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# Binding of Flavivirus Nonstructural Protein NS1 to C4b Binding Protein Modulates Complement Activation

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**The complement system plays a pivotal protective role in the innate immune response to many pathogens including flaviviruses. Flavivirus nonstructural protein 1 (NS1) is a secreted nonstructural glycoprotein that accumulates in plasma to high levels and is displayed on the surface of infected cells but absent from viral particles. Previous work has defined an immune evasion role of flavivirus NS1 in limiting complement activation by forming a complex with C1s and C4 to promote cleavage of C4 to C4b. In this study, we demonstrate a second mechanism, also involving C4 and its active fragment C4b, by which NS1 antagonizes complement activation. Dengue, West Nile, or yellow fever virus NS1 directly associated with C4b binding protein (C4BP), a complement regulatory plasma protein that attenuates the classical and lectin pathways. Soluble NS1 recruited C4BP to inactivate C4b in solution and on the plasma membrane. Mapping studies revealed that the interaction sites of NS1 on C4BP partially overlap with the C4b binding sites. Together, these studies further define the immune evasion potential of NS1 in reducing the functional capacity of C4 in complement activation and control of flavivirus infection.** *The Journal of Immunology*, **2011, 187: 424–433.**

The complement system is a key component of the early innate immune response to pathogens. Three activation pathways, the classical (CP), lectin, and alternative pathways (AP), may become engaged to control invading microorganisms. Each complement activation pathway is initiated by a distinct set of recognition molecules and converges at the cleavage of C3 to C3a and C3b. The CP is initiated by the binding of C1q upon IgM or IgG engaging Ag. The lectin pathway is triggered by the binding of mannose-binding lectin or ficolins to defined sugar moieties on microbial surfaces. Both pathways activate C4 and C2, resulting in generation of the common C3 convertase C4b2a that cleaves C3 to C3b. The AP is constitutively activated by spontaneous hydrolysis of C3, resulting in generation of C3b, which deposits on pathogen surfaces. Association of factor B with C3b enables the serine protease factor D to cleave factor B into Bb, which then forms the C3bBb AP C3 convertase. The binding of C3b to C4b2a and C3bBb convertases converts them into the CP (C4b2a3b) and AP (C3bBb3b) C5 convertases, respectively. Cleavage of C5 promotes formation of the membrane attack complex C5b-9 that lyses pathogens or infected cells. Beyond its lytic capacity, complement protects against viral infections by priming adaptive B and T cell responses, triggering leukocyte chemotaxis through the release of anaphylatoxins (C3a and C5a), and opsonizing viruses for phagocytosis and destruction by macrophages (reviewed in Refs. 1, 2).

In response to these protective functions, many viral pathogens have evolved evasion strategies to limit complement-mediated control, including the display of surface proteins that bind the Fc domain of Abs to prevent C1q-dependent complement activation, secretion of viral proteins that mimic or recruit host complement regulators, expression of viral proteins that degrade key complement components, direct incorporation of host complement negative regulatory proteins onto the virion surface, and upregulation of complement regulatory proteins on the surface of infected cells (reviewed in Refs. 1–3).

C4b binding protein (C4BP) is the primary fluid-phase regulator of the CP and lectin pathways. The 570-kDa glycoprotein has a large spider-like structure and is composed of seven identical 70-kDa α-chains and one 45-kDa β-chain that are linked by disulfide bonds at their C-terminal domains (4). C4BP is synthesized and secreted primarily by hepatocytes and accumulates to high levels (150–300 μg/ml) in human plasma (5). Similar to other regulators of complement activation for which genes are encoded on the long arm of chromosome 1, the α- and β-chains of C4BP contain eight and three complement control protein (CCP) modules, respectively (6). C4BP regulates complement activation by interacting with the noncatalytic subunit C4b of the CP and lectin pathway C3 and C5 convertases; it inhibits convertase assembly, enhances convertase decay, and has cofactor activity

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Abbreviations used in this article: AP, alternative pathway; BHK, baby hamster kidney fibroblast; C4BP, C4b binding protein; C4BP-dp HS, C4BP-depleted human serum; CCP, complement control protein; CP, classical pathway; DENV, dengue virus; DENV2-Rep, DENV-2 subgenomic replicon; DVB, dextrose veronal buffered saline; NHS, normal human serum; NS1, nonstructural protein 1; proC1s, C1s proenzyme; rWT, recombinant wild-type; WNV, West Nile virus; WNV-Rep, WNV subgenomic replicon; YFV, yellow fever virus; YFV-Rep, YFV subgenomic replicon.



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for the serine protease factor I, which cleaves and thereby inactivates fluid-phase and cell-bound C4b (7–10).

Although C4BP is a plasma complement inhibitor, several studies have shown that it also binds to the surface of cells. Binding of C4BP to CD40 on B lymphocytes induces proliferation and activation (11). The complex of C4BP/CD154/CD40 promotes survival of epithelial cells by inhibiting apoptosis (12). Interaction of the α-chain of C4BP with heparin-sulfate proteoglycans in conjunction with CD91 induces cellular uptake of C4BP (13). C4BP can act as a bridging molecule that facilitates the uptake of adenoviruses by liver cells through the interaction with cell-surface heparin-sulfate proteoglycans (14). C4BP binds to certain types of malignant cell lines via its α-chain and can act as a cofactor for factor I to inactivate C4b on the cell surface (15). Finally, C4BP in concert with protein S binds to phosphatidylserine exposed on the outer membrane of apoptotic and necrotic cells to inhibit DNA release and complement-mediated anaphylatoxin generation and lysis (16, 17).

Flaviviruses are single-stranded positive-sense enveloped RNA viruses that cause extensive global morbidity and mortality in humans and include insect-transmitted viruses such as dengue virus (DENV), West Nile virus (WNV), yellow fever virus (YFV), Japanese encephalitis, and tick-borne encephalitis viruses. The 11-kb flavivirus RNA genome encodes a polyprotein that is cleaved by viral and host proteases to generate three structural (capsid, membrane, and envelope) and seven nonstructural proteins including nonstructural protein 1 (NS1), NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (18). Flavivirus NS1 is a 48-kDa nonstructural glycoprotein that is absent from the virion. NS1 is an essential gene as it is required for efficient viral RNA replication (19–21). In infected mammalian cells, NS1 is synthesized as a soluble monomer, dimerizes after posttranslational modification in the lumen of the endoplasmic reticulum, and is transported to the cell surface and accumulates extracellularly as a hexamer (22–25). Soluble NS1 also binds back to the plasma membrane of cells through interactions with specific sulfated glycosaminoglycans (26).

