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The Src Family Kinase c-Yes Is Required for Maturation of West Nile Virus Particles

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The role of cellular genes in West Nile virus (WNV) replication is not well understood. Examination of cellular transcripts upregulated during WNV infection revealed an increase in the expression of the src family kinase (SFK) c-Yes. WNV-infected cell lines treated with the SFK inhibitor PP2 demonstrated a 2- to 4-log decrease in viral titers, suggesting that SFK activity is required for completion of the viral replication cycle. RNA interference mediated knock-down of c-Yes, but not c-Src, and similarly reduced virus yield, specifically implicating c-Yes in WNV production. Interestingly, PP2 treatment did not reduce intracellular levels of either viral RNA or protein, suggesting that the drug does not act on the early stages of replication. However, endoglycosidase H (endoH) digestion of the viral envelope (E) glycoprotein revealed that the acquisition of endoH-resistant glycans by E, but not endogenous major histocompatibility complex class I, was reduced in PP2-treated cells, demonstrating that E specifically does not traffic beyond the endoplasmic reticulum in the absence of SFK activity. Electron microscopy further revealed that PP2-treated WNV-infected cells accumulated an increased number of virions in the ER compared to untreated cells. Therefore, we conclude that inhibition of SFK activity did not interfere with virus assembly but prevented transit of virions through the secretory pathway. These results identify c-Yes as a cellular protein that is involved in WNV assembly and egress.

West Nile virus (WNV) is a newly emerging pathogen that has become a significant threat to the U.S. population. First detected in Uganda in 1937, the virus appeared in the United States in the summer of 1999, and since that time it has reemerged each year over an increasing geographical area (1). Typical of flaviviruses, WNV contains a single-stranded positive-sense 11-kb RNA genome. The genome is translated into a single polyprotein, which is cleaved by host and virus-encoded proteases into 10 functional subunits (5, 20). The WNV particle is composed of the subunits derived from the amino terminus of the polyprotein: capsid (C), precursor membrane/membrane (prM/M), and envelope (E). The remaining seven proteins are nonstructural and function in virus replication. WNV infection results in proliferation and reorganization of intracellular membranes into several unique structures that colocalize with specific subsets of viral proteins, suggesting a distinct spatial segregation of the stages of virus replication (24, 48).

WNV virion assembly appears to occur at the rough endoplasmic reticulum (ER), presumably by budding of the nucleocapsid (NC) into the ER lumen (5, 20, 28). In the context of viral infection, packaging of the genomic RNA requires NS2A and the replication of the RNA by the viral RNA-dependent RNA polymerase NS5 (16, 21). The mechanism of NC budding into the ER and acquisition of the viral membrane and glycoproteins is unknown, although a hydrophobic region in the C protein has been proposed to be important in these interactions (23). The NC is not required for formation and secretion of particles, as subviral particles containing only E and M will form in the absence of the NC (28). Virions are hypothesized to transit from the ER to the Golgi and *trans*-Golgi network (TGN) and reach full maturity in the TGN following cleavage of the prM protein into the M form by the host protease furin (37).

Several host cell factors have been implicated in the replication of flaviviruses. Recently, entry of WNV was reported to be mediated by $\alpha_V\beta_3$ integrin, which leads to internalization of virus via a clathrin-dependent endocytic pathway (6, 7). Several other cellular proteins have also been implicated in flavivirus replication, including cellular RNA binding proteins that bind to the 3′ stem-loop (SL) of the viral RNA. One of these cellular proteins, translation elongation factor 1α (EF-1α), has been shown to bind to the genomic WNV 3′ SL (2). Other cellular proteins, such as EF-1α, La, and polypyrimidine-tract binding protein (PTB), were shown to bind to dengue virus RNA (9). Additionally, the RNA binding proteins TIAR and TIA-1 bind to the 3′ SL of the WNV minus-strand RNA (19). Mutations in the viral genome that interfere with binding of these proteins, or growth of virus in cells lacking individual RNA binding proteins, reduce viral replication, demonstrating the importance of these interactions in the viral replication cycle.

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The host factors that are involved in virion assembly and trafficking are largely unknown. In this study, we identified a member of the src kinase family, c-Yes, as an important host factor in the WNV replication cycle. Our results demonstrate that c-Yes is involved in the process of trafficking of the assembled virion from the ER.

MATERIALS AND METHODS

Cells and viruses. Vero cells were propagated in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Gibco), 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (Gibco). SK-N-MC cells and HEK 293 cells were propagated in minimum essential medium (MEM) supplemented as above. Infectious WNV was obtained by transfection of viral RNA [WNV (NY99) derived from an infected crow] into Vero cells (15). Infectious progeny was propagated once on Vero cells, and aliquots were stored at −80°C. Viral titer was determined by plaque assay.

Viral growth curves and plaque assays. Cell cultures were infected at a multiplicity of 5 in growth medium supplemented with 2% FBS. Virus was allowed to adsorb for 2 h, then washed once in 40 mM Na-citrate, 150 mM NaCl, pH 3.2, for 1 min and twice in phosphate-buffered saline (PBS), and refed with growth medium supplemented with 2% FBS. PP2 (Calbiochem) in dimethyl sulfoxide (DMSO) was added to the indicated concentrations. An equal volume of DMSO was added to control wells. Supernatant was harvested at the indicated time points. In order to isolate intracellular virus, cells were washed three times with PBS and harvested in PBS followed by three rounds of freeze-thaw. For plaque assays, 10^6 Vero cells were plated per well of a 12-well tissue culture dish. Serial dilutions of virus were plated in duplicate and allowed to adsorb for 2 h, followed by overlay with 0.5% carboxymethyl-cellulose (CMC; Sigma). After 72 h, cells were fixed in 10% buffered formalin and plaques were visualized by staining with crystal violet.

Tissue culture (50% tissue culture infective dose [$TCID_{50}$]) assays. Vero cells were plated at 40,000 cells per well in 96-well tissue culture dishes. Virus stocks were serially diluted from 10^{-3} to 10^{-10}, and each dilution was plated across 12 wells (100 μl per well) in DMEM–2% FBS containing the indicated concentrations of PP2 or PP3. At 6 days postinfection, the wells were fixed in 10% buffered formalin, stained with crystal violet, and scored for the presence of cytopathic effect (CPE). Titers were calculated as previously described (8).

Toxicity assays. A total of 1×10^5 SK-N-MC cells were plated per well in six-well tissue culture dishes in MEM supplemented with 2% FBS. PP2 was added to the indicated concentrations, and the cells were incubated for 48 h. Each concentration was performed in triplicate. Cells were removed from the plate by trypsin digestion, resuspended in MEM–2% FBS, and diluted in 0.2% trypan blue. Viable cells were counted using a hemocytometer. For the XTT {sodium 3′-[l-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate} assay, 10,000 Vero cells were plated per well in a 96-well plate and maintained in DMEM supplemented with 2% FBS. PP2 was added at the indicated concentrations (in triplicate), and cells were incubated for 48 h. A standard curve was generated by plating fivefold serial dilutions of cells from 100,000 to 800 cells/well. Metabolically active cells were quantified using the Cell Proliferation Kit II (XTT) (Roche) according to the manufacturer's protocol.

siRNA transfections. Vero cells were plated in 96-well tissue culture dishes so as to be ~30% confluent on the day of transfection. Cells were washed and fed with 50 μl Opti-MEM (Invitrogen) per well. Each well was transfected with 0.1875 μl Oligofectamine (Invitrogen) and 0.3125 μl small interfering RNAs (siRNAs) (20 μM) in a total volume of 12.5 μl Opti-MEM according to the manufacturer's protocol. Cells were transfected a second time 24 h after the first transfection. At 24 h after the second transfection, the cells were infected and growth on transfected cells was analyzed using the $TCID_{50}$ protocol described above. The c-Yes-specific duplex sequence was 5′-UUAUGAAGCUAGAACU ACAdTdT-3′, and the c-Src-specific duplex was 5′-GGAACAAAGUCGCCGU CAAdTdT-3′. siRNAs were obtained from Dharmacon. The control siRNA was a commercially available duplex that contains at least four mismatches with all known human genes (Dharmacon). Additional cells transfected in parallel were harvested and analyzed by Western blotting to ascertain knock-down of the target. The extent of the knock-down was measured using IPLab Gel software (Signal Analytics Corp.).

