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Association between NS3 and NS5 Proteins of Dengue Virus Type 2 in the Putative RNA Replicase Is Linked to Differential Phosphorylation of NS5

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Abstract

Dengue virus type 2, a member of the family Flaviviridae, encodes a single polyprotein precursor consisting of 3391 amino acid residues that is processed to at least 10 mature proteins by host and viral proteases. The NS3 protein contains a domain commonly found in cellular serine proteinases that in cooperation with NS2B is involved in polyprotein processing. In addition, NS3 and NS5 proteins contain conserved motifs found in several RNA helicases and RNA-dependent RNA polymerases, respectively. Both enzymatic activities have been suggested to be involved in viral RNA replication. In this report, we demonstrate that the NS3 and NS5 proteins interact *in vivo* in dengue virus type 2-infected monkey kidney (CV-1) cells and in HeLa cells coinfected with recombinant vaccinia viruses encoding these proteins as shown by coimmunoprecipitations and immunoblotting methods. We also show by immunofluorescence, metabolic labeling, and two-dimensional peptide mapping that NS5 is a nuclear phosphoprotein and that phosphorylation occurs on serine residues at multiple sites. Furthermore, NS5 exists in differentially phosphorylated states in the nuclear and the cytoplasmic fractions, and only the cytoplasmic form of NS5 is found to coimmunoprecipitate with NS3, suggesting that differential phosphorylation may control the interaction between these proteins and its function in the viral RNA replicase.

Abbreviations: DEN-2, dengue virus type 2; NS, nonstructural protein denoting the virus-specific proteins expressed only in the infected cells as opposed to being a component of the virion; PBS, phosphate-buffered saline; vv, vaccinia virus; PAGE, polyacrylamide gel electrophoresis; Ni NTA, nickel-nitrilotriacetic acid.

Introduction

Dengue virus type 2 (DEN-2), a member of the family of Flaviviridae, has a single-stranded RNA genome of positive-strand polarity containing 10,723 nucleotides (in New Guinea C strain; Reference 1). The 5′ end of the genomic RNA has a type I cap, and the 3′ end is devoid of a poly(A) tail (for reviews, see 2, 3, 4, 5, 6). The genomic RNA has a single open reading frame containing 10,173 nucleotides encoding a polyprotein of 3391 amino acid residues with a gene order of 5′-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′, which is processed into three structural proteins that are components of the virion (C, prM, and E) and at least seven nonstructural proteins, NS1 to NS5, which are expressed in the infected cells (6).

The processing of the structural region of the polyprotein is carried out by the host signal peptidase associated with the endoplasmic reticulum (7, 8, 9, 10). The processing of the nonstructural protein precursors at 2A-2B, 2B-3, 3-4A, and 4B-5 sites containing two basic amino acid residues (such as RR, RK, and KR) followed by a G, A, or S is well characterized, and it requires both NS2B and the N-terminal 180 amino acid residues of NS3 containing the conserved catalytic triad of the serine proteinase family (11, 12, 13, 14, 15, 16, 17, 18, 19, 20). However, functions of the viral nonstructural proteins in genome replication are not well defined.

The region immediately C-terminal to the catalytic triad of the serine protease domain of NS3 contains conserved segments of NTP-binding proteins and DEAD family of RNA helicases (12, 21, 22), and in this regard, NS3 appears to be a bifunctional protein. The RNA helicase function of NS3 has not yet been demonstrated, but it has been implicated in an unwinding step of genomic RNA replication, which is thought to occur through the formation of a double-stranded RNA replicative intermediate (23, 24, 25, and 42; for a review, see reference 5). NS5, the largest protein of the virus, is the putative RNA-dependent RNA polymerase involved in viral RNA replication based on the conserved motif GDD found in several RNA-dependent RNA polymerases (26, 27, 28 ; for a review, see references 6 and 21) and is thought to be involved in the formation of this replicative intermediate form. Thus, NS3 and NS5 are thought to be the components of the putative viral RNA replicase complex. We have overproduced NS3 and NS5 proteins using the recombinant vaccinia virus expression system (29, 30) with a focus on the characterization of these two proteins for their enzymatic activities and for their role in viral RNA replication.

In this report, we demonstrate that NS3 and NS5 proteins interact *in vivo* in cells infected with either DEN-2 or with recombinant vaccinia viruses encoding these proteins. The NS3 protein also binds *in vitro* to a C-terminally modified form of NS5 protein immobilized to an affinity matrix. Localization by immunofluorescence using NS3- or NS5-specific antibodies shows that NS3 is localized in the cytoplasm and perinuclear region, whereas NS5 is localized predominantly in the nucleus of DEN-2 infected cells, although diffuse cytoplasmic localization of NS5 is also observed. Immunoprecipitation analysis of the cytoplasmic and nuclear extracts from these cells shows two forms of NS5 in the cytoplasm separable by their electro-

phoretic mobilities, and only one of these forms is predominantly located in the nucleus. NS3 coimmunoprecipitated with only one form of NS5 in the cytoplasmic extract. Furthermore, we show that NS5 is phosphorylated *in vivo* at serine residues at multiple sites. The electrophoretic mobilities of the two forms of NS5 are altered upon treatment with a phosphatase, indicating that NS5 exists in differentially phosphorylated states.