DENV NS1 has been implicated in the pathogenesis of severe infections (dengue hemorrhagic fever/dengue shock syndrome), although the mechanism remains uncertain. High levels of NS1 are detected in the serum of DENV-infected patients and correlate with severe disease (27, 28). NS1 has been proposed to facilitate immune complex formation (28), elicit autoantibodies that react with platelet and extracellular matrix proteins (29–31), damage endothelial cells via Ab-dependent complement-mediated cytolysis (32), and directly enhance infection (33). Anti-flavivirus NS1 Abs protect mice against lethal virus challenge (34–45) and restrict viral spread by complement-mediated lysis of infected cells (46). Flavivirus NS1 also exhibits immune-evasion functions. WNV NS1 attenuates the AP of complement activation by binding the complement regulatory protein factor H (47). Recently, DENV, WNV, and YFV NS1 were shown to antagonize the CP and lectin pathways by forming a tripartite complex between C4–NS1–C1s/C1s proenzyme (proC1s), which promotes degradation of native C4 (48).

In this study, we describe a second mechanism by which flavivirus NS1 antagonizes the CP and lectin pathways of complement activation through a direct interaction with C4BP. Soluble NS1 recruits C4BP to inactivate C4b in solution and on the plasma membrane of cells.

## Materials and Methods

### *Purified NS1, complement proteins, and sera*

DENV, WNV, and YFV NS1 were produced and isolated from the supernatants of baby hamster kidney fibroblast (BHK) cells that stably propagate DENV-2 (BHK-DENV-2 subgenomic replicon [DENV2-Rep]), WNV (BHK-WNV subgenomic replicon [WNV-Rep]), or YFV (BHK-YFV subgenomic replicon [YFV-Rep]) subgenomic replicons, respectively (48). A two-step purification of flavivirus NS1 was performed using immunoaffinity and size-exclusion chromatography as described previously (26, 48). Purified recombinant wild-type (rWT) C4BP or mutants lacking individual CCP domains were generated and purified as previously described (8). All other purified human complement proteins were purchased from Complement Technologies. Normal human serum (NHS) and C4BP-deficient serum were prepared as previously reported (49).

### *NS1 and C4BP binding ELISA*

Maxi-Sorp microtiter plates (Nunc) were adsorbed with 50 μl purified C4BP or BSA (5 μg/ml in PBS) at 4˚C overnight. After five washes with PBS, nonspecific binding sites were blocked with 150 μl 2% heat-inactivated BSA in PBS for 1.5 h at 37˚C followed by five washes with PBS. Purified DENV, WNV, and YFV NS1 (50 μl diluted in PBS containing 0.1% BSA) at specified concentrations were added to each well and incubated for 2 h at room temperature. Plates were then washed five times with PBS containing 0.05% Tween 20 followed by a 1-h incubation with 1 μg/ml DENV NS1-specific polyclonal Ab, an anti-WNV NS1 mAb (3NS1) (34), or a 1:700 dilution of ascites fluid containing YFV-specific anti-NS1 mAb (4E3) (43). After washing, biotinylated goat anti-mouse IgG (1 μg/ml; Sigma-Aldrich) and HRP-conjugated streptavidin (1 μg/ml; Vector Laboratories) were added sequentially at room temperature. After six final washes with PBS, signal was developed by adding 50 μl tetramethylbenzidine substrate (DakoCytomation) and 25 μl 2 N $H_2SO_4$ stop solution to each well. The OD at 450 nm was determined by a 96-well plate reader (Genio Pro; Tecan Instruments). In some experiments, purified NS1, anti-NS1 specific Abs, biotinylated goat anti-mouse IgG, and HRP-conjugated streptavidin were diluted in 25 mM phosphate buffer (pH 7.4) containing 75, 150, 300, 600, or 1200 mM NaCl and 0.1% BSA. Plates were washed with phosphate buffer (25 mM) (pH 7.4) containing 75, 150, 300, 600, or 1200 mM NaCl and 0.05% Tween 20.

### *Coimmunoprecipitation of NS1 and C4BP*

Serum-free supernatants containing NS1 from BHK-DENV2-Rep or BHK-WNV-Rep cells (1 ml, clarified at 20,000 × g, 10 min) were incubated with purified human C4BP (15 μg), NHS, or C4BP-depleted human serum (C4BP-dp HS) (70 μl) at 4˚C overnight and then precleared with normal mouse IgG (2 μg; Santa Cruz Biotechnology) and protein A Sepharose 4B (40 μl 50% slurry; Zymed) for 3 h at 4˚C followed by immunoprecipitation with anti-DENV NS1 mAb (2G6)-Sepharose or anti-WNV NS1 mAb (9NS1)-Sepharose (20 μl 50% slurry) at 4˚C overnight. After six washes with 1 ml DMEM containing 0.05% Tween 20, bound proteins were eluted in SDS reducing (with 5% [v/v] 2-ME) or nonreducing sample buffer and separated by 4–12% gradient SDS-PAGE (NuPAGE; Invitrogen). Western blotting was performed using a rabbit anti-human C4BP polyclonal Ab (Quidel) followed by a 1:5000 dilution of HRP-conjugated donkey anti-rabbit IgG (GE Healthcare).

### *NS1 binding to C4BP deletion mutants*

Purified rWT C4BP or mutants lacking individual CCP domains (ΔCCP1, ΔCCP2, ΔCCP3, ΔCCP4, ΔCCP5, ΔCCP6, ΔCCP7, and ΔCCP8) (8) (1 μg) were separated by 4% SDS-PAGE or 12% SDS-PAGE without or with 5% (v/v) 2-ME, respectively, followed by silver staining using a ProteoSilverSilver Stain Kit (Sigma-Aldrich) to assess purity and quality of the recombinant proteins. Maxi-Sorp microtiter plates (Nunc) were coated with 20 μg/ml purified rWT C4BP or mutants lacking an individual CCP domain or BSA at 4˚C overnight. Nonspecific binding sites were blocked with 2% BSA in PBS for 1.5 h at 37˚C. After five washes with 0.05% Tween 20 in PBS, 50 μl clarified (20,000 × *g*, 10 min) serum-free cell supernatant from BHK-DENV2-Rep or BHK-WNV-Rep cells was added to each well and incubated for 1 h at room temperature. Bound NS1 was detected as outlined above. In parallel, additional wells coated with wild-type and mutant C4BP were incubated with a 1:5000 dilution of rabbit anti-C4BP polyclonal Ab followed by HRP-conjugated donkey anti-rabbit IgG (1:5000) prior to addition of tetramethylbenzidine substrate.