RNA isolation and real-time PCR. RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Two micrograms of total RNA was reverse transcribed using Omniscript reverse transcriptase (RT; QIAGEN) and 1 μM random hexamer primers. One-tenth of the RT reaction mixture was used as a template for real-time PCR (Taqman) using TaqMan Universal PCR master mix (Applied Biosystems). WNV NS3-specific primers were forward, 5′-GCACTGAGAGGACTGCCCAT-3′, and reverse, 5′-TGGGTGAGGGTAGCATGACA-3′, and fluorescently labeled probe 5′–6-carboxyfluorescein–TACCAGACATCCGCAGTGCCCAGA–tetramethyl carboxyrhodamine–3′ (4) was used. An in vitro-transcribed RNA was used as a standard for quantitation.

Immunoprecipitation and purification of supernatant virus. For each condition, a 10-cm dish of HEK 293 cells was infected at a multiplicity of 5 with WNV (NY99). PP2 was added to 20 μM, and control plates received an equal volume of DMSO. At 20 h postinfection, the dishes were washed with PBS and refed with DMEM containing 0.5 mCi of ^{35}S-Express protein labeling mix (Perkin-Elmer). Culture supernatants and cells were harvested at 25 h postinfection. Cells were lysed in 0.4 ml buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, and 1 mM phenylmethylsulfonyl fluoride. To immunoprecipitate WNV E, 5×10^7 cpm of labeled lysate was incubated in a 0.5-ml total volume of lysis buffer with 1 μl anti-E monoclonal antibody (MAb 8150; Chemicon) and 10 μl protein A/G agarose (Santa Cruz Biotechnology) at 4°C for 16 h. Beads were washed three times in lysis buffer and boiled for 2 min in 2× Laemmli sample buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography. To purify supernatant virus, supernatants from labeled cells (3 ml) were first precleared at $10{,}000 \times g$ for 10 min in an Eppendorf microcentrifuge. Cleared supernatants were layered over 1 ml 20% sucrose in 50 mM Tris, pH 7.4, and spun at 30,000 rpm for 1.5 h at 20°C in a Beckman SW50.1 rotor. Pellets were resuspended in Laemmli sample buffer, and subjected to SDS-PAGE, and proteins were visualized by autoradiography.

Western blotting and glycosylation analysis. Cells were lysed in buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP-40, and 1% sodium deoxycholate. Proteins were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). The following antibodies were used: mouse anti-Yes (610375; BD Transduction Labs), mouse anti-β-actin (A5441; Sigma), mouse anti-WNV E (MAb 8150; Chemicon), rabbit anti-src (sc-19; Santa Cruz), mouse anti-major histocompatibility complex class I (anti-MHC I) (38), and horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies (Amersham). Blots were visualized by Supersignal West Pico chemiluminescent substrate (Pierce) according to the manufacturer's protocol. For glycosylation analysis, WNV-infected cells were lysed in 1× glycoprotein denaturation buffer (New England Biolabs) at 95°C for 10 min. Fifty micrograms of denatured lysate was digested with 1,000 U of endoglycosidase H (endoH; NEB) or 1,000 U of peptide-*N*-glycosidase F (PNGase F; NEB) for 1 h at 37°C. Proteins were resolved by SDS-PAGE and detected by Western blotting with anti-WNV E or anti-MHC I.

EM. For electron microscopy (EM), Vero cells were infected at a multiplicity of 5 and treated with 10 μM PP2 or DMSO as a control. At 24 h postinfection, medium was carefully removed from the cells and replaced with fixative (100 mM sodium cacodylate [pH 7.2], 2.5% glutaraldehyde, 1.6% paraformaldehyde, 0.064% picric acid, 0.1% ruthenium red) for 30 min. After removal of fixative, cells were gently washed in PBS, scraped into PBS, and pelleted in Eppendorf tubes. Cell pellets were postfixed for 1 h in 1% osmium tetroxide plus 0.8% potassium ferricyanide in 100 mM sodium cacodylate, pH 7.2. After thorough rinsing in water, cells were prestained in 4% uranyl acetate for 1 h, thoroughly rinsed, dehydrated, infiltrated overnight in 1:1 acetone-Epon 812, infiltrated for 1 h with 100% Epon 812 resin, and embedded in the resin. After polymerization, 60- to 80-nm thin sections were cut on a Reichert ultramicrotome, stained 5 min in lead citrate, rinsed, poststained 30 min in uranyl acetate, rinsed, and dried. EM was performed at 60 kV on a Philips CM120/Biotwin equipped with a 1,024 by 1,024 Gatan multiscan charge-coupled device, and images were collected at original magnifications of ×28,000 to ×36,000. Virions (40- to 50-nm electron-dense, spherical, enveloped particles) were quantitated in 14 13.125-μm^2 fields each for PP2- and DMSO-treated cells.

RESULTS

Induction of c-Yes expression in WNV-infected cells. In order to identify host genes that are important for viral replication, we performed cDNA microarray analysis on WNV-infected SK-N-MC neuroblastoma-derived cells, reasoning such genes may be differentially regulated during the course of infection. We observed that the level of mRNA encoding the src family kinase (SFK) c-Yes was increased approximately

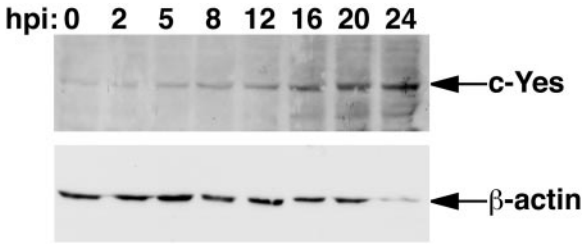

FIG. 1. Expression of c-Yes protein during WNV replication. SK-N-MC cells were infected with WNV at a multiplicity of 10 and harvested at the indicated times postinfection, and expression of c-Yes and β-actin was analyzed by Western blotting.

fivefold over the levels in mock-infected cells by 15 h postinfection (data not shown) and that there was a corresponding increase in c-Yes protein during infection (Fig. 1A). Cellular β-actin protein levels remained unchanged throughout infection, indicating that the observed effect is not a general increase of macromolecular synthesis within infected cells (Fig. 1B).