Experimental Procedures

Materials and Cell Lines — [^{32}P]Orthophosphate (carrier-free, 285 Ci/mg phosphate) and trans ^{35}S-label (1000 Ci/mmol) were obtained from ICN Radiochemicals. The cellulose-coated thin-layer plates were purchased from EM Sciences (Cherry Hill, NJ). The phosphoamino acid standards, iodoacetamide, phenylmethylsulfonyl fluoride, potato acid phosphatase, tosylphenylalanyl chloromethyl ketone-treated trypsin, bovine serum albumin, and the methionine-free Dulbecco's modified Eagle's medium were purchased from Sigma.

Monkey kidney (CV-1), BSC-1 cells, and human TK$^-$ (143B) cells were grown in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%). HeLa cells were grown in suspension culture in Dulbecco's modified Eagle's medium supplemented with 7% mixed serum (3.5% new born bovine serum and 3.5% bovine serum). To prepare a monolayer of HeLa cells, HeLa cells growing in a suspension culture were pelleted, washed with Dulbecco's modified Eagle's medium containing fetal bovine serum (10%), and then plated in a suitable flask. DEN-2 virus (New Guinea strain C) was originally obtained from Walter Reed Army Institute of Research (Washington, D. C.) and was propagated in CV-1 cells. A laboratory strain of vaccinia virus, WR strain, as well as the recombinant vaccinia virus encoding the T7 RNA polymerase, vTF7-3, were originally obtained from Dr. Bernard Moss and then propagated in HeLa cells (31).

Construction of Recombinant Vaccinia Viruses Encoding NS3, NS5, and NS5 with a C-terminal Histidine Tag — To construct recombinant vaccinia viruses encoding NS3 and NS5, CV-1 cells were infected with the wild-type (WR strain) vaccinia virus, followed by transfection with the expression plasmids encoding full-length NS3 and NS5 cloned into the T7 hybrid expression vector pTM1 (30). To construct NS5 with a C-terminal histine tag, we used a modified pTM-1 vector (pJK3) (J. Kusukawa and R. Padmanabhan, unpublished results) containing a region encoding the synthetic FLAG (DYKDDDDK) epitope (32), for which a commercial monoclonal antibody is available (Kodak Scientific Imaging Systems, New Haven, CT), and six histidine residues (33). pJK3 was digested with *Cla*I and *Xho*I and a 4.7-kilobase pair fragment was purified. pLZNS5 (19) was digested with *Cla*I and *Eag*I and a 3-kilobase pair fragment was purified. A third fragment was obtained by polymerase chain reaction using pLZNS5 as template and the primers, 5′-GAAAGACGGCCGCGTACT and 5′-TAGTCCTCGAGCCACAGGACTCCTGCCTC. The three fragments were ligated to yield the pNS5.H$_6$. To construct the recombinant virus, this expression plasmid was transfected into CV-1 cells as described above. Cell lysates were prepared by subjecting the transfected cells to five freeze-thaw cycles. The recombinant viruses encoding the NS3, NS5, and NS5.H$_6$ (vvNS3, vvNS5, and vvNS5.H$_6$) having the TK$^-$ phenotype were selected by infecting a human TK$^-$ cell line (HuTK$^-$ 143B) in the presence of the 5-bromodeoxyuridine and screened by Southern hybridization. The recombinant virus stocks were prepared as described (31). For immunoprecipitations and immunoblot analysis, rabbit polyclonal antibody against NS5 and NS3 were used (19, 34).

Indirect Immunofluorescence — For subcellular localization of NS3 and NS5 by immunofluorescence, CV-1 cells were infected with DEN-2 virus at 50 plaque forming units/cell. At 48 h postinfection, the cells were detached from the monolayers by trypsin treatment and plated onto glass coverslips. The infected cells were washed briefly with cold phosphate-buffered saline (PBS), pH 7.2, fixed in cold acetone (kept at –20 °C) for 20 min, and air-dried for 15 min (35). Cells were briefly rehydrated in cold PBS and incubated with 200 μl of rabbit polyclonal antibody (1:30) in PBS at 37 °C for 1 h. Subsequently, they were subjected to three 10-min washes with cold PBS and incubated with 200 μl of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:30) in PBS at 37 °C for 1 h. Cells were finally washed 3 times (10 min each) with cold PBS, coated with 90% glycerol in PBS, and observed under a microscope (Nikon Diaphot) with an epifluorescence attachment.

Metabolic Labeling with ^{32}P and ^{35}S — Monolayers of HeLa cells were coinfected with vTF7-3 and vvNS3 or vvNS5 at 5 plaque forming units/cell for each virus. In experiments in which both NS3 and NS5 were expressed, the vTF7-3, vvNS3, and vvNS5 were used at 5, 2.5, and 2.5 plaque forming units/cell, respectively. For *in vivo* labeling, HeLa cells infected with vvNS3 and vvNS5 at 13 h postinfection (or CV-1 cells infected with DEN-2 at 36 h postinfection) were washed and incubated for 1 h in methionine-free medium supplemented with 5% dialyzed fetal bovine serum. The medium was replaced with the one containing [^{35}S]methionine (at a final concentration of 100 μCi/ml) and incubated for 4 h at 37 °C. For ^{32}P labeling, cells were incubated for 1 h with phosphate-free Joklik's modified Eagle's medium supplemented with 5% dialyzed fetal bovine serum and 20 mM HEPES, pH 7.5, and then in a medium containing [^{32}P]orthophosphate (carrier-free, 285 Ci/mg phosphate, and 1 mCi/flask) for 4 h.