### *C4b cofactor activity assay*

*After NS1-C4BP coimmunoprecipitation.* These assays were performed in low-salt buffer (25 mM phosphate buffer [pH 7.4] and 25 mM NaCl). Biotinylated C4b (25 ng) and factor I (50 ng) were mixed, and immunoprecipitates from 2G6 anti-DENV NS1 or 9NS1 anti-WNV NS1-Sepharose 4B were added in a total of 60 μl and incubated at 37˚C for 15 min. Reducing SDS sample buffer was added, C4b fragments were separated on a 4–12% gradient SDS-PAGE, and Western blot analysis was performed.

In some experiments, unlabeled C4b (300 ng) was used instead of biotinylated C4b, and Western blot of C4b fragments was performed using a 1:2000 dilution of anti-C4d mAb (Quidel) followed by a 1:5000 dilution of HRP-conjugated anti-mouse IgG.

*On the cell surface.* Serum-free supernatants from control BHK or BHK-DENV2-Rep cells (200 μl) were incubated with C4BP (1.25 or 2.5 μl/ml) for 1 h on ice. Adherent BHK cells were detached after incubation with PBS supplemented with 8 mM EDTA and 10% FBS. Cells ($1 \times 10^6$) in suspension were added to the C4BP solution (± NS1) and incubated for 1 h on ice. After three washes with 1 ml DMEM at 4˚C, cells were washed with 1 ml dextrose veronal buffered saline (DVB; 2.5 mM veronal buffer pH 7.35, 25 mM NaCl, and 240 mM glucose) at 4˚C. C4b (400 ng) and factor I (200 ng) diluted in DVB were added to cell pellets in a total volume of 50 μl. After a 15-min incubation at 37˚C, cells were pelleted ($3000 \times g$), and supernatants were mixed with SDS sample buffer supplemented with 2-ME (5% v/v) and subjected to 4–12% gradient SDS-PAGE. C4b fragmentation was analyzed by Western blot using an anti-human C4d mAb as outlined above. In some experiments, after incubation with C4BP in the absence or presence of DENV NS1, BHK cells were washed three times to remove unbound C4BP followed by a 15-min incubation at 37˚C with an equivalent volume of DVB as performed in the cofactor experiments above, but without addition of factor I and C4b. Subsequently, the supernatants and cells were separately subjected to a cofactor assay as described above.

### *Recruitment of C4BP by soluble NS1 to the cell surface*

Adherent BHK cells were detached as described above. Employing these cells ($1 \times 10^6$/condition), in one set of experiments, purified DENV NS1 or BSA (30 μg/ml) was mixed with various concentrations of C4BP and immediately added to the cell suspension. In the other, supernatants from BHK or BHK-DENV2-Rep cells (200 μl) [the latter containing NS1 (26)] were mixed with C4BP and the cells. After a 1-h incubation on ice, the cells were washed three times with 1 ml DMEM at 4˚C. Surface-bound NS1 and C4BP were detected after adding mouse anti-DENV NS1 2G6 mAb (50) or mouse anti-human C4BP MK 104 mAb (51) followed by 4 μg/ml Alexa Fluor 647-conjugated goat anti-mouse IgG (Invitrogen) and 0.5 μg/ml propidium iodide (Molecular Probes) to exclude dead cells. In some experiments, BHK cells were incubated first with purified DENV NS1 or BSA (10 μg/ml) or serum-free supernatants from BHK or BHK-DENV2-Rep cells (200 μl) for 1 h on ice. After three washes, the cells were incubated with various concentrations of C4BP for 1 h on ice followed by three washes with DMEM at 4˚C. Subsequently, bound NS1 and C4BP were detected as described above. The levels of surface NS1 and C4BP were assessed by flow cytometry (FACSCalibur; BD Biosciences), and data were processed with FlowJo software (Tree Star).

### *Statistical analysis*

Data sets were compared by a two-tailed, unpaired *t* test. Multiple comparisons were performed using an ANOVA test using Prism software (GraphPad). Statistical significance was achieved when *p* values were $<0.05$.

## Results

### *DENV, WNV, and YFV NS1 directly interact with C4BP*

Previous studies have shown that flavivirus NS1 attenuates complement activation by targeting C4, a component common to the CP and lectin pathways, for proteolysis to C4b via a tripartite interaction with C1s (48). An alternative way to restrict C4 activation on a specific target is to recruit a negative complement regulatory protein of the CP and lectin pathways to the surface of pathogens (reviewed in Ref. 52). To begin to address whether flaviviruses use NS1 to control C4b activation by recruiting C4BP, we evaluated if NS1 bound human C4BP. Microtiter plates were adsorbed with purified human C4BP or control protein BSA (Fig. 1). Increasing concentrations of purified DENV NS1 (Fig. 1*A*),