Effect of inhibition of SFK activity on WNV replication. To determine whether WNV induction of c-Yes protein synthesis is important for viral replication, we examined the effect of an SFK-specific inhibitor, PP2 (13), on WNV replication in cultured SK-N-MC cells. Cells were infected at a multiplicity of 5 and treated with PP2. Infectious virus was collected at the indicated time points and quantified by plaque assay. As shown, treatment of SK-N-MC cells following WNV infection with PP2 resulted in a decrease of infectious virus recovered from cell culture supernatants as well as cell-associated virus at 16, 24, and 48 h postinfection (Fig. 2A and B). Similar results were obtained with HEK 293 cells (Fig. 2C and D), Huh7 hepatocellular carcinoma cells, primary human foreskin fibroblasts, Vero cells, and primary rat neurons (not shown). Toxicity of PP2 was assessed by quantitation of the number of viable cells by trypan blue exclusion following 48 h of treatment with PP2 (Fig. 3A) or by measurement of cleavage of the tetrazolium salt XTT (Fig. 3B). These assays did not indicate significant cytotoxicity of the compounds at the concentrations used in these experiments. A second inhibitor of SFKs, SU6656 (3), exerted a similar effect on WNV replication, as did the general tyrosine kinase inhibitor genestein (data not shown). These data demonstrate that SFKs (possibly including the identified target, c-Yes) are indeed involved in the replication of WNV.

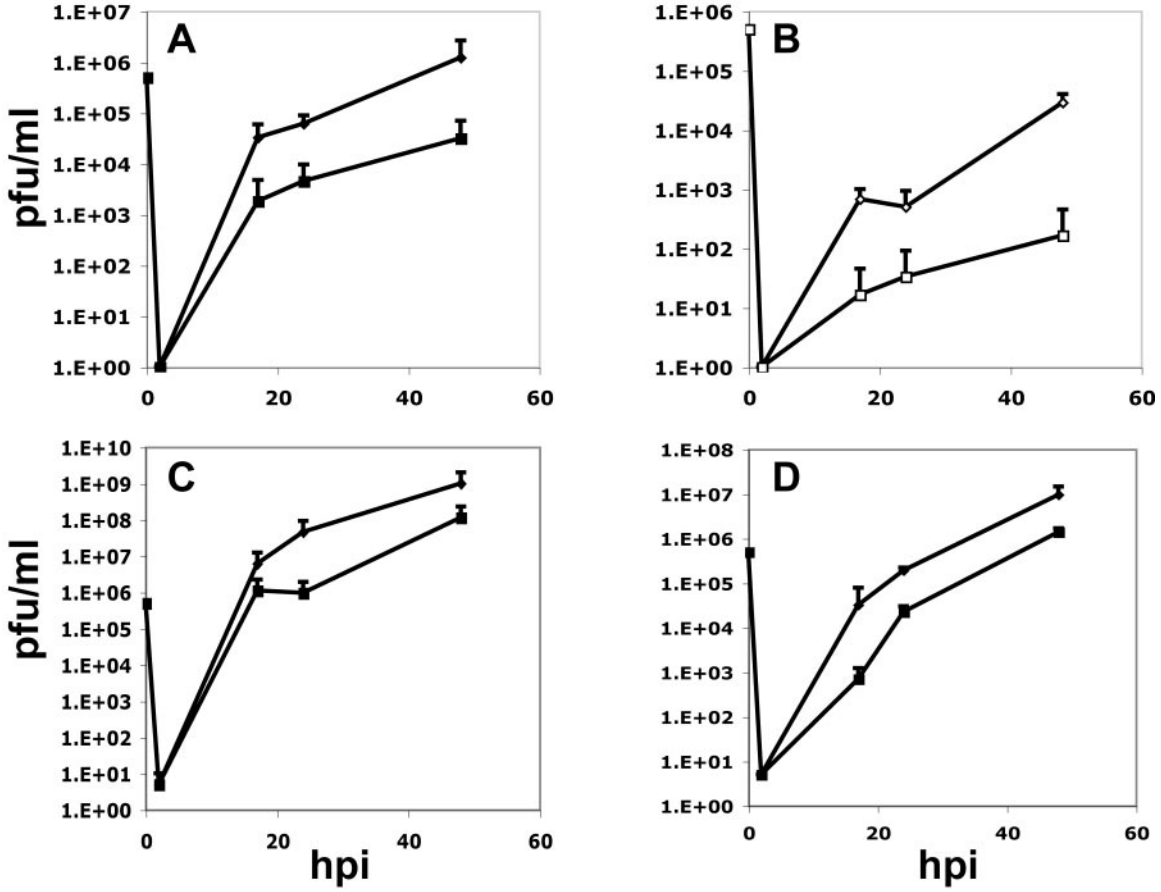

FIG. 2. Effect of the SFK inhibitor PP2 on WNV growth. WNV-infected SK-N-MC (A and B) or HEK 293 (C and D) cells were treated with DMSO (diamonds) or 20 μM PP2 (squares). At the indicated times postinfection, supernatant (A and C) and cell-associated virus (B and D) were quantitated by plaque assay on Vero cells. Results shown are the averages of three independent infections. Error bars represent standard deviations.

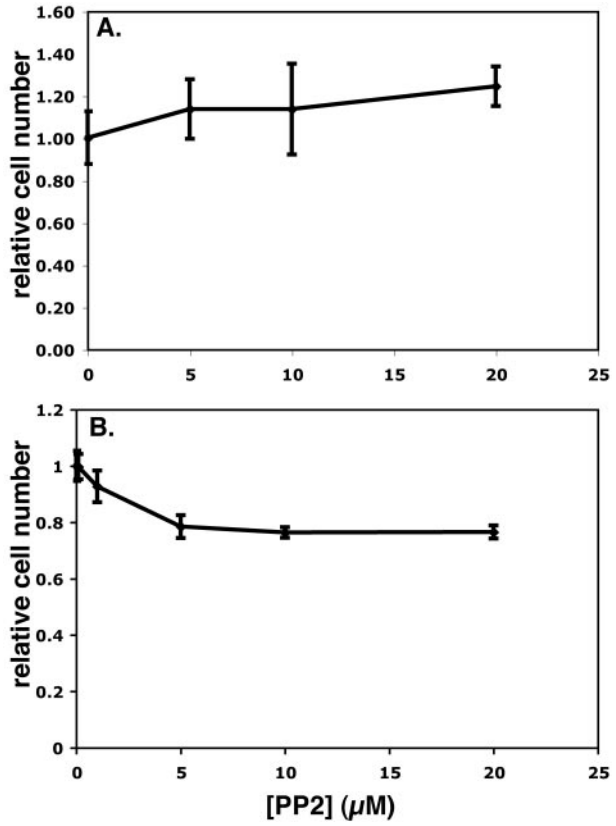

FIG. 3. PP2 treatment does not result in significant cellular toxicity through 48 h. (A) SK-N-MC cells were treated with the indicated concentrations of PP2 for 48 h. Viable cells were counted using trypan blue exclusion. Cell number is expressed relative to the number of viable cells in DMSO controls. Numbers represent the averages of three wells for each condition. (B) Vero cells were treated with the indicated concentrations of PP2 for 48 h. Numbers of metabolically active cells were quantified by measuring conversion of XTT tetrazolium salt to a formazan dye. Cell number is expressed relative to the number of viable cells in DMSO controls. Numbers represent the averages of three wells for each condition.