Cell Lysis and Immunoprecipitation — Cells were lysed in 1 ml of a buffer containing 50 mM Tris-HCl, pH 7.4, 0.25 M NaCl, 0.1% Triton X-100, 5 mM EDTA, 1 mM iodoacetamide, and 1 mM phenylmethylsulfonyl fluoride. The cell lysates were treated with micrococcal nuclease for 45 min at 37 °C to digest nucleic acids, precleared with normal rabbit serum, and then immunoprecipitated as described before (36). For the analysis of labeled phosphoproteins, the immunoprecipitation buffer was supplemented with 0.2% SDS. The immunoprecipitated protein was dissociated from protein A-agarose by boiling for 5 min in the sample buffer containing 65 mM Tris-HCl, pH 7.0, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromphenol blue and electrophoresed on 6 or 8% SDS-polyacrylamide gels (37). The dried gels containing radiolabeled proteins were detected by autoradiography using a Kodak XAR-5 x-ray film at –70 °C. The nuclear and cytoplasmic fractions from vTF7-3- and vvNS5- or vvNS3-coinfected HeLa cells or from DEN-2-infected CV-1 cells were isolated as described previously (38). For examining the interaction between NS3 and NS5 *in vitro*, the extracts prepared from vTF7-3 and vvNS5.H$_6$-coinfected HeLa cells were incubated with nickel-nitrilotriacetic acid (Ni NTA) beads (Qiagen) in 0.25 M Tris-Cl, pH 8.0, and 1 mM phenylmethylsulfonyl fluoride for 10 h at 4 °C with gentle rocking. The beads were then washed twice with PBS, pH 7.4, containing 0.3 M NaCl, followed by two more washings with PBS, pH 6.8, containing 0.3 M NaCl, to remove unbound NS5 and nonspecific proteins. NS5-bound beads were then incubated for 2 h at 4 °C with extracts prepared from either vTF7-3-infected, or vTF7-3- and vvNS3-coinfected HeLa cells. The beads were washed as described above, and the proteins that were bound to NS5-Ni NTA beads were analyzed by SDS-PAGE and immunoblotting methods. The immunoblot was probed firstly with anti-NS3 antibody and then with goat anti-rabbit coupled to alkaline phosphatase.

Phosphoamino Acid Analysis and Two-dimensional Peptide Mapping — ^{32}P-Labeled proteins were resolved on 6% SDS-polyacrylamide gels and transferred onto nitrocellulose membrane (Bio-Rad). A small portion of the membrane was subjected to immunoblot analysis to identify the band of interest. The remaining untreated membrane was subjected to autoradiography, and the proteins were eluted from the membrane by digestion with tosylphenylalanyl chloromethyl ketone-treated trypsin as described (39). Phosphoamino acid analysis was carried out as described (40, 41). Migration of the unlabeled standards was determined by ninhydrin staining, and labeled phosphoamino acids were visualized by autoradiography. Phosphopeptide analysis by two-dimensional finger printing was performed as described (40, 41).

In Vitro Dephosphorylation of NS5 — [^{35}S]Methionine-labeled cytoplasmic and nuclear extracts obtained from DEN-2-infected cells were immunoprecipitated with anti-NS5 antibody and protein A-agarose beads. The beads were washed once with the immunoprecipitation wash buffer and twice with dephosphorylation buffer (0.15 M sodium acetate buffer, pH 5.0). The beads were resuspended in the dephosphorylation buffer, treated with 0.5 unit of potato acid phosphatase for 1 h at 37 °C, and analyzed by SDS-PAGE, followed by autoradiography.

Results

Evidence That NS3 and NS5 Interact in Vivo and in Vitro — NS3 and NS5 proteins are thought to be the components of the putative viral replicase complex. To examine whether there is a physical interaction between NS3 and NS5, DEN-2-infected cells were metabolically labeled with [^{35}S]methionine and analyzed by immunoprecipitation using a rabbit polyclonal antibody against NS3, NS5, or rabbit preimmune serum. Immunoprecipitates were then subjected to SDS-PAGE and blotted on to a nitrocellulose membrane. Autoradiography

