plays an important role in the primary transcription of VSV genome and that its requirement during this process is selective for VSV-NJ compared with VSV-IND.

CypA interacts with VSV N protein intracellularly and is incorporated into VSV virions

Since viruses are known to incorporate specific host proteins into their virions (Choudhary et al., 2000; Franke et al., 1994; Gupta et al., 1998; Thali et al., 1994), we next examined whether CypA was present in purified VSV virions by subjecting equal amounts (6 μg protein) of RNP purified from VSV-IND and VSV-NJ virions to Western blot analysis with anti-CypA antibody. As shown in Fig. 5(A), both VSV-IND (lane 3) and VSV-NJ (lane 2) incorporated CypA into its virions, while CypA was not detected in RNP (10 μg protein) isolated from HPIV-3 virions (lane 4). BHK cell lysate was used as the control (lane 1). Similar amounts of VSV-IND and VSV-NJ viral proteins were present in the RNP preparation as deduced by SDS-PAGE and Western blot analysis with VSV anti-P antibody (Fig. 5B, C). SDS-PAGE analysis of purified RNP (2 μg protein) isolated from VSV-IND (Fig. 5B, lane 1) and VSV-NJ (Fig. 5B, lane 2) revealed that equal amounts of N/P proteins were present in both preparations when equal

(A) Cell lysates obtained 36 h post-transfection from cells transiently transfected with empty vector (lane 1) or mutant CypA cDNA plasmid (lane 2) were subjected to Western blot analysis with anti-CypA antibody. (B) At 24 h p.i., the medium supernatant from VSV-IND-infected (grey bars) or VSV-NJ-infected (black bars) cells transfected with either control or mutant CypA plasmid was subjected to plaque assay analysis. Data represent the mean of three independent experiments.

Fig. 3. Effect of catalytically inactive cyclophilin A (CypA) mutant (Mut) overexpression on VSV-IND and VSV-NJ infection. (A) Cell lysates obtained 36 h post-transfection from cells transiently transfected with empty vector (lane 1) or mutant CypA cDNA plasmid (lane 2) were subjected to Western blot analysis with anti-CypA antibody. (B) At 24 h p.i., the medium supernatant from VSV-IND-infected (grey bars) or VSV-NJ-infected (black bars) cells transfected with either control or mutant CypA plasmid was subjected to plaque assay analysis. Data represent the mean of three independent experiments.
amounts of protein (2 µg protein) were loaded. It is difficult to differentiate between VSV N and P proteins in the gel shown in Fig. 5(B), since both N and P proteins of VSV have similar migratory patterns. Nevertheless, Western blot analysis of equal amounts (500 ng protein) of VSV-NJ (Fig. 5C, lane 1) and VSV-IND (Fig. 5C, lane 2) RNP preparations with their corresponding anti-P antibody revealed that similar amounts of VSV P were present in both RNP preparations. Moreover, the majority of CypA in purified VSV preparations was bound to viral RNP, since there was no difference in CypA protein levels in purified viruses compared with the RNP isolated from these viruses as deduced by Western blot analysis with anti-CypA antibody (data not shown). Thus, it seems that both VSV serotypes package CypA bound to viral RNP; however, it is preferentially required by the New Jersey serotype for its multiplication.

Finally, to investigate directly the intracellular interaction of CypA with VSV protein(s) in infected cells, co-immunoprecipitation analysis of VSV-infected cell lysates was performed using anti-CypA and VSV anti-N
antibodies. We chose to immunoprecipitate with the VSV N protein antibody because the N protein is the major component of the viral RNP and the possible site of CypA interaction (Fig. 5A). Mock-infected cells or cells infected with VSV-NJ (Fig. 5D) or VSV-IND (Fig. 5E) for 8 h were lysed and the lysate was immunoprecipitated with VSV anti-N antibody or control normal rabbit serum and subjected to Western blot analysis with anti-CypA, as described in Methods. As shown in Fig. 5, VSV-NJ N protein precipitated CypA from infected cells following treatment with VSV-IND N antibody, the antibody that cross-reacts with VSV-NJ N protein (Fig. 5D, lane 3) (Banerjee et al., 1984; Frazier & Shope, 1979). Likewise, VSV-IND N protein precipitated CypA from infected cells (Fig. 5E, lane 2). No CypA was detected following immunoprecipitation of either mock-infected cell lysate with anti-N antibody (Fig. 5D, lane 2; Fig. 5E, lane 3) or VSV-infected cell lysate with control normal rabbit serum (Fig. 5D, lane 4; Fig. 5E, lane 4). Similar amounts of viral proteins were present in the VSV-NJ- and VSV-IND-infected cell lysates following the 8 h infection, as deduced by SDS-PAGE and Western blot analysis (data not shown). These results suggest that in VSV-IND- and VSV-NJ-infected cells CypA interacts with the N protein. This CypA-bound N protein presumably constitutes the intracellular RNP complex that is eventually packaged into the mature virions. However, in contrast to the interaction of CypA with VSV N protein in infected cells, no association of CypA was noted with VSV P protein as deduced by co-immunoprecipitation analysis of VSV-infected cell lysates with VSV P and CypA antibodies (data not shown). It is interesting to note that, although both VSV-NJ and VSV-IND N proteins bind with intracellular CypA, interaction of VSV-NJ N protein with CypA in infected cells led to efficient transcription of the viral genome. The reasons for this apparent differential role of CypA for two serotypes of VSV are discussed below.