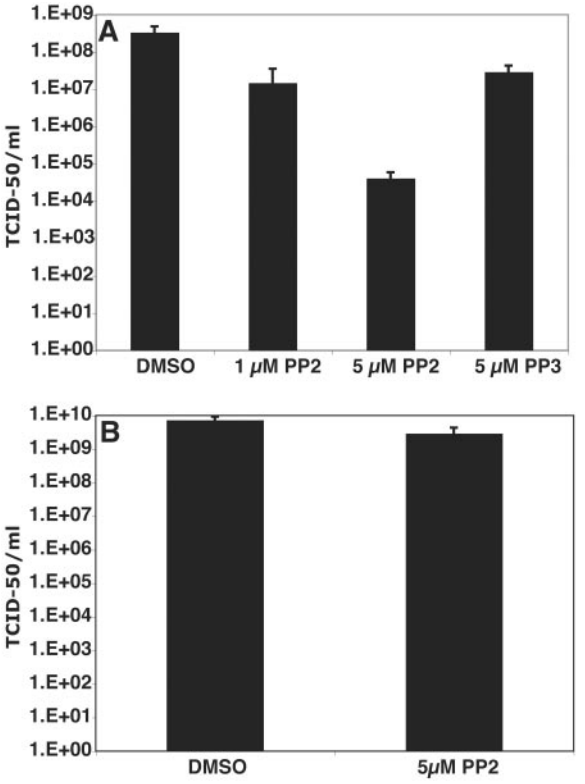

FIG. 4. $TCID_{50}$ determination of WNV and HSV in the presence of SFK inhibitors. (A) $TCID_{50}$s of WNV on Vero cells in the presence of the indicated concentrations of PP2, PP3, or DMSO. (B) $TCID_{50}$s of Vero cells infected with HSV in the presence of PP2 or DMSO. Results shown are the averages of three independent infections. Error bars represent standard deviations.

As a second approach to examining the effect of SFK inhibitors on WNV replication, we employed a $TCID_{50}$ assay. For this assay, 10-fold serial dilutions of WNV were plated on 96-well plates in the presence of the indicated concentrations of PP2. After 5 to 6 days, wells were scored for CPE and we determined the dilution at which 50% of the wells were infected (8). Viral titers (expressed as $TCID_{50}$/ml) in the presence or absence of PP2 were calculated as the inverse of this dilution. Because the virus undergoes multiple rounds of replication in this assay, the effect of the drug is amplified, making this assay particularly sensitive. Therefore, we were able to discern effects of lower concentrations of PP2 on WNV replication in this assay compared to single-step growth curves. As shown in Fig. 4A, the $TCID_{50}$ titer was reduced approximately 25-fold in the presence of 1 μM PP2 and 10,000-fold in the presence of 5 μM PP2. PP3, an molecular analog of PP2 that lacks activity against src kinases (44), has only a modest effect on WNV growth at 5 μM. Infection of Vero cells by herpes simplex virus (HSV) was only slightly decreased by 5 μM PP2 (Fig. 4B), compared to the effect on WNV (Fig. 4A). Additionally, one-step growth curves performed with HSV in the presence of 20 μM PP2 showed that viral replication was unaffected (data not shown). These results suggest a specific role for SFK activity in WNV replication, as opposed to a generalized effect rendering the cells unable to support viral replication.

siRNA-mediated knock-down of c-Yes reduces WNV growth. Although the compounds used in the above studies are specific for SFKs, the inhibitors do not discriminate between SFK members. In order to identify which SFK was responsible for PP2 sensitivity of WNV replication, siRNAs were used to specifically knock down expression of c-Yes and c-Src in WNV-infected cells. Transfection with the c-Yes- and c-Src-specific siRNAs resulted in knock-down of protein levels to 14% and 30% of control cells, respectively (Fig. 5A). siRNA-transfected cells were subsequently infected with serial dilutions of WNV, and the $TCID_{50}$ titer was determined for each transfection condition. Cells transfected with the c-Yes-specific siRNA, but not the c-Src-specific siRNA, showed a 50-fold decrease in viral titers (Fig. 5B). These data suggest that the effect of PP2 on WNV replication is due to the inhibition of c-Yes activity, further supporting the hypothesis that this protein plays an important role in the viral life cycle.

SFK inhibition does not affect synthesis of WNV RNA or protein. The effects of the SFK inhibitors on WNV may be

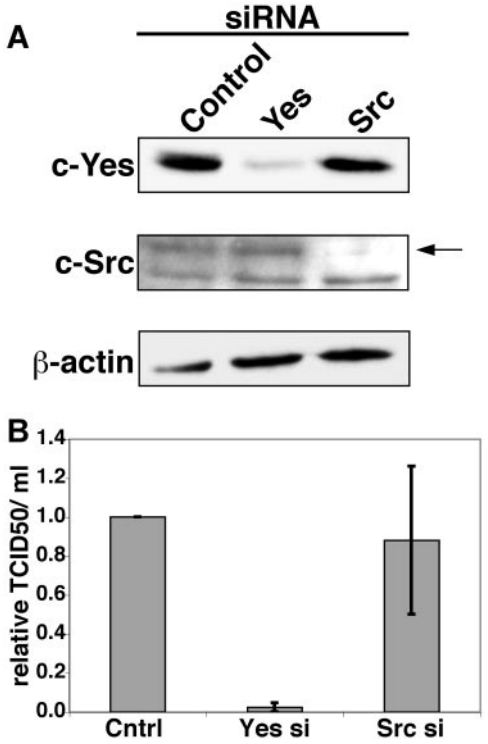

FIG. 5. WNV growth in the presence of siRNA-mediated knockdown of c-Yes and c-Src. (A) Vero cells were transfected with siRNA duplexes specific for c-Yes, c-Src, or a control siRNA duplex. Lysates of transfected cells were analyzed by Western blotting for expression of c-Yes, c-Src, and β-actin. The arrow in the second panel indicates the 60-kDa c-Src band. (B) $TCID_{50}$s were determined on Vero cells transfected with the indicated siRNA duplexes. Results shown are the averages of three independent experiments.

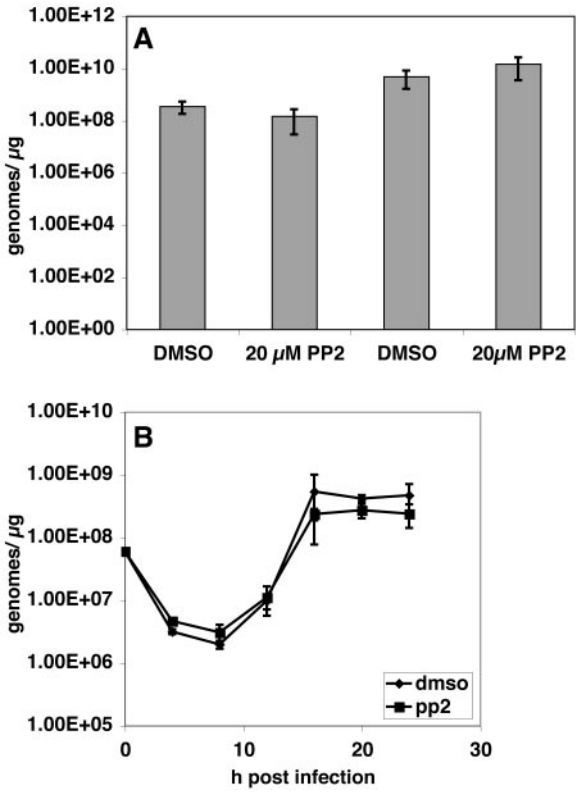

FIG. 6. WNV RNA levels in PP2-treated SK-N-MC and Vero cells. (A) SK-N-MC cells were infected at a multiplicity of 10 and treated with 20 μM PP2 or DMSO. RNA was harvested at the indicated times postinfection and quantified using quantitative real-time PCR. RNA quantities are expressed as viral genomes/μg total cellular RNA. (B) Vero cells were infected at a multiplicity of 5 and treated with 20 μM PP2 (squares) or DMSO (diamonds). RNA was harvested and quantified as above. In both panels, values represent the averages of three infections.

exerted at several stages of the viral life cycle, including RNA replication, virion assembly and maturation, or viral egress from the cell. In order to determine if SFK inhibitors affect RNA replication, we examined the levels of WNV RNA within infected SK-N-MC cells using quantitative RT-PCR. At 24 h and 48 h postinfection, times at which a significant decrease in infectious virus titers was observed, we found that viral RNA levels were equivalent in PP2-treated and control cells (Fig. 6A). In addition, RNA levels were measured in infected Vero cells at 4-h intervals from the start of infection up to 24 h postinfection (Fig. 6B). Again, PP2 treatment did not result in a difference compared to untreated controls. The function of SFKs during WNV replication, therefore, occurs at a step following viral RNA replication.