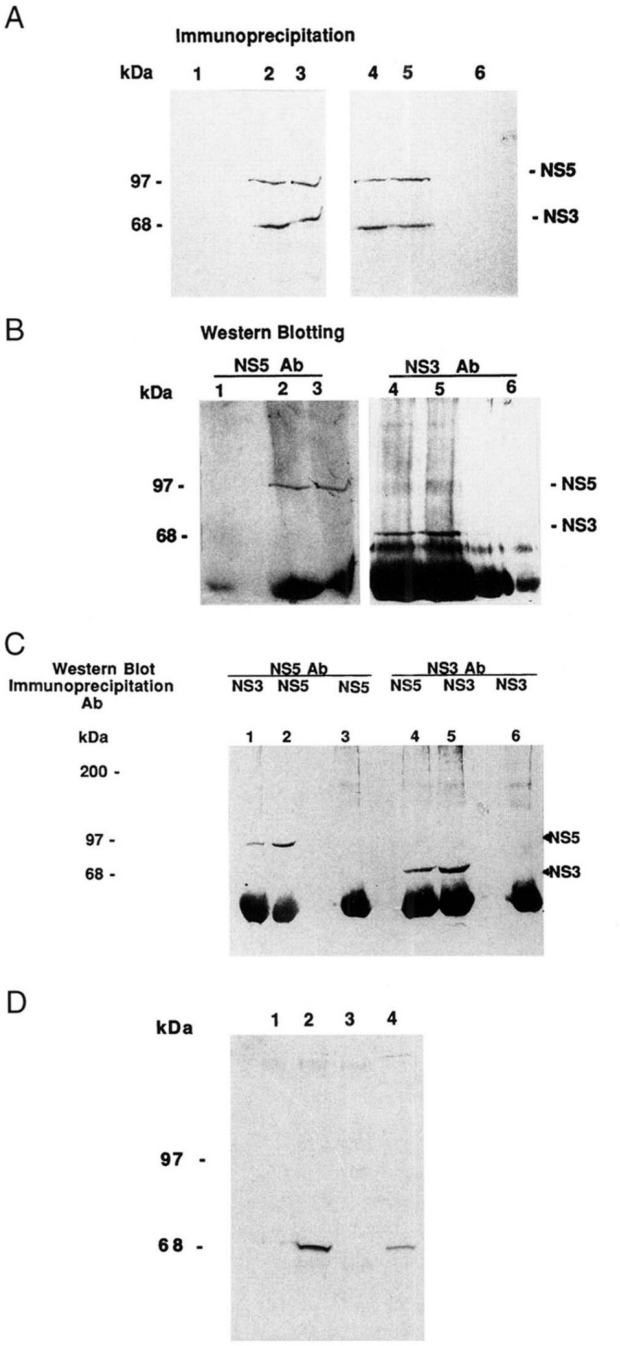

Figure 1: NS3 and NS5 interact *in vivo*. [^{35}S]Methionine-labeled DEN-2-infected cells were lysed and subjected to immunoprecipitation followed by Western blot analysis (**B**) and autoradiography (**A**) of the same blot. *Lanes 1 & 6*, immunoprecipitation using preimmune serum; *lanes 2 & 4*, immunoprecipitation using NS3 antibody (*Ab*); *lanes 3 & 5*, immunoprecipitation using NS5 antibody. For Western blot analysis, *lanes 1*, *2*, & *3* were probed with anti-NS5 antibody, and *lanes 4*, *5*, & *6* were probed with anti-NS3 antibody. **C)** HeLa cells coinfected with recombinant vaccinia viruses encoding the T7 RNA polymerase gene (vTF7-3) and NS5 (vvNS5) were lysed, and the cell lysates were immunoprecipitated with anti-NS5 antibody (*lanes 2* and *4*) and anti-NS3 antibody (*lanes 1 & 5*). In *lanes 3 & 6*, the vTF7-3-infected cell lysates were immunoprecipitated with anti-NS5 and anti-NS3 antibody, respectively. Immunoprecipitates were subjected to SDS-PAGE and Western blot analysis. *Lanes 1–3* were probed with anti-NS5 antibody, and *lanes 4–6* were probed with anti-NS3 antibody. **D**) The *in vitro* interaction between NS5.H_6 and NS3 is shown. Extracts prepared from HeLa cells coinfected with vTF7-3 and vvNS5.H_6 were incubated with Ni NTA beads (Qiagen), and the beads were washed as described under "Experimental Procedures." The immobilized NS5 containing beads were incubated with an extract prepared from HeLa cells either infected with the vTF7-3 alone (*lane 3*) or coinfected with vTF7-3 and vvNS3 (*lane 4*). *Lane 2* was loaded with the extract from vvNS3-infected HeLa cells without any treatment, and *lane 1* was loaded with the sample prepared from the binding of NS3 extract to Ni NTA beads in the absence of NS5.H_6. Binding to Ni NTA beads, washing conditions, SDS-PAGE, and immunoblotting analyses were carried out as described under "Experimental Procedures."

of the blot shows that neither NS5 nor NS3 was immunoprecipitated with preimmune serum (Figure 1A, lanes 1 and 6), but NS3 was co-immunoprecipitated with NS5 when anti-NS5 (Figure 1A, lanes 3 and 5) or anti-NS3 antibody was used for immunoprecipitation (Figure 1A, lanes 2 and 4), suggesting a physical interaction between these two proteins. To further verify these results and to eliminate the possibility that the antisera against one protein may be cross-reacting with the other protein, the same blot used for autoradiography was analyzed by immunoblotting (Figure 1B). NS5 could be visualized in both anti-NS3 and anti-NS5 immunoprecipitates when the blot was probed with anti-NS5 antibody (Figure 1B, lanes 2 and 3). In the same way, NS3 could be detected in the anti-NS5 and NS3 immunoprecipitates when the blot was probed with anti-NS3 antibody (Figure 1B, lanes 4 and 5). However, neither protein could be visualized when preimmune serum was used for immunoprecipitations (Figure 1B, lanes 1 and 6).