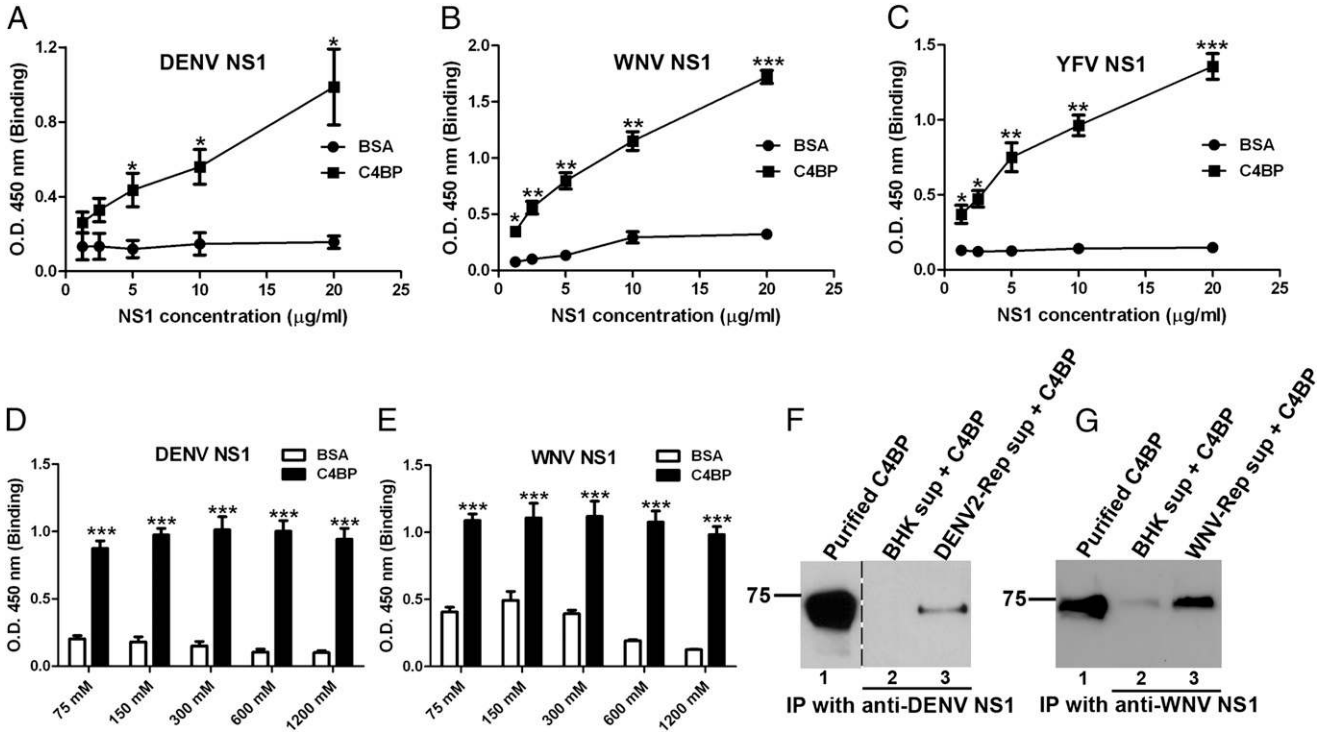


**FIGURE 1.** Flavivirus NS1 binds to C4BP. *A–C*, ELISA. Microtiter plates were coated with BSA or C4BP (5 μg/ml). After incubation with increasing concentrations of purified DENV NS1 (*A*), WNV NS1 (*B*), or YFV NS1 (*C*), bound NS1 was detected with specific mAbs. Error bars indicate SEM from three independent experiments, and asterisks indicate statistical difference from the control BSA. *D* and *E*, Binding of NS1 with C4BP is not affected by salt concentration. Microtiter plates were coated with BSA or C4BP (5 μg/ml). After incubation with 15 μg/ml purified DENV NS1 (*D*) or WNV NS1 (*E*), bound NS1 was detected with NS1-specific mAbs. Error bars indicate SEM from three independent experiments, and asterisks indicate statistical difference from the control BSA. *F* and *G*, Coimmunoprecipitation studies. Serum-free supernatants from BHK-DENV2-Rep, BHK-WNV-Rep, or control BHK cells were incubated with purified C4BP (15 μg/ml), and Western blots were performed after immunoprecipitation with anti-DENV NS1 2G6 mAb-Sepharose (*F*) or anti-WNV NS1 9NS1 mAb-Sepharose (*G*). Immunoprecipitates were probed with a rabbit polyclonal anti-human C4BP Ab. Western blot results are representative of two to three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.0001$.

WNV NS1 (Fig. 1*B*), or YFV NS1 (Fig. 1*C*) were added to C4BP- or BSA-coated wells, and bound NS1 was detected with specific mAbs. A dose-dependent interaction between all three NS1 and C4BP was identified. Increasing ionic strength of the buffer did not appreciably affect the NS1–C4BP interaction, suggesting a nonionic interaction between C4BP and DENV or WNV NS1 (Fig. 1*D*, 1*E*). Coimmunoprecipitation experiments confirmed the interaction between NS1 and C4BP (Fig. 1*F*, 1*G*).

To determine the region(s) of C4BP that interact(s) with NS1, we evaluated a set of C4BP deletion mutants lacking individual CCP domains (Fig. 2*A*). Purity of the recombinant proteins was assessed by silver staining after separation by 4% SDS-PAGE or 12% SDS-PAGE under non-reducing (Fig. 2*B*) or reducing conditions (Fig. 2*C*), respectively. Notably, all recombinant proteins formed multimers in solution as the pattern of migration shifted from larger than 250 kDa to ~75 kDa in the absence and presence of a reducing agent 2-ME, respectively. Microtiter plate wells were adsorbed with rWT α-chain of C4BP or deletion mutants lacking a CCP repeating unit or BSA. Approximately equivalent amounts of wild-type and mutant C4BP were adsorbed as judged by a C4BP-specific polyclonal Ab (Fig. 2*D*). Serum-free supernatants from BHK cells that stably propagate DENV-2 (BHK-DENV2-Rep) or WNV (BHK-WNV-Rep) subgenomic replicons and secrete high levels (up to 4 μg/ml) of NS1 (26) or control BHK cells were added to complement protein-coated wells. DENV and WNV NS1 bound to wild-type α-chain or the ΔCCP1, ΔCCP6, and ΔCCP7 mutants, but there was significantly reduced binding to the ΔCCP2, ΔCCP3, ΔCCP4, ΔCCP5, and ΔCCP8 mutants (Fig. 2*E*, $p < 0.0001$). These results suggest that the binding site for NS1 is located on CCP2, CCP3, CCP4, CCP5, and CCP8 of the α-chain of C4BP.

### *DENV NS1 and WNV NS1 recruit C4BP to degrade C4b in solution*

One of the major regulatory functions of C4BP is to serve as a cofactor for the serine protease factor I to inactivate C4b, also termed cofactor activity (Fig. 3*A*). In the presence of a cofactor (in this case, C4BP), factor I cleaves the α′-chain of C4b at two sites yielding the final large fragment C4c (146 kDa) and the small fragment C4d (45 kDa). Cofactor assays were performed to determine if NS1 associates with C4BP to trigger factor I-mediated cleavage of C4b. Experiments were initially conducted in the fluid phase using biotinylated human C4b. Serum-free culture supernatants from BHK-DENV2-Rep or BHK-WNV-Rep cells containing DENV (Fig. 3*B*, 3*C*) or WNV (Fig. 3*D*, 3*E*) NS1 or from control BHK cells were incubated with C4BP followed by immunoprecipitation with anti-NS1 mAb-Sepharose. After extensive washing, purified factor I and biotinylated C4b were added. C4b cleavage fragments were separated by SDS-PAGE and analyzed by Western blot (Fig. 3*B*, 3*D*). Anti-NS1 mAb-Sepharose incubated with supernatants containing NS1 recruited C4BP cofactor activity as indicated by the enhanced generation of the 45 kDa-cleavage product C4d (Fig. 3*B*, *lane 2*, 3*D*, *lane 2*) and the lower levels of intact α′-chain (Fig. 3*C*, 3*E*). The 70-kDa partial cleavage fragment, α3-C4d, was detected in conditions with or without NS1, suggesting a small nonspecific background interaction of C4BP with the Sepharose resin (Fig. 3*B*, *lane 1*, 3*D*, *lane 1*).