We next assessed the effect of PP2 on viral protein levels. Immunoprecipitation of the E protein from lysates of ^{35}S-labeled cells showed equal levels of E in treated and untreated cells (Fig. 7A). Furthermore, both samples showed coprecipitation of the expected 25-kDa band, consistent with the size of the prM protein. In addition, direct visualization of the E protein in infected cell lysates by Western blotting also indicated approximately equal protein levels in PP2-treated and untreated cells (Fig. 8A). These data indicate that SFK activity does not affect protein levels, nor does it affect the association of the membrane glycoproteins.

Inhibition of SFK activity results in a decrease in particle release. We next pelleted virus from the supernatants of the ^{35}S-labeled, WNV-infected cells used above. As shown in Fig. 7B, PP2 results in a decrease in the amount of pelleted E protein, indicating that PP2-treated cells release fewer viral particles than their DMSO-treated counterparts. Furthermore, the 25-kDa prM band is not visible in either lane, showing that processing of prM by furin has been completed. Therefore, those particles that are released in the presence of PP2 appear to be of the mature form. These data rule out the possibility that the observed decrease in infectious titer was due to an increased production of defective particles. Because we see a reduction in intracellular infectious virus in the presence of PP2, we conclude that SFK activity is needed for virus particles to form, at least to the point of obtaining infectivity. Alternatively, particles may assemble in the ER but remain associated with the membrane so as to be insoluble and, therefore, excluded from the cell lysate used to analyze intracellular infectivity. The reduced number of virions that do assemble (or leave the ER) appear to be able to transit to the TGN, where prM is cleaved by furin, and exit the cell. It is possible that residual SFK activity that exists in the presence of PP2 is sufficient for the production/transit of this residual number of

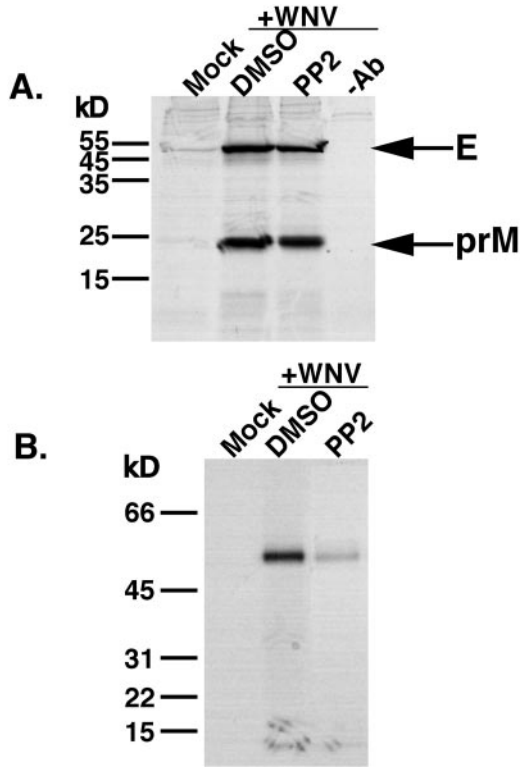

FIG. 7. PP2 does not inhibit WNV protein synthesis but prevents release of viral particles. (A) WNV E protein was immunoprecipitated from ^{35}S-labeled lysates from mock-treated, WNV-infected and DMSO-treated, or WNV-infected and PP2 (20 μM)-treated cells. Arrows indicate the expected sizes of the E and prM proteins. In lane 4, an infected cell lysate was subjected to the immunoprecipitation procedure without E-specific antibody. (B) Culture supernatants from ^{35}S-labeled mock-infected, WNV-infected and DMSO-treated, or WNV-infected and PP2 (20 μM)-treated cells were centrifuged through a 20% sucrose cushion, and pelleted virus was analyzed by SDS-PAGE.

virions. Alternatively, these results may indicate the existence of a secondary pathway of virus assembly and maturation.

Inhibition of SFK activity prevents maturation of the E glycoprotein. Inhibition of SFKs may result in mis-localization or aberrant trafficking of WNV proteins, thereby interfering with formation of infectious particles. To test this hypothesis, we treated lysates from WNV-infected cells (with or without PP2) with endoH or PNGase F. EndoH removes immature high-mannose glycans from proteins. High-mannose sugars are added to proteins in the ER. These sugars are trimmed and further modified in the Golgi, conferring resistance to endoH. Sensitivity to endoH is therefore indicative of proteins that have not advanced beyond the endoplasmic reticulum in the secretory pathway (11). PNGase F removes all glycan moieties regardless of modification. Vero cells were infected with WNV and treated with PP2 (or DMSO as a control) 1 h postinfection. At 24 h postinfection, cells were lysed and treated with endoH or PNGase F. Samples were resolved by SDS-PAGE, and WNV E was detected by Western blotting. As shown in Fig. 8, treatment of cell lysates with endoH revealed the presence of roughly equivalent populations of resistant samples (equal mobility to untreated samples) and sensitive samples (equal mobility to PNGase F-digested proteins). In contrast, in PP2-treated samples, the proportion of endoH-resistant population was decreased. These data suggest that in the absence of SFK activity transit of the E protein to post-ER compartments of the secretory pathway is impaired. Importantly, MHC I, a cellular glycoprotein, was endoH resistant in PP2-treated cells. Consequently, we conclude that SFK inhibition does not generally impair trafficking of proteins from the ER but either acts specifically on the transit of WNV glycoproteins or, at least, on a subset of proteins that include WNV E (but exclude MHC I).

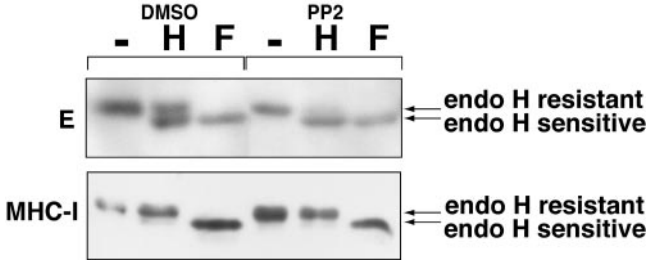

FIG. 8. WNV E protein maturation is impaired in PP2-treated cells. Vero cells were infected with WNV at a multiplicity of 5 and treated with 10 μM PP2 (or DMSO as a control) 1 h postinfection. Cells were lysed at 24 h postinfection. Lysates were denatured and treated with endoH (H lanes) or PNGase F (F lanes). Samples were resolved by SDS-PAGE, and WNV E and MHC I were detected by Western blotting.