The results of the experiments described above could not address the possibility that other DEN-2 proteins might be involved in facilitating the interaction between NS3 and NS5. In order to explore this possibility, HeLa cells were coinfected with recombinant vaccinia viruses encoding NS3 and NS5, and the lysates from these infected cells were used for immunoprecipitations and immunoblotting experiments. As the results indicate, NS5 could be coimmunoprecipitated with NS3 when either anti-NS3 or anti-NS5 antibody was used for immunoprecipitation (Figure 1C, lanes 1 and 4, respectively). As a negative control, when vTF7-3 (encoding only T7 RNA polymerase)-infected extracts were used for immunoprecipitation with NS3 or NS5 antibody, no specific bands were seen (Figure 1C, lanes 3 and 6). These experiments clearly demonstrated that NS3 and NS5 proteins interact *in vivo* in DEN-2-infected CV-1 cells or in HeLa cells infected with recombinant vaccinia viruses encoding NS3 and NS5 proteins. The results of the latter experiments (Figure 1*C*) show that no other viral protein is required for this interaction. In order to examine whether NS3 and NS5 proteins interact *in vitro*, we constructed the recombinant vaccinia virus encoding the NS5 with the C-terminal modification (vvNS5.H_6) containing the FLAG epitope (32) and the histidine tag (33). The modified NS5 (NS5.H_6) protein expressed in HeLa cells coinfected with vTF7-3 and vvNS5.H_6 was immobilized to Ni NTA affinity beads, which were then washed as described under "Experimental Procedures" to remove any unbound NS5.H_6 as well as nonspecific proteins. The NS5 attached to the beads was incubated with either the extract prepared from the vTF7-3-infected HeLa cells as negative control or the NS3-containing extracts prepared from HeLa cells coinfected with vTF7-3 and vvNS3. The beads were washed to remove any unbound NS3 as well as nonspecific proteins, and the bound proteins were analyzed by SDS-PAGE and immunoblotting using anti-NS3 antibodies as described under "Experimental Procedures." As shown in Figure1 D, NS3 was retained in the NS5-bound Ni NTA beads (lane 4), whereas little or no NS3 was bound to Ni NTA beads in the absence of immobilized NS5 protein (lane 1) or when the NS5-bound beads were incubated with the vTF7-3 extract (lane 3). The NS3 extract was loaded into lane 2 as positive control. These experiments indicate that the NS3 and NS5 proteins interact *in vitro*.

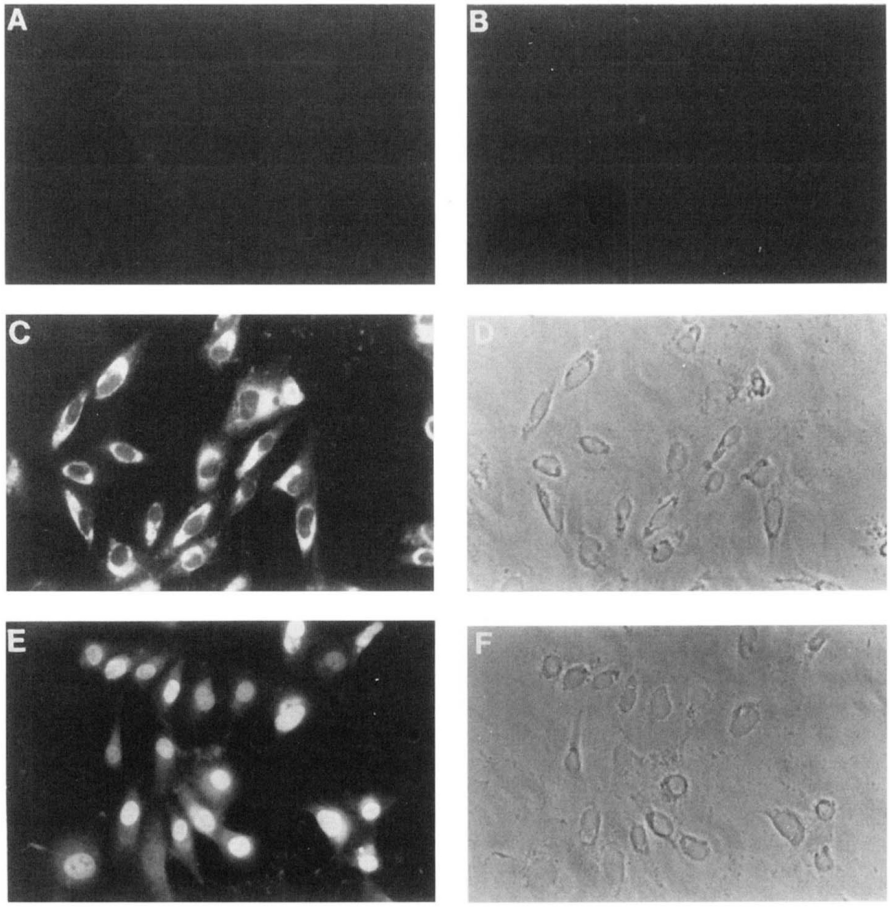

Figure 2: Subcellular localization of NS3 and NS5 in DEN-2-infected cells. Cells were infected with DEN-2 (50 plaque forming units/cell) at room temperature for 2 h. 36-48 h postinfection, cells were trypsinized and plated on coverslips. Indirect immunofluorescence was performed using anti-NS3 antibody (**C**) or anti-NS5 antibody (**E**) as described under "Experimental Procedures." **D** and **F** are the respective phase contrast pictures. Mock-infected cells were used as negative controls for anti-NS3 and anti-NS5 antibodies (**A** and **B**, respectively).