To corroborate these studies, control or DENV NS1-containing supernatants were incubated with NHS or C4BP-dp HS prior to immunoprecipitation with anti-NS1 mAb-Sepharose. Cofactor assays were performed by mixing immunoprecipitates with unlabeled human C4b and factor I. Anti-NS1 mAb-Sepharose incubated with supernatants containing NS1 precipitated a protein or proteins from NHS with cofactor activity for C4b cleavage as

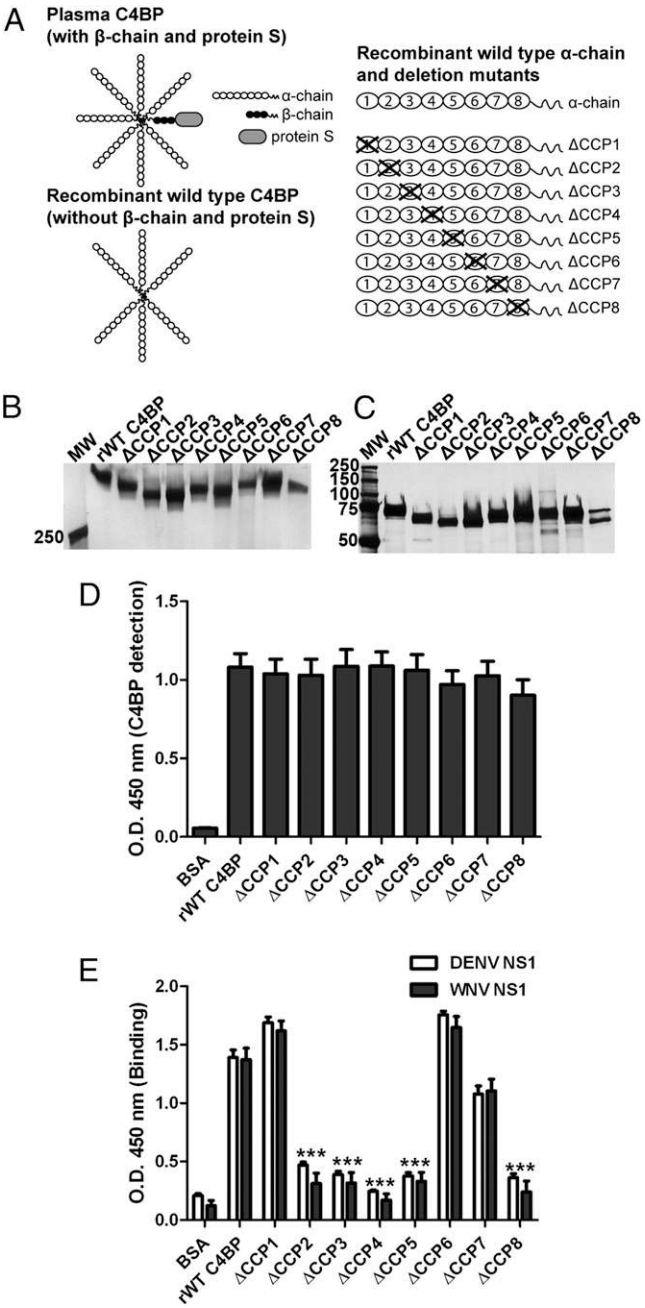


**FIGURE 2.** Flavivirus NS1 binds to the α-chain of C4BP. *A*, Schematic diagrams of different forms of C4BP used in this study. Plasma-purified C4BP mostly consists of seven α-chains, one β-chain, and one molecule of protein S bound to the β-chain. rWT C4BP consists of six α-chains, but lacks a β-chain and protein S (7, 17). The α-chain is composed of eight CCP domains with the C-terminal region that is responsible for multimerization of a single C4BP molecule. Deletion mutants lacking an individual repeating CCP domain are depicted as ΔCCP. *B* and *C*, Purity and electrophoretic mobility of rWT and mutant C4BP. Purified rWT C4BP and deletion mutants lacking a single repeating CCP domain (ΔCCP1–ΔCCP8) (1 μg) were separated by nonreducing 4% SDS-PAGE (*B*) or reducing 12% SDS-PAGE (*C*) followed by silver staining. *D*, Microtiter plates were coated with BSA, rWT C4BP, or deletion mutants lacking an individual CCP domain. Adsorbed proteins were detected using a rabbit polyclonal anti-C4BP Ab. *E*, Adsorbed rWT C4BP or deletion mutants lacking individual CCP domains were incubated with serum-free supernatants from BHK, BHK-DENV2-Rep, or BHK-WNV-Rep cells; bound DENV and WNV NS1 were detected with specific mAbs. The data represent the mean ± SE for three independent experiments, and asterisks indicate statistical difference from the rWT C4BP. ***$p < 0.0001$.