SFK inhibition allows WNV virion assembly but retains particles in the ER. The observation that E protein was retained in the ER in PP2-treated cells suggests one of two possibilities: (i) the virus assembles but is unable to transit further in the cell secretory pathway or (ii) the virus fails to assemble, and free prM-E heterodimers remain in the ER. In order to distinguish between these possibilities, we performed transmission electron microscopy on WNV-infected Vero cells in the presence or absence of PP2 (Fig. 9). If virions were observed associated with the ER, this observation would support the first hypothesis, and if particles were not observed this finding would support the second hypothesis. As shown, infected cells in both DMSO and PP2-treated samples had extensive virus-induced membranous structures (designated "paracrystalline arrays" or "convoluted membranes") that are indicative of flavivirus infection (30, 49). Additionally, the PP2-treated cells possessed large numbers of "pea pod-like" structures that appeared identical to structures previously identified as virions within the lumen of engorged ER (30) (Fig. 9D and E). Virions were also found in electron micrographs of DMSO-treated infected cells, although in smaller numbers (Fig. 9C). Quantitation of virions in multiple fields of PP2- and DMSO-treated cells (14 fields for each condition) showed an approximately 2.5-fold increase in total intracellular virions and an approximately 5-fold increase in ER-associated virions in PP2-treated cells (Fig. 9F). Therefore, inhibition of SFK activity does not prevent assembly of the WNV virion but leads to an accumulation of virus particles within the ER. The fact that we observed a reduction of infectious virus within the infected cells following PP2 treatment (Fig. 2B and D) suggests that these virions are noninfectious, consistent with the immature state of virions that are expected to be found in the ER (37, 46)

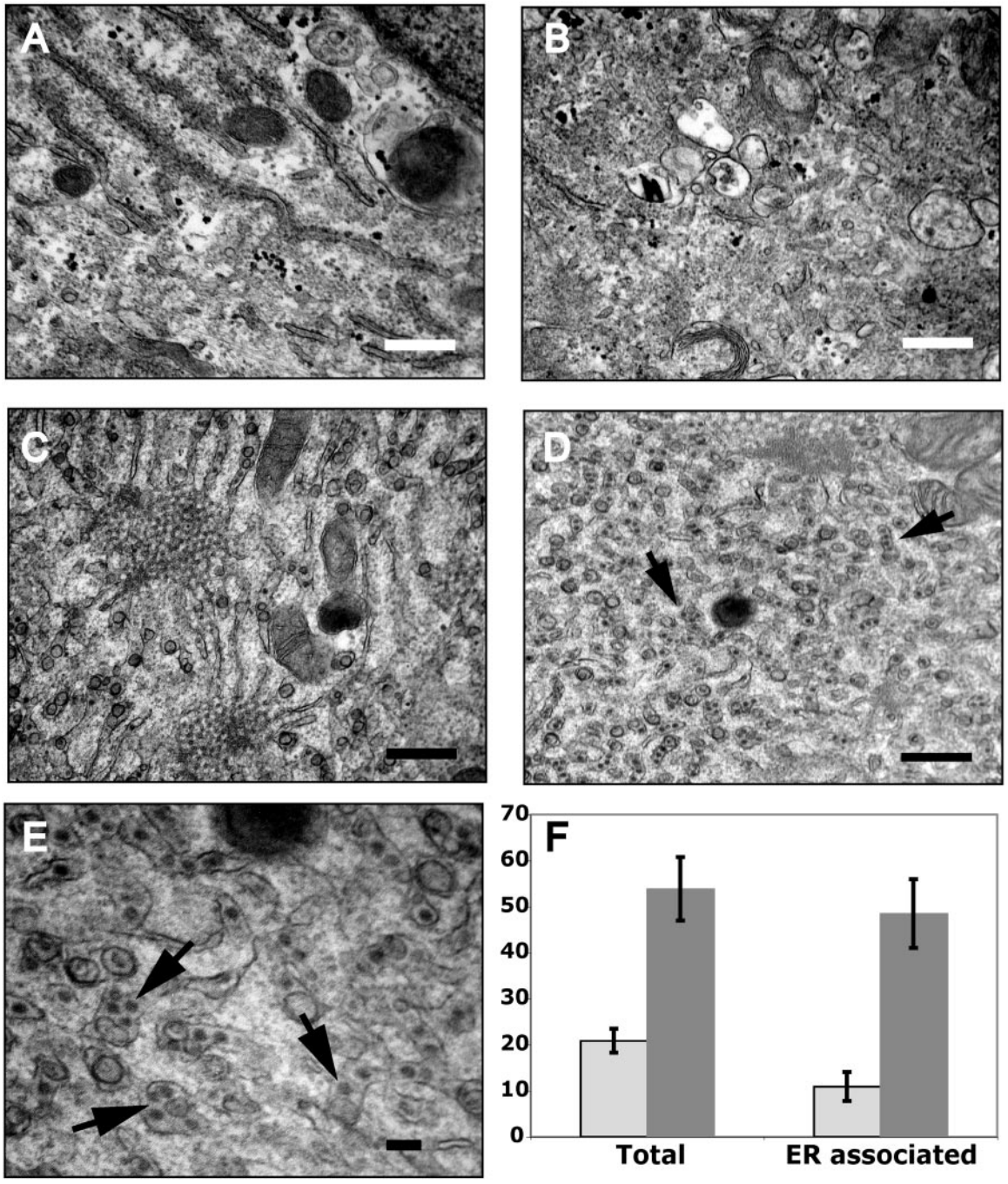

FIG. 9. ER-associated particles are found in increased numbers in PP2-treated cells. Mock-infected (A and B) or WNV-infected (C, D, and E) Vero cells treated with DMSO (A and C) or 10 μM PP2 (B, D, and E) were processed for electron microscopy at 24 h postinfection. Representative ER-localized virions are indicated by arrows. Bars, 500 nm (A to D) or 100 nm (E). (F) Quantitation of total or ER-localized virus particles per field (13.125 μm^2) ± standard error of the mean. Totals represent the average of 14 fields each. Light gray bars, DMSO-treated cells; dark gray bars, PP2-treated cells.

or, as previously suggested, remain in an insoluble, membrane-associated fraction following cell lysis. Additionally, we note that the increase of ER-associated virions observed in PP2-treated cells does not fully account for the loss of infectious virus seen in cells or culture supernatants. This may indicate that virion assembly is slowed in these cells, or that virions "stuck" in the ER are subject to degradation.

DISCUSSION

In the current study, we have observed that the src family kinase c-Yes is upregulated during WNV infection and that inhibition of SFK activity reduces the production of infectious virus. The role of c-Yes in the production of infectious WNV was specifically confirmed using an RNAi-mediated knock-

down. c-Yes activity does not affect synthesis of viral RNA or protein, but rather it plays a role in the trafficking of the assembled virion from the ER, through the cellular secretory pathway.

A recent report observed that tyrosine kinase inhibitors, including PP2, reduced neurotoxicity in Japanese encephalitis virus (JEV)-infected neuronal cultures (33). Analysis of JEV-infected cells treated with the SFK inhibitors revealed that the compounds did not affect production of either viral RNA or protein. However, those authors did not analyze production of infectious virus. These observations are largely in agreement with our results with WNV. Interestingly, we observed a reduction of JEV titers similar to that observed for WNV in PP2-treated Vero cells (data not shown).

The host cell response to flavivirus infection has been characterized in several studies (10, 27, 40, 45). As might be expected, many of the host genes differentially regulated during infection are proinflammatory genes, such as cytokines or interferon response factors. Additionally, JEV has been shown to induce genes of the unfolded protein response in certain cell types (40). The process by which cells detect double-stranded RNA or excessive ER protein loads and alter gene expression have been largely defined, but c-Yes has not been implicated in either of these pathways. The mechanism of c-Yes mRNA upregulation during WNV remains unknown. It seems likely that transcriptional upregulation of c-*yes* is achieved via the indirect activation of one or more cellular transcription factors, since WNV is a cytoplasmic virus. However, it has been shown that the C and NS4B proteins of Kunjin virus (a WNV subtype) translocate into the nucleus, suggesting the possibility of a direct effect on host cell transcription (47).