**DISCUSSION**

In the current study we have identified a host protein, CypA, as one of the cellular co-factors critically required for VSV-NJ replication but not for replication of the serologically distinct VSV-IND. Our findings were established based on the following evidence: (i) two compounds, CsA and SDZ-211-811, which inhibit the prolyl-isomerase chaperonin function of CypA, strongly inhibited VSV-NJ infectivity; (ii) overexpression of the prolyl-isomerase mutant form of CypA reduced VSV-NJ infectivity; and (iii) inhibition of the prolyl-isomerase function of CypA with CsA severely inhibited VSV-NJ primary transcription. Interestingly, CypA interacted with both VSV-NJ and VSV-IND N protein *in vivo* and was incorporated into the virions bound to the viral RNP.

It is important to note that in previous studies with HIV-1 a lower concentration of CsA and SDZ-211-811 (1–10 μM) was used to inhibit virus replication in immune cells (Braaten & Luban, 2001; Braaten *et al.*, 1996a, b) compared with the concentrations used in our studies, where, in BHK cells, maximal inhibition of virus replication occurred in the presence of 25 μM CsA or SDZ-211-811. The requirement for a higher concentration of these drugs to inhibit VSV-NJ replication in non-immune cell culture could be due to the difference in the amount of intracellular CypA present in BHK cells compared with the immune cells (T-lymphocyte Jurkat cells). Moreover, as for BHK cells, 25 μM CsA and SDZ-211-811 was required to inhibit VSV-NJ but not VSV-IND replication efficiently in two