Subcellular Localization of NS3 and NS5 — The results of the coimmunoprecipitation experiments showed an association between NS3 and NS5 *in vivo* in both DEN-2-infected CV-1 cells as well as in HeLa cells coinfected with recombinant vaccinia viruses encoding NS3 and NS5. The subcellular localization of these proteins in DEN-2-infected CV-1 cells as determined by indirect immunofluorescence showed that NS3 was predominantly localized in the cytoplasmic and perinuclear regions of the infected cells (Figure 2C), whereas the NS5 was distributed predominantly in the nucleus, although some diffuse cytoplasmic staining was also detected (Figure 2E). Uninfected cells showed no reactivity to the NS3 and NS5 antibodies (Figure 2, A and B). Similar localization patterns of these proteins were observed in CV-1 cells infected with recombinant vaccinia NS3 and NS5 viruses (data not shown).

Different Forms of NS5 Exist in Nuclear and Cytoplasmic Fractions — The results obtained from the immunofluorescence and coimmunoprecipitation experiments support a working hypothesis that NS3 and NS5 interact to form a complex in the cytoplasm or in the perinuclear region where NS3 is predominantly localized and a different form of NS5 is localized in the nucleus, which is free of NS3. In order to investigate this hypothesis, DEN-2-infected cells metabolically labeled with [^{35}S]methionine were fractionated into cytoplasmic and nuclear fractions, and the fractions were used for immunoprecipitation analyses using anti-NS5 and anti-NS3 antibody or preimmune serum (normal rabbit serum) as a negative control (Figure 3). When cytoplasmic extracts were immunoprecipitated with anti-NS5 antibody, two closely migrating bands for NS5 were detected (Figure 3, lane 1), whereas the nuclear extracts predominantly contained the slower migrating form of the protein (Figure 3, lane 2). Furthermore, NS3 could be coimmunoprecipitated with NS5

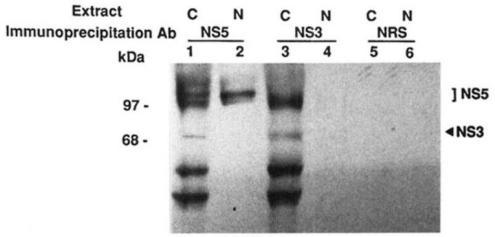

Figure 3: NS5 exists in multiple forms. Cytoplasmic (C) and nuclear extracts (N) from [^{35}S]methionine-labeled DEN-2-infected cells were immunoprecipitated, and the immunoprecipitates were subjected to SDS-PAGE and autoradiography. Lanes 1 and 2, anti-NS5 antibody; lanes 3 and 4, anti-NS3 antibody; lanes 5 and 6, preimmune serum as negative controls.

only from the cytoplasmic extracts (69-kDa band in Figure 3, lane 1). When NS3 antibody was used for immunoprecipitation, the faster migrating form of NS5 could be predominantly coimmunoprecipitated (Figure 3, lane 3). No NS3 was observed in the nuclear extracts, consistent with its subcellular localization observed by immunofluorescence (Figure 2B). Lanes 5 and 6 of Figure 3 show negative controls where preimmune serum was used for immunoprecipitation. We also made an interesting observation that the two forms of NS5 were readily detectable only when DEN-2-infected cells were subjected to a medium containing low serum (2.5%), whereas under normal serum concentration (10%), only one form was obtained (data not shown). The ratio of NS5 to NS3 in the immunoprecipitates was also increased under low serum concentration compared with that obtained under normal medium (compare Figure3, lanes 1 and 3 with Figure 1A, lanes 2-5, respectively). Also, two faster migrating pro-

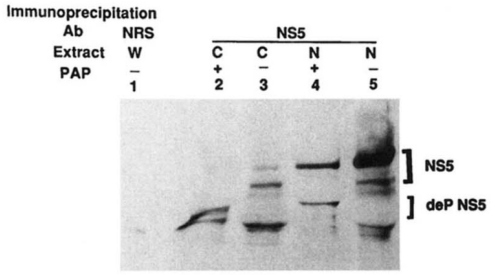

Figure 4: Multiple forms of NS5 are due to differential phosphorylation. Cytoplasmic (C) and nuclear (N) extracts from [^{35}S]methionine-labeled DEN-2-infected cells were used for immunoprecipitation with anti-NS5 antibody (Ab). Immunoprecipitated NS5 was either treated with potato acid phosphatase (lanes 2 and 4) or left untreated (lanes 3 and 5). Lane 1 is a negative control where DEN-2-infected whole cell extract was used for immunoprecipitation with preimmune serum. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

teins coimmunoprecipitated with NS3 and NS5 from cytoplasmic extracts of DEN-2-infected cells. The identity of these proteins in the NS3/NS5 immune complexes is unknown at present.

NS5 Is Post-translationally Modified — The results of the immunoprecipitations with anti-NS5 antibody suggested that the two forms of NS5 could be due to post-translational modification. In order to investigate whether NS5 was modified by phosphorylation, NS5 was immunoprecipitated from cytoplasmic and nuclear extracts of ^{35}S-labeled DEN-2-infected cells. The immunoprecipitated protein was then treated with potato acid phosphatase, and phosphatase-treated and untreated controls were analyzed by SDS-PAGE. The results shown in Figure 4 indicate that after treatment with potato acid phosphatase both the cytoplasmic and the nuclear forms of NS5 shift to faster migrating forms (compare untreated cytoplasmic and nuclear extracts loaded on to lanes 3 and 5 of Figure 4 with the treated samples on lanes 2 and 4). The fastest migrating bands present in the cytoplasmic and nuclear extracts immunoprecipitated by the anti-NS5 antibody were sensitive to potato acid phosphatase treatment, but the identity of these bands is unknown at present. The negative control for these immunoprecipitations is shown in Figure 4, lane 1, in which the unfractionated cell extract was used for immunoprecipitation with preimmune serum (*NRS*).