Flavivirus assembly is believed to occur by budding of the RNA-containing NC into the ER lumen. This process may involve the interaction of exposed hydrophobic residues on the NC with the ER membrane, although the mechanism of coordination of the NC with the envelope glycoproteins is unknown (28). Following assembly in the ER, virions are hypothesized to be transported by vesicles into the Golgi apparatus, with subsequent movement through the *trans*-Golgi, accumulation in vesicles, and release by exocytosis (reviewed in reference 14). Using cryoimmunoelectron microscopy of Kunjin virus-infected cells, Mackenzie and Westaway (25) identified single virions being transported from the ER in vesicles. This step is sensitive to inhibition by Brefeldin A, which interferes with vesicular transport from the ER to Golgi. Our observations suggest that c-Yes may be involved in this process. In contrast, Ng et al. observed formation of nucleocapsids at the plasma membrane as well as in large vesicles in Vero cells infected with WNV strain Sarafend (31, 32). It is possible that these observations represent two distinct pathways by which the virus can exit the cell. Because we observed a reduction, but not total elimination, of infectious virus in cells treated with PP2 or transfected with c-Yes-specific siRNA, we believe our results are consistent with the existence of a second pathway independent of c-Yes. Indeed, the fact that a cellular glycoprotein, MHC I, traffics normally (Fig. 8B) in the presence of PP2 indicates that movement from the ER to the Golgi apparatus and beyond can still occur under these conditions. Therefore, when the c-Yes-dependent virus-specific pathway is blocked, the virus may be able to traffic via a default secretory pathway, albeit at reduced levels.

The src kinases are a family of protein tyrosine kinases that have been implicated in a variety of cellular processes, such as cell division and cytoskeletal organization (reviewed in reference 43). c-Yes is the most closely related of the SFKs to the prototypical family member, c-Src, with the two proteins possessing greater than 80% homology (41), and both are expressed ubiquitously (43). Additionally, these kinases are able to compensate for one another to effect entry into mitosis (34). Functional redundancy appears to occur between Src and Yes in vivo, since knockout mice lacking either Src or Yes survive, while homozygous knockout mice null for both SFKs develop lethal defects prior to birth (39). However, the individual phenotypes of mice lacking Src or Yes are dramatically different, clearly indicating that these kinases play nonredundant roles in vivo as well. c-Yes$^{-/-}$ mice do not display any overt abnormality, except for reduced trans-cytosis of the poly(immunoglobulin A) receptor (22). In contrast, src$^{-/-}$ mice demonstrate defects in osteoclast function, poor cell motility, and decreased neurite outgrowth (36). In this study, we have found that transit of WNV virions is not a shared function of c-Src and c-Yes but is specific to the c-Yes protein. Several cellular functions have been attributed to c-Yes, but not c-Src (42). Notably, c-Yes, alone of the SFKs, is found in lipid rafts enriched in caveolin in MDCK cells. Some groups hypothesize that this observation may reflect a role for c-Yes in vesicular trafficking or protein sorting (35, 42).

SFKs have been shown to be involved in various aspects of the replicative cycles of several types of virus, such as hepatitis B virus, vaccinia virus, mouse polyoma virus, and Rous sarcoma virus (RSV) (12, 17, 18, 26, 29). In the case of RSV, c-Yes is hypothesized to play a role in the budding and release of particles. Residues critical for virus budding (the late [L] domain) of the RSV gag protein have been shown to bind the Yes-associated protein (Yap) in vitro, and the region of Yap that binds the RSV gag L domain is capable of blocking viral egress in vivo. However, a direct role for c-Yes itself in this process has not been demonstrated definitively.

The results of this study demonstrate that WNV trafficking is dependent on c-Yes activity during egress from the ER. However, the WNV E and prM proteins are lumenal, while c-Yes is localized to the cytoplasmic leaflet of intracellular membranes. Moreover, NS5 is the only flavivirus protein known to be phosphorylated, and this event occurs exclusively on serine residues. This suggests that c-Yes does not act directly on the WNV particle but is one component of a larger pathway that controls WNV trafficking. Nevertheless, c-Yes may represent a novel target for therapeutic intervention.