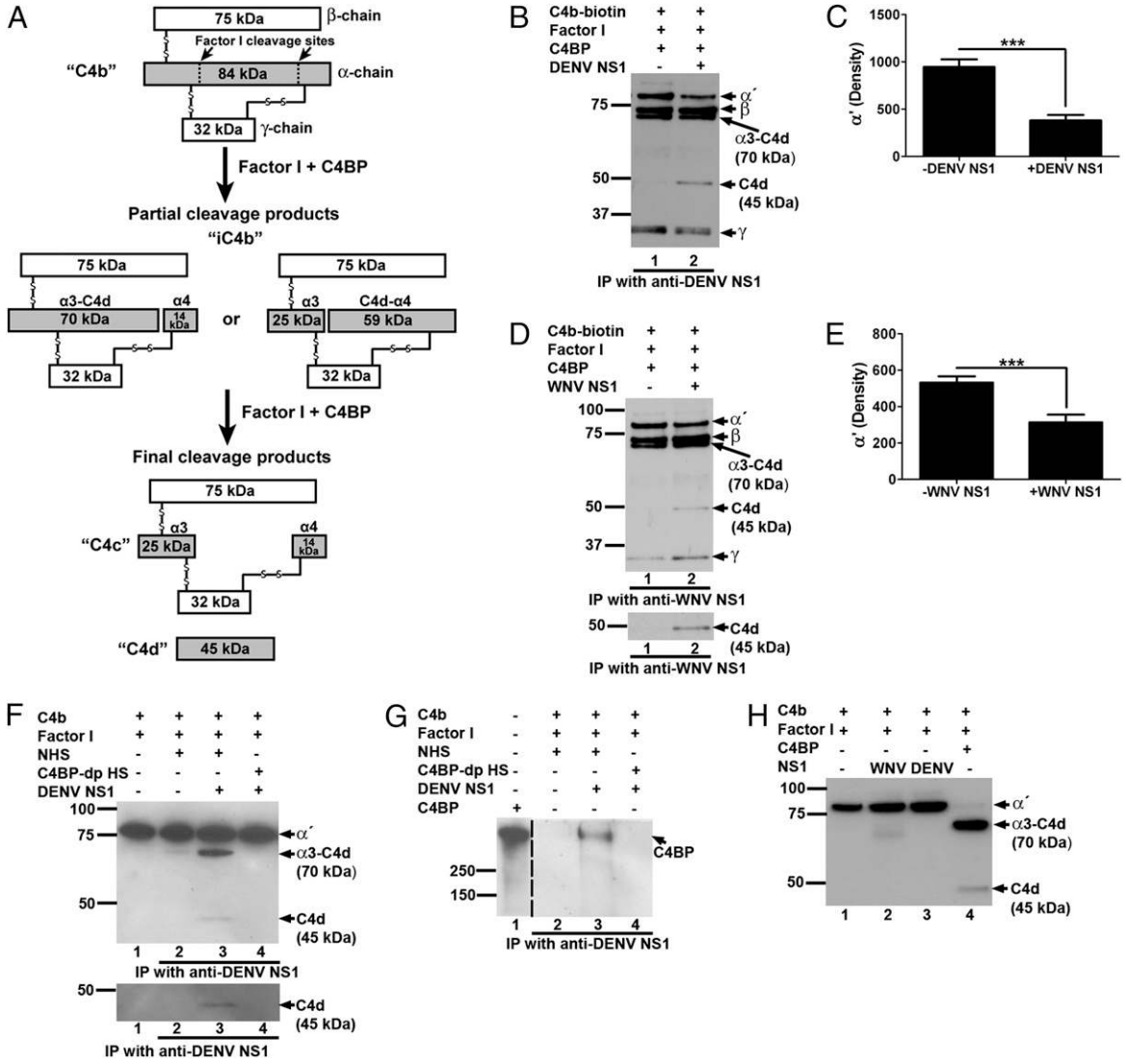


**FIGURE 3.** DENV and WNV NS1 recruit C4BP to degrade C4b in solution. *A*, Schematic depiction of the C4BP cofactor activity for factor I-mediated cleavage of C4b. Cleavage of C4b by factor I requires the presence of a specific cofactor, in this case C4BP. Factor I cleaves the α′-chain of C4b at two sites indicated by arrows. Cleavage at either one of the two sites generates fragments α3-C4d (70 kDa) and α4 (14 kDa) or α3 (25 kDa) and C4d-α4 (59 kDa), which are seen after SDS-PAGE under reducing conditions. Further cleavage of the α′-chain of C4b yields the large soluble C4c (146 kDa) fragment and the small C4d (45 kDa) fragment that remains associated with targets. *B–E*, DENV and WNV recruit C4BP to degrade C4b. Serum-free supernatants from control BHK, BHK-DENV2-Rep (*B*), or BHK-WNV-Rep (*D*) cells were incubated with purified C4BP (15 μg/ml) and immunoprecipitated with anti-DENV NS1 2G6 mAb-Sepharose or anti-WNV NS1 9NS1 mAb-Sepharose, respectively. The NS1-charged beads were washed extensively prior to sequential addition of biotinylated C4b and factor I. Reactions were stopped after 15 min with SDS-reducing sample buffer. Cleavage of C4b was analyzed by 4–12% SDS-PAGE under reducing conditions and Western blot using HRP-conjugated extravidin. A longer exposure of the C4d fragment is shown in the *bottom panel* (*D*). Results are representative of three independent experiments. Intensity of the bands of the α′-chain of C4b in *B* for DENV NS1 (*C*) and *D* for WNV NS1 (*E*) from each individual experiment was measured using quantitative densitometry. Asterisks denote statistical differences from the control. *F* and *G*, DENV NS1 recruits C4BP in human serum to degrade C4b. Serum-free supernatants from control BHK or BHK-DENV2-Rep were incubated with 7% NHS or matched C4BP-dp HS followed by immunoprecipitation with anti-DENV NS1 2G6 mAb-Sepharose. After extensive washing, immunoprecipitates were divided: one half was mixed with unlabeled C4b and factor I and incubated for 15 min. C4b cleavage fragments were separated by 4–12% SDS-PAGE under reducing conditions followed by Western blotting using an anti-C4d mAb (*F*). A longer exposure of the C4d fragment is shown in the *bottom panel* (*F*). The second half of the immunoprecipitate was subjected to 4–12% SDS-PAGE under nonreducing conditions followed by Western blotting using a rabbit anti-human C4BP Ab (*G*). *H*, DENV and WNV NS1 lack cofactor activity for factor I to degrade C4b. Purified DENV or WNV NS1 (20 μg/ml) (*lanes 2* and *3*), or C4BP (0.25 μg/ml, *lane 4*) was incubated with C4b (8.9 μg/ml) followed by addition of factor I (4.4 μg/ml). Reactions were stopped after 1 h with SDS-reducing sample buffer. Cleavage of C4b was analyzed as described in *F*. C4b fragments are labeled at the right of the gel. Cofactor activity was confirmed by the appearance of α3-C4d (70 kDa) or C4d (45 kDa). Results are representative of two to three independent experiments. ***$p < 0.001$.

indicated by the cleavage fragments α3-C4d and C4d (Fig. 3*F*, *lane 3*). Analogously, when C4BP-dp HS was used, C4b degradation was no longer observed (Fig. 3*F*, *lane 4*). Western blot analysis with an anti-C4BP Ab revealed the presence of plasma C4BP in the immunoprecipitates in which NS1 was present (Fig. 3*G*, *lane 3*). However, NS1 itself lacked cofactor activity for factor I as addition of purified DENV or WNV NS1 to factor I, and C4b did not generate C4b cleavage fragments (Fig. 3*H*) (48). Collectively, these experiments establish that NS1 recruits C4BP and through cofactor activity degrades C4b in solution.