The sensitivity of immunoprecipitated nuclear and cytoplasmic forms of NS5 to the phosphatase treatment suggested that the multiple forms of NS5 may be due to differential phosphorylation. In order to verify this possibility more directly, CV-1 cells infected with DEN-2 and HeLa cells infected with recombinant vaccinia virus encoding NS5 were metabolically labeled using [^{32}P]orthophosphate. Labeled extracts were immunoprecipitated and analyzed by SDS-PAGE followed by autoradiography (Figure 5). As shown in Figure 5, ^{32}P-labeled NS5 could be immunoprecipitated with anti-NS5 antibody from both the recombinant vaccinia NS5 virus-infected HeLa cell extracts (lane 2) and the DEN-2-infected CV-1 cell extracts (lane 3). As a positive control, NS5 was immunoprecipitated using anti-NS5 antibody from ^{35}S-labeled DEN-2-infected cell extract (Figure 5, lane 1), and as negative controls, extracts of HeLa cells infected with only the T7 RNA polymerase gene-encoded recombinant vaccinia virus vTF7-3 were immunoprecipitated with anti-NS5 antibody (lane 6) or the extracts used in lanes 2 and 3 with normal rabbit serum (lanes 5 and 4, respectively). These results confirm that NS5 is a phosphoprotein. The identity of the phosphorylated form of NS5 was also verified by immunoblot analysis of the anti-NS5 immunoprecipitate prepared from ^{32}P-labeled DEN-2-infected cell extracts, followed by autoradiography of the blot (data not shown).

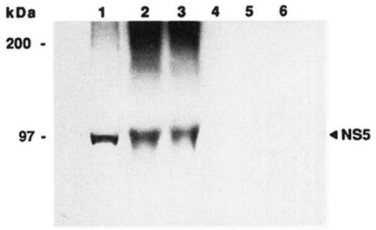

Figure 5: Evidence for Phosphorylation of NS5. CV-1 cells infected with DEN-2 or HeLa cells infected with vvNS5 were metabolically labeled with [^{32}P]orthophosphate. Cell lysates were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. [^{35}S]Methionine-labeled DEN-2-infected CV-1 cell lysate (positive control) with anti-NS5 antibody (lane 1); vTF7-3- and vvNS5-coinfected cell lysate with anti-NS5 antibody (lane 2) or with preimmune serum (lane 5); DEN-2-infected cell lysate with anti-NS5 antibody (lane 3) or with preimmune serum (lane 4); vTF7-3 infected cell lysate with anti-NS5 antibody (lane 6).

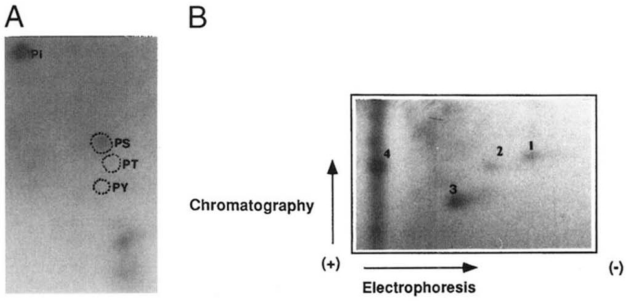

Figure 6: Phosphoamino acid analysis and phophopeptide mapping. A) ^{32}P-labeled NS5 from recombinant vaccinia NS5-infected cells was acid hydrolyzed and mixed with unlabeled phosphoserine, phosphothreonine, and phosphotyrosine as standards. The mixture was subjected to two-dimensional electrophoresis on thin-layer cellulose-coated plates using buffers at pH 1.9 and 3.5 for the first and second dimensions, respectively. Labeled phosphoamino acids were detected by autoradiography, and the migration of unlabeled phosphoamino acids was determined by ninhydrin staining (shown as dotted circles). **PS**, phosphoserine; **PT**, phosphothreonine; **PY**, phosphotyrosine. Partially hydrolyzed NS5 is seen as a smear below the phosphoamino acids. **B)** ^{32}P-labeled NS5 was digested with tosylphenylalanyl chloromethyl ketone-treated trypsin. Tryptic digests were subjected to electrophoresis in the first dimension and ascending chromatography in the second dimension on thin-layer cellulose plates. Phosphopeptides were detected by autoradiography.

Phosphoamino Acid Analysis and Phosphopeptide Mapping — In order to analyze the amino acid residues that were modified by phosphorylation, phosphoamino acid analyses of the 32P-labeled NS5 produced in DEN-2-infected CV-1 cells and in recombinant vaccinia NS5 virus-infected HeLa cells were carried out. The results shown in Figure 6A for recombinant NS5 indicate that phosphate groups are associated only with serine residues (Figure 6A), similar to the pattern observed for native NS5 (data not shown). In order to determine whether a single or multiple serines were modified in NS5, two-dimensional phosphopeptide mapping was performed using 32P-labeled NS5 obtained from recombinant vaccinia NS5 virus-infected HeLa cells (Figure 6B). The results show that there are at least four distinct phosphorylation sites in addition to a few minor ones.