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REFERENCES

1. **Anonymous.** 2005. West Nile virus. [Online.] http://www.cdc.gov/ncidod/dvbid/westnile/index.html.
2. **Blackwell, J. L., and M. A. Brinton.** 1997. Translation elongation factor-1 alpha interacts with the 3′ stem-loop region of West Nile virus genomic RNA. J. Virol. **71:**6433–6444.
3. **Blake, R. A., M. A. Broome, X. Liu, J. Wu, M. Gishizky, L. Sun, and S. A. Courtneidge.** 2000. SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. Mol. Cell. Biol. **20:**9018–9027.
4. **Briese, T., W. G. Glass, and W. I. Lipkin.** 2000. Detection of West Nile virus sequences in cerebrospinal fluid. Lancet **355:**1614–1615.
5. **Brinton, M. A.** 2002. The molecular biology of West Nile virus: a new invader of the western hemisphere. Annu. Rev. Microbiol. **56:**371–402.
6. **Chu, J. J., and M. L. Ng.** 2004. Infectious entry of West Nile virus occurs through a clathrin-mediated endocytic pathway. J. Virol. **78:**10543–10555.
7. **Chu, J. J., and M. L. Ng.** 2004. Interaction of West Nile virus with alpha v beta 3 integrin mediates virus entry into cells. J. Biol. Chem. **279:**54533–54541.
8. **Cottey, R., C. A. Rowe, and B. S. Bender.** 2003. Measurement of tissue culture influenza virus infectious dose ($TCID_{50}$), p. 19.11. *In* J. E. Colgan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.), Current protocols in immunology. John Wiley & Sons, New York, N.Y.
9. **De Nova-Ocampo, M., N. Villegas-Sepulveda, and R. M. del Angel.** 2002. Translation elongation factor-1α, La, and PTB interact with the 3′ untranslated region of dengue 4 virus RNA. Virology **295:**337–347.
10. **Fredericksen, B. L., M. Smith, M. G. Katze, P. Y. Shi, and M. Gale, Jr.** 2004. The host response to West Nile virus infection limits viral spread through the activation of the interferon regulatory factor 3 pathway. J. Virol. **78:**7737–7747.
11. **Freeze, H. H.** 1999. Use of glycosidases to study protein trafficking, p. 15.2.1–15.2.26. *In* J. S. Bonifacino, M. Dasso, J. B. Harford, J. Lippincott-Schwartz, and K. M. Yamada (ed.), Current protocols in cell biology. John Wiley and Sons, Inc., Philadelphia, Pa.
12. **Garnier, L., J. W. Wills, M. F. Verderame, and M. Sudol.** 1996. WW domains and retrovirus budding. Nature **381:**744–745.
13. **Hanke, J. H., J. P. Gardner, R. L. Dow, P. S. Changelian, W. H. Brissette, E. J. Weringer, B. A. Pollok, and P. A. Connelly.** 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J. Biol. Chem. **271:**695–701.
14. **Heinz, F. X., and S. L. Allison.** 2003. Flavivirus structure and membrane fusion. Adv. Virus Res. **59:**63–97.
15. **Jordan, I., T. Briese, N. Fischer, J. Y. Lau, and W. I. Lipkin.** 2000. Ribavirin inhibits West Nile virus replication and cytopathic effect in neural cells. J. Infect. Dis. **182:**1214–1217.
16. **Khromykh, A. A., A. N. Varnavski, P. L. Sedlak, and E. G. Westaway.** 2001. Coupling between replication and packaging of flavivirus RNA: evidence derived from the use of DNA-based full-length cDNA clones of Kunjin virus. J. Virol. **75:**4633–4640.
17. **Klein, N. P., M. J. Bouchard, L. H. Wang, C. Kobarg, and R. J. Schneider.** 1999. Src kinases involved in hepatitis B virus replication. EMBO J. **18:**5019–5027.
18. **Klein, N. P., and R. J. Schneider.** 1997. Activation of Src family kinases by hepatitis B virus HBx protein and coupled signaling to Ras. Mol. Cell. Biol. **17:**6427–6436.
19. **Li, W., Y. Li, N. Kedersha, P. Anderson, M. Emara, K. M. Swiderek, G. T. Moreno, and M. A. Brinton.** 2002. Cell proteins TIA-1 and TIAR interact with the 3′ stem-loop of the West Nile virus complementary minus-strand RNA and facilitate virus replication. J. Virol. **76:**11989–12000.
20. **Lindenbach, B. D., and C. M. Rice.** 2001. *Flaviviridae:* the viruses and their replication, p. 991–1041. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
21. **Liu, W. J., H. B. Chen, and A. A. Khromykh.** 2003. Molecular and functional analyses of Kunjin virus infectious cDNA clones demonstrate the essential roles for NS2A in virus assembly and for a nonconservative residue in NS3 in RNA replication. J. Virol. **77:**7804–7813.
22. **Luton, F., M. Verges, J. P. Vaerman, M. Sudol, and K. E. Mostov.** 1999. The SRC family protein tyrosine kinase p62yes controls polymeric IgA transcytosis in vivo. Mol. Cell **4:**627–632.
23. **Ma, L., C. T. Jones, T. D. Groesch, R. J. Kuhn, and C. B. Post.** 2004. Solution structure of dengue virus capsid protein reveals another fold. Proc. Natl. Acad. Sci. USA **101:**3414–3419.
24. **Mackenzie, J. M., A. A. Khromykh, M. K. Jones, and E. G. Westaway.** 1998. Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. Virology **245:**203–215.
25. **Mackenzie, J. M., and E. G. Westaway.** 2001. Assembly and maturation of the flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively. J. Virol. **75:**10787–10799.
26. **Messerschmitt, A. S., N. Dunant, and K. Ballmer-Hofer.** 1997. DNA tumor viruses and Src family tyrosine kinases, an intimate relationship. Virology **227:**271–280.
27. **Moreno-Altamirano, M. M., M. Romano, M. Legorreta-Herrera, F. J. Sanchez-Garcia, and M. J. Colston.** 2004. Gene expression in human macrophages infected with dengue virus serotype-2. Scand. J. Immunol. **60:**631–638.
28. **Mukhopadhyay, S., R. J. Kuhn, and M. G. Rossmann.** 2005. A structural perspective of the flavivirus life cycle. Nat. Rev. Microbiol. **3:**13–22.
29. **Newsome, T. P., N. Scaplehorn, and M. Way.** 2004. SRC mediates a switch from microtubule- to actin-based motility of vaccinia virus. Science **306:**124–129.
30. **Ng, M. L., and J. H. Chu.** 2002. Interaction of West Nile and Kunjin viruses with cellular components during morphogenesis. Curr. Top. Microbiol. Immunol. **267:**353–372.
31. **Ng, M. L., J. Howe, V. Sreenivasan, and J. J. Mulders.** 1994. Flavivirus West Nile (Sarafend) egress at the plasma membrane. Arch. Virol. **137:**303–313.
32. **Ng, M. L., S. H. Tan, and J. J. Chu.** 2001. Transport and budding at two distinct sites of visible nucleocapsids of West Nile (Sarafend) virus. J. Med. Virol. **65:**758–764.
33. **Raung, S. L., S. Y. Chen, S. L. Liao, J. H. Chen, and C. J. Chen.** 2005. Tyrosine kinase inhibitors attenuate Japanese encephalitis virus-induced neurotoxicity. Biochem. Biophys. Res. Commun. **327:**399–406.
34. **Roche, S., S. Fumagalli, and S. A. Courtneidge.** 1995. Requirement for Src family protein tyrosine kinases in G_2 for fibroblast cell division. Science **269:**1567–1569.
35. **Sargiacomo, M., M. Sudol, Z. Tang, and M. P. Lisanti.** 1993. Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. J. Cell Biol. **122:**789–807.
36. **Soriano, P., C. Montgomery, R. Geske, and A. Bradley.** 1991. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell **64:**693–702.
37. **Stadler, K., S. L. Allison, J. Schalich, and F. X. Heinz.** 1997. Proteolytic activation of tick-borne encephalitis virus by furin. J. Virol. **71:**8475–8481.
38. **Stam, N. J., T. M. Vroom, P. J. Peters, E. B. Pastoors, and H. L. Ploegh.** 1990. HLA-A- and HLA-B-specific monoclonal antibodies reactive with free heavy chains in Western blots, in formalin-fixed, paraffin-embedded tissue sections and in cryo-immuno-electron microscopy. Int. Immunol. **2:**113–125.
39. **Stein, P. L., H. Vogel, and P. Soriano.** 1994. Combined deficiencies of Src, Fyn, and Yes tyrosine kinases in mutant mice. Genes Dev. **8:**1999–2007.
40. **Su, H. L., C. L. Liao, and Y. L. Lin.** 2002. Japanese encephalitis virus infection initiates endoplasmic reticulum stress and an unfolded protein response. J. Virol. **76:**4162–4171.
41. **Sukegawa, J., K. Semba, Y. Yamanashi, M. Nishizawa, N. Miyajima, T. Yamamoto, and K. Toyoshima.** 1987. Characterization of cDNA clones for the human c-yes gene. Mol. Cell. Biol. **7:**41–47.
42. **Summy, J. M., M. Sudol, M. J. Eck, A. N. Monteiro, A. Gatesman, and D. C. Flynn.** 2003. Specificity in signaling by c-Yes. Front. Biosci. **8:**s185–s205.
43. **Thomas, S. M., and J. S. Brugge.** 1997. Cellular functions regulated by Src family kinases. Annu. Rev. Cell Dev. Biol. **13:**513–609.
44. **Traxler, P., G. Bold, J. Frei, M. Lang, N. Lydon, H. Mett, E. Buchdunger, T. Meyer, M. Mueller, and P. Furet.** 1997. Use of a pharmacophore model for the design of EGF-R tyrosine kinase inhibitors: 4-(phenylamino)pyrazolo[3,4-d]pyrimidines. J. Med. Chem. **40:**3601–3616.
45. **Warke, R. V., K. Xhaja, K. J. Martin, M. F. Fournier, S. K. Shaw, N. Brizuela, N. de Bosch, D. Lapointe, F. A. Ennis, A. L. Rothman, and I. Bosch.** 2003. Dengue virus induces novel changes in gene expression of human umbilical vein endothelial cells. J. Virol. **77:**11822–11832.
46. **Wengler, G.** 1989. Cell-associated West Nile flavivirus is covered with E+pre-M protein heterodimers which are destroyed and reorganized by proteolytic cleavage during virus release. J. Virol. **63:**2521–2526.
47. **Westaway, E. G., A. A. Khromykh, M. T. Kenney, J. M. Mackenzie, and M. K. Jones.** 1997. Proteins C and NS4B of the flavivirus Kunjin translocate independently into the nucleus. Virology **234:**31–41.
48. **Westaway, E. G., J. M. Mackenzie, M. T. Kenney, M. K. Jones, and A. A. Khromykh.** 1997. Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. J. Virol. **71:**6650–6661.
49. **Westaway, E. G., J. M. Mackenzie, and A. A. Khromykh.** 2002. Replication and gene function in Kunjin virus. Curr. Top. Microbiol. Immunol. **267:**323–351.