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*Fig. 4. Intracellular VSV-IND and VSV-NJ genome and primary transcription in the presence of cyclosporin A (CsA).* (A,B) BHK cells were infected with either VSV-IND (grey bars) (A) or VSV-NJ (black bars) (B) in the absence or presence of 25 μM CsA. At 4 h p.i., the cells were incubated with actinomycin D (5 μg ml⁻¹) for 2 h at 37 °C in the presence or absence of CsA. Following actinomycin D treatment, the cells were washed and incubated with [³H]uridine (100 μCi ml⁻¹) in the presence or absence of CsA along with actinomycin D for an additional 4 h at 37 °C. The lyastes obtained from the labelled cells were precipitated with cold 10% TCA and the washed acid-insoluble pellets were counted on a gamma counter. The percentage transcription was calculated based on the ratio of the amount of acid-precipitable radioactivity obtained from VSV-infected cells in the presence of CsA compared with the amount of acid-precipitable radioactivity obtained from VSV-infected cells in the absence of CsA. A value of 100% represents cells infected with VSV in the absence of CsA. The amount of radioactivity obtained from mock-infected cells in the presence of actinomycin D was subtracted from the values obtained from cells infected with VSV in the presence or absence of CsA. The results represent the average of three independent experiments and standard deviations are indicated as error bars. (C) Total RNA extracts (10 μg) from cells mock infected (lanes 1 and 4) or infected with VSV-IND (lanes 2, 3, 5 and 6) in the presence (lanes 2 and 5) or absence (lanes 3 and 6) of cycloheximide (CHX, 10 μg ml⁻¹) and in the absence (lanes 1–3) or presence (lanes 4–6) of 25 μM CsA were subjected to Northern blot analysis with a VSV-IND N mRNA riboprobe. (D) The VSV-IND N mRNA transcripts from (C) were quantified using a PhosphorImager and represented as the percentage of N mRNA present in cell extracts. A value of 100% N mRNA represents cells infected with VSV-NJ in the absence of CsA.
Fig. 5. Incorporation of cyclophilin A (CypA) into VSV virions and association of CypA with VSV N protein in infected cells. (A) Cell lysate (lane 1) and purified RNP prepared from VSV-NJ (lane 2; 6 μg), VSV-IND (lane 3; 6 μg) and HPIV-3 (lane 4; 10 μg) virions were subjected to Western blot analysis with anti-CypA antibody. (B) 10% SDS-PAGE analysis of purified RNP (2 μg) isolated from VSV-IND (lane 1) and VSV-NJ (lane 2) virions. (C) RNP isolated from VSV-NJ (lane 1) and VSV-IND (lane 2) virions was subjected to Western blot analysis with VSV-NJ and VSV-IND anti-P antibody, respectively. (D) Cell lysates obtained from mock-infected (lane 2) or VSV-NJ-infected cells (lanes 3 and 4) were immunoprecipitated (IP) with either VSV anti-N antibody (lanes 2 and 3) or control normal rabbit serum (lane 4) in the presence of protein A–Sepharose. Following incubation for 8 h at 4 °C, the beads were washed extensively and the bound proteins were subjected to SDS-PAGE and Western blot analysis with anti-CypA antibody. BHK cell lysate (lane 1) was included in the Western blot analysis to serve as a control. (E) Cell lysates obtained from mock-infected (lane 3) or VSV-IND-infected (lanes 2 and 4) cells were immunoprecipitated with either VSV anti-N antibody (lanes 2 and 3) or control normal rabbit serum (lane 4) in the presence of protein A–Sepharose. Following incubation for 8 h at 4 °C, the beads were washed extensively and the bound proteins were subjected to SDS-PAGE and Western blot analysis with anti-CypA antibody. BHK cell lysate (lane 1) was included in the Western blot analysis to serve as a control.
additional non-immune cell lines, HeLa (Kopecky et al., 2001) and A549 (data not shown). Nevertheless, the effects of CsA and SDZ-211-811 on VSV infection were specific, since CsA and SDZ-211-811 at a concentration of 10–50 μM failed to inhibit the replication of a related negative-strand RNA virus, HPIV-3, as evaluated by plaque assay (data not shown). CsA is known to inactivate a cytosolic phosphatase, calcineurin, in addition to inhibiting CypA (Alexanian & Bamburg, 1999). However, the antiviral effect of CsA on VSV-NJ replication was not due to calcineurin inhibition, since neither expression of a calcineurin inhibitor, cain, via adenovirus (Taigen et al., 2000) nor treatment of cells with a calcineurin inhibitory drug, cypermethrin (Alexanian & Bamburg, 1999), had any effect on VSV-NJ and VSV-IND replication (data not shown). In addition, the treatment of cells with these drugs (50 μM) was not toxic within the time frame of our experiments (24 h maximum) as deduced by the trypan blue exclusion viability test (data not shown). It is noteworthy that, although in our current studies we used only one subtype of each virus (Mudd–Summers strain for VSV-IND and Ogden strain for VSV-NJ), it is possible that other subtypes of VSV-IND and/or VSV-NJ may have different requirements for CypA during replication. This line of investigation dealing with subtype-specific requirements for a host protein demands further studies.

CypA belongs to the family of immunophilins and is a peptidyl prolyl-isomerase that acts as a chaperone to maintain proper protein conformation by catalysing the cis-trans isomerization of peptide bonds N-terminal to proline residues (Gotzel & Marahiel, 1999; Takahashi et al., 1989). Several cellular proteins (Steinmann et al., 1991; Lodish & Kong, 1991; Schneuwly et al., 1989; Helekar & Patrick, 1997) have been shown to be substrates of CypA in vivo. Recently, CypA has also been shown to interact with the HIV-1 capsid and the nucleocapsid domain of HIV-1 Gag polyprotein (Colgan et al., 1996; Luban et al., 1993) and is also incorporated into the virions (Franke et al., 1994; Thali et al., 1994). However, to date, no definitive stages of the HIV-1 life-cycle that require CypA have been established. Nevertheless, loss of interaction of CypA with HIV-1 Gag results in loss of infection, suggesting an important role for CypA in the HIV-1 life-cycle (Braaten & Luban, 2001; Saphire et al., 1999). CypA binds to the capsid protein of HIV-1 to provide functional conformation required for HIV-1 infection (Agresta & Carter, 1997; Dietrich et al., 2001; Streblow et al., 1998) following proline isomerization. Our current studies have demonstrated that functional CypA is also required for VSV infection and that in infected cells, CypA binds to the N protein, which encapsidates the viral genome, similar to the CypA-binding capsid protein of HIV-1 (Agresta & Carter, 1997). Based on these similarities, one may speculate that CypA binds to the VSV N protein to isomerize the proline residues required for proper functional folding of the N protein.