*Soluble NS1 recruits C4BP to the surface of cells to inactivate C4b on the plasma membrane*

Soluble DENV NS1 can bind back to the plasma membrane of some cells through interactions with specific sulfated glycosaminoglycans (26). We reasoned that the interaction of soluble NS1 with C4BP could be advantageous to flaviviruses if NS1 recruited C4BP to the surface of cells to inhibit complement activation. To assess this, serum-free supernatants from control BHK or DENV2-Rep-BHK cells containing DENV NS1 were mixed with increasing concentrations of C4BP and $1 \times 10^6$ BHK cells. Deposition of NS1 and C4BP on the cells was analyzed by flow cytometry using mAbs specific to DENV NS1 (Fig. 4*A*) or C4BP (Fig. 4*B–E*). In the presence of DENV NS1 (Fig. 4*B–E*, *right panels*), up to 10-fold higher ($p < 0.05$) percentage of cells was positive for surface C4BP compared with those lacking NS1 (Fig. 4*B–E*, *left panels*). When purified DENV NS1 was used, up to 4-fold higher percentage of cells was positive for surface C4BP compared with the BSA control ($p < 0.05$) (Fig. 4*F–J*). To test whether NS1 that has already bound to the cell surface can subsequently recruit C4BP, BHK cells were treated with serum-free supernatants containing DENV NS1 to allow deposition of NS1 on the plasma membrane prior to incubation with C4BP. Incubation of cells with increasing concentrations of C4BP, however, did not show specific binding between C4BP and NS1 that had already deposited on the surface of cells (Supplemental Fig. 1). Thus, soluble but not surface-bound DENV NS1 recruited C4BP to the cell membrane.

To determine the functional significance of surface-bound C4BP, we evaluated whether DENV NS1-recruited C4BP could act as a cofactor for factor I to inactivate C4b on the plasma membrane. BHK cells were incubated with C4BP in the presence or absence of soluble DENV NS1. After extensive washing to remove soluble unbound C4BP, factor I and C4b were added to the cells to induce cleavage of the C4b α′-chain. Subsequently, supernatants were collected, and C4b cleavage fragments were analyzed by Western blot. C4BP that was recruited to the cell surface by NS1 enhanced factor I-mediated cleavage of C4b, as greater amounts of the cleavage fragments α3-C4d and C4d and lesser amounts of α′-

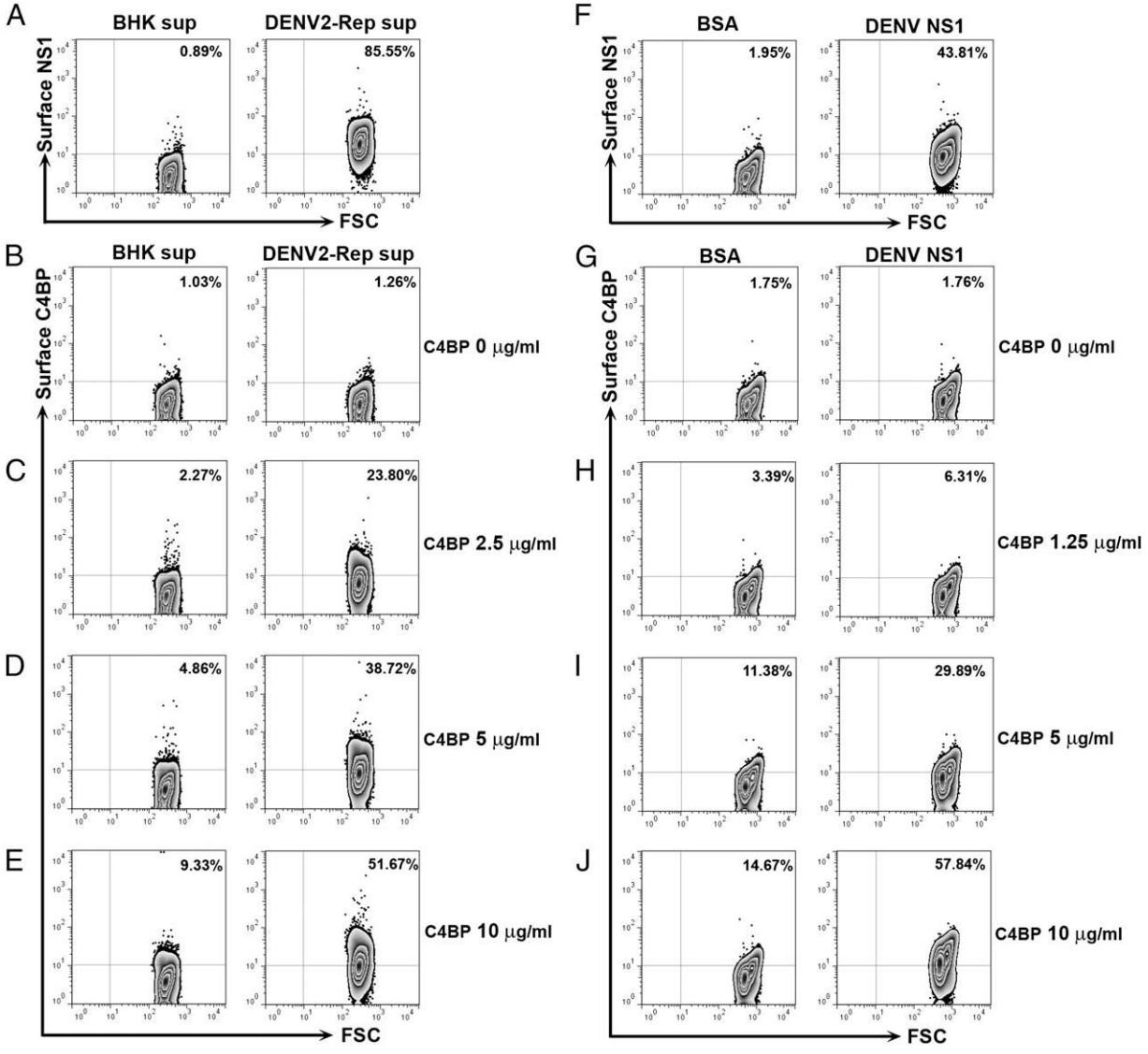


**FIGURE 4.** Soluble DENV NS1 recruits C4BP to the cell surface. Serum-free supernatants from BHK or BHK-DENV2-Rep cells (*A–E*) and BSA or purified DENV NS1 (30 μg/ml) (*F–J*) were incubated with the indicated concentrations of C4BP and $1 \times 10^6$ BHK cells. Cell-surface NS1 and C4BP were assessed by staining with mAbs to DENV NS1 (*A*, *F*) or C4BP (*B–E*, *G–J*) and analyzed by flow cytometry. Examples of contour plots from three independent experiments are shown. The *y*-axis indicates the levels of surface-associated NS1 or C4BP, and the *x*-axis shows the forward scatter (FSC) of the cell population.