Discussion

The experimental evidence presented in this report indicates that the two non-structural proteins, NS3 and NS5, interact *in vivo* both in DEN-2-infected CV-1 cells and HeLa cells infected with recombinant vaccinia viruses encoding these proteins. Using the NS5.H_6 im-

mobilized to an affinity matrix (Ni NTA beads), we show that these two proteins also interact *in vitro*. This interaction between NS3 and NS5 supports the notion that they are components of the putative viral replicase postulated in earlier studies (6, 42) based on the presence of conserved motifs of a RNA helicase in NS3 and the G-D-D motif in NS5 characteristic of RNA-dependent RNA polymerases (12, 26, 28). The data obtained from the *in vivo* and *in vitro* interaction studies using the recombinant vaccinia virus-infected cell extracts indicate that no other flavivirus protein is required for this interaction. However, a requirement of a cellular protein(s) for this interaction or participation of other viral and cellular proteins in stabilization of this complex cannot be ruled out.

Flavivirus genome replication is thought to occur through the formation of double-stranded RNA replicative intermediate (23, 24, 25, 42). Subcellular localization studies revealed that virus-specific double-stranded RNA appeared to be associated in the perinuclear region of the infected cells (5, 43, 45). The cell fractionation studies also supported these results and further indicated that viral RNA synthesis appeared to be confined principally to the membranes of the perinuclear endoplasmic reticulum (5, 46, 47, 48, 49, 50). Both NS3 and NS5 proteins of flavivirus are predicted to have enzymatic roles in flavivirus RNA replication. Although both proteins have been found to be present in the subcellular fractions containing the RNA polymerase activity, direct interaction between these two proteins has not been demonstrated previously.

Previous studies have reported that NS5 of two other flaviviruses is localized in the nucleus and cytoplasm of infected cells (51, 52). This study, as shown by immunofluorescence, subcellular fractionation, and immunoprecipitation methods, shows that there are two forms of NS5 present in the cytoplasmic fraction that are separable by SDS-PAGE. Only the form with the slower mobility is predominantly located in the nuclear fraction. The multiple forms of NS5 are due to differential phosphorylation, and the hyperphosphorylated form is located predominantly in the nucleus. The presence of two forms of NS5 in DEN-2-infected cells was serentipitously discovered by incubating the infected cells with a medium containing low serum (2.5%). The slower migrating form seems to be the hyperphosphorylated form when compared with the faster migrating and hypophosphorylated cytoplasmic form. Under normal growth conditions containing 10% serum, only one form of NS5 was observed. The result that only the cytoplasmic form of NS5 was found to form a complex with NS3 suggests that differential phosphorylation may regulate the interaction between NS3 and NS5 and thus their participation as components in viral RNA replication.

Based on these results we propose a model as shown in Figure 7. According to this model, the RNA replicase complex containing NS3 and NS5 participate in viral RNA replication associated with the endoplasmic reticulum membrane. In a postreplicative event, NS5 becomes hyperphosphorylated, dissociates from NS3, and is transported to the nucleus. It is known that post-translational modification by phosphorylation regulates protein-protein interactions in eukaryotic cells (for reviews, see references 51 and 52). For instance, in the case of the retinoblastoma gene product (Rb), its state of phosphorylation is important for its interaction with the cellular transcription factor E2F (for a review, see reference 53 and the references therein). The phosphorylation state of Rb varies in different stages of the cell cycle. The protein is in a hypophosphorylated stage at the G_0/G_1 phase, during which it is in a complex with E2F. During the G_1/S phase, Rb gets hyperphosphorylated and dissociates from E2F. Similarly, phosphorylation of IκB results in the dissociation of NFκB-IκB complex and translocation of NFκB to the nucleus (54, 55).

The poliovirus RNA-dependent RNA polymerase, $3D^{pol}$ is a phosphoprotein (56), but the role of phosphorylation in the function of $3D^{pol}$ is unknown. For NS5, phosphorylation may play a role in its nuclear transport, because only the slower migrating hyperphosphorylated form is present in the nucleus. In this regard, it is worth noting that phosphorylation has been shown to play a role in the nuclear transport of SV40 large T antigen (57, 58). The role of NS5 in the nucleus is unknown at present. An interesting speculation is that NS5 may regulate the expression of cellular genes in response to viral infection.

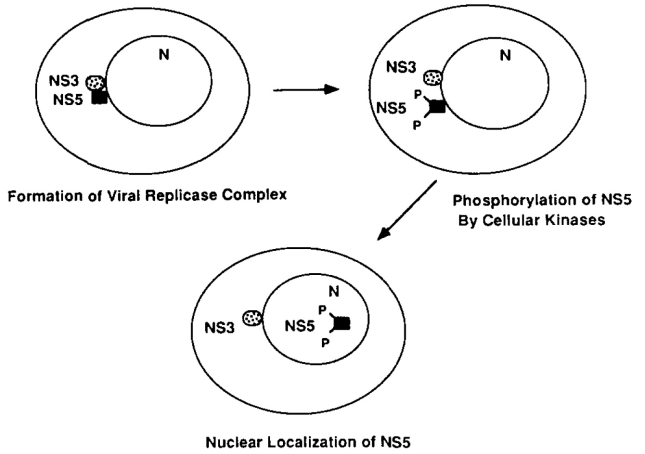

Figure 7: Model for interaction of NS3 and NS5. NS3 and NS5 interact in the perinuclear region and function as the components of putative viral replicase. Phosphorylation of NS5 by cellular kinase(s) results in the disruption of NS3-NS5 interaction and the transport of NS5 to the nucleus.

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