One intriguing observation of the current investigation is the striking difference in requirement for CypA by VSV-NJ compared with VSV-IND. Functional CypA is definitely required for VSV-NJ infection and primary transcription, whereas the CypA requirement for VSV-IND is minimal compared with VSV-NJ, although both viruses incorporate CypA into their virions. It is interesting to note that a similar correlation exists between the HIV-1 isolates in relation to their requirement for CypA for infection. For example, HIV-2 and SIV, which belong to the same family of viruses as HIV-1, do not require CypA for infection (Braaten et al., 1996b). In addition, among the HIV-1 subtypes, group main (M) HIV-1, but not group outlier (O) HIV-1, requires CypA, although isolates of group O HIV-1 incorporate CypA into their virions (Braaten et al., 1996b). Most interestingly, the replication of a primary wild-type isolate of HIV-1-Eli was found to be more sensitive to the presence of intracellular CypA compared with the laboratory strain HIV-1-NL4-3, although both isolates interacted with CypA (Braaten & Luban, 2001). Based on our findings that VSV-NJ (the prevailing virulent VSV strain in the wild; Rodriguez & Nichol, 1999) critically requires functional CypA for replication, in contrast to VSV-IND (a less virulent and less prevailing VSV serotype in the wild; Rodriguez & Nichol, 1999), it is tempting to speculate that the two viruses have evolved from an ancestral VSV that was initially dependent on CypA for replication. During evolutionary divergence from the ancestral lineages, VSV-IND may have adapted to reduce its dependency on CypA, possibly as a result of evolutionary pressure. Indeed, phylogenetic analysis of these two serotypes of VSV has revealed that VSV-NJ has diverged less from the VSV ancestor than VSV-IND (Bilsel & Nichol, 1990). It is thus possible that, due to this adaptation, VSV-IND utilizes host chaperones other than CypA, including heat-shock proteins (HSPs) and other immunophilins.

Our results suggest that the requirement for CypA in VSV infection could be at the level of primary transcription. It is plausible that by virtue of CypA’s interaction with VSV N protein the nucleocapsids are folded into a transcriptionally competent conformation. Such stringent structural requirements (a helical extended structure) of the VSV N protein for transcription have been previously demonstrated (De et al., 1982; Heggeness et al., 1980). Thus, the interaction of N with CypA could result in the formation of the correct structure required for optimal transcription efficiency of the VSV-NJ genome. This scenario could be similar to the interaction of CypA with the Gag protein of HIV-1. The binding of CypA to the HIV-1 capsid (which, like the N protein, encapsidates the HIV-1 genome RNA) protein oligomers led to the formation of an elongated compact structure, the functional conformation required for efficient HIV-1 infection (Agresta & Carter, 1997; Braaten et al., 1996a; Streblow et al., 1998).

Finally, the role of chaperones in the transcription of the virus genome has been documented for several viruses. For example, binding of the chaperonin complex HSP 90, 70 and...
p23 to the N protein of hepatitis B virus (Hu et al., 1997, 2002) results in the optimal conformation of N required for efficient reverse transcription and RNP formation. It is interesting to note that earlier studies have reported cyclophilins functioning as a cellular chaperonin complex along with HSPs (Jakob & Buchner, 1994; Sanchez & Ding, 1996; Uittenbogaard et al., 1998). For example, the HSP and cyclophilin complex have been shown to be involved in correct folding of steroid receptors (Jakob & Buchner, 1994; Sanchez & Ding, 1996). Whether CycA along with HSP or other chaperonin protein(s) forms a complex to facilitate VSV genome transcription remains to be elucidated. Coupled with the fact that HIV-1 packages CycA (Franke et al., 1994; Thali et al., 1994) and HSP 70 (Gurer et al., 2002), earlier studies have demonstrated that VSV-NJ also incorporates HSP 70 into its virions (Sagara & Kawai, 1992) and that the N protein of VSV interacts with HSP in infected cells (Garry et al., 1983). Thus, these observations, together with our current studies of CycA, ascertain the importance of cellular chaperones in VSV infection. Our current study has thus highlighted the essential role of the chaperone protein CycA, a member of the cellular protein-folding machinery in VSV transcription and infection. Since VSV-NJ is the highly virulent strain of VSV known to cause disease and high mortality among economically important livestock, the requirement for CycA for its replication could serve as a potential target for antiviral agents.

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