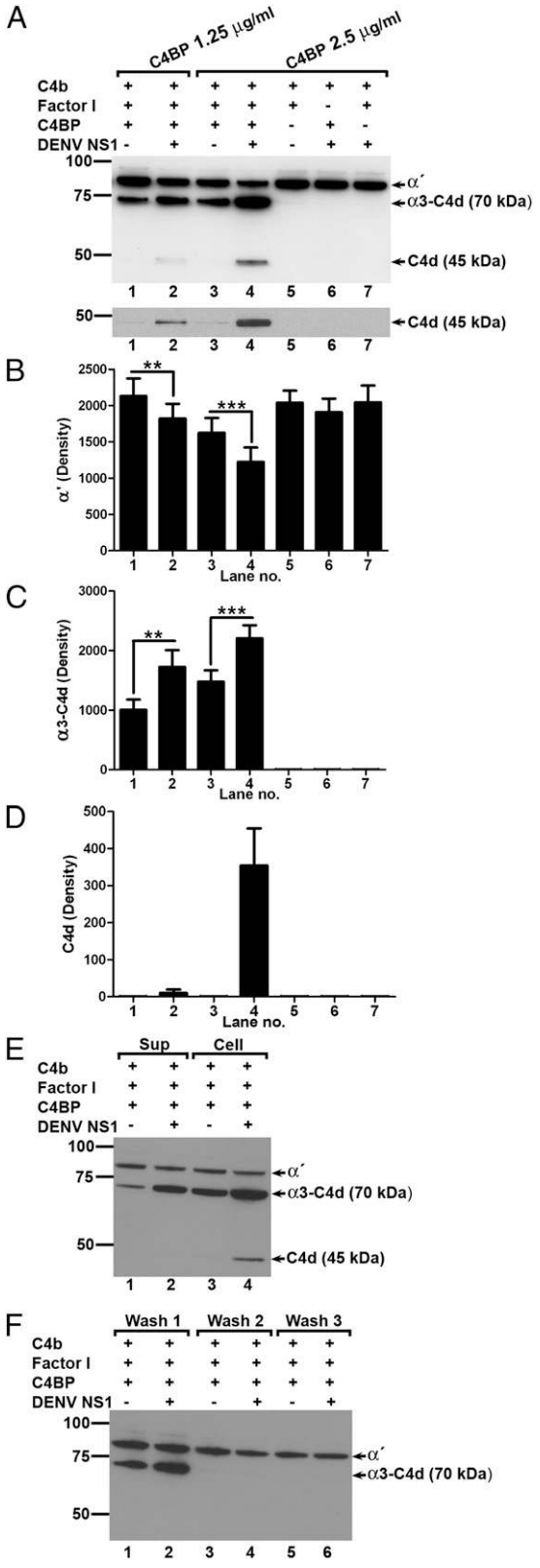


**FIGURE 5.** Soluble DENV NS1 recruits C4BP to degrade C4b on the cell surface. *A*, Serum-free supernatants from BHK (*lanes 1*, *3*, and *5*) and BHK-DENV2-Rep cells (*lanes 2*, *4*, *6*, and *7*) were preincubated with 1.25 μg/ml (*lanes 1* and *2*) or 2.5 μg/ml (*lanes 3*, *4*, and *6*) C4BP. The mixture of DENV NS1 and C4BP was incubated with BHK cells. After three washes, the cells were incubated with C4b and factor I. After 15 min, cofactor activity was determined in the supernatants by the appearance of C4b cleavage products, a 70-kDa α3-C4d and a 45-kDa C4d after 4–12% SDS-PAGE under reducing condition followed by immunoblotting with anti-C4d mAb. A longer exposure of C4d fragments is shown in the *bottom panel* (*A*). Results are representative of three independent experiments. Intensity of the bands of substrate α′-chain (*B*), the incomplete cleavage fragment α3-C4d (*C*), and the final cleavage product C4d (*D*) from each individual experiment was measured using quantitative densitometry. Asterisks denote statistical difference from the control. *E* and *F*, BHK cells were incubated with the mixture of DENV NS1 and C4BP (2.5 μg/ml) followed by three washes with medium. A small volume of washing buffer after each wash (wash 1, wash 2, and wash 3) was collected and subjected to a cofactor assay (*F*). Cells were subsequently incubated with an equal volume of buffer as in *A*, but without C4b and factor I. After 15 min, cofactor activity was determined in both supernatant and cell fractions as above (*E*). Results are representative of three independent experiments. $^{**}p < 0.005$, $^{***}p < 0.0001$.

chain of C4b were detected compared with the conditions in which NS1 was absent (Fig. 5*A*, *lanes 1–4*, 5*B–D*). Cofactor activity required the recruited plasma membrane-bound C4BP, as incubation of cells with factor I and C4b alone did not cause C4b degradation (Fig. 5*A*, *lanes 5* and *7*, 5*B*). Analogous to that observed in solution (Fig. 3*H*), surface-bound DENV NS1 lacked inherent cofactor activity for factor I-mediated cleavage of C4b (Fig. 5*A*, *lane 7*, 5*B*). Moreover, cleavage of the α′-chain of C4b was not due to nonspecific proteolysis; without factor I, cleavage of C4b α′-chain was not observed (Fig. 5*A*, *lane 6*, 5*B*).

We next assessed whether the cofactor activity observed in these samples was caused by cell-bound C4BP or C4BP that dissociated from the plasma membrane during the cofactor assay. After incubation with C4BP in the absence or presence of DENV NS1, BHK cells were washed three times to remove unbound C4BP followed by a similar 15-min incubation with an equivalent volume of buffer as performed in the cofactor experiments above, but without addition of factor I and C4b. Subsequently, the supernatants and cells were separately subjected to a cofactor assay. The cofactor activity of C4BP was observed in both supernatant and cell fractions (Fig. 5*E*). However, C4b degradation was more pronounced in the cell fraction as judged by enhanced α3-C4d and C4d generation (Fig. 5*E*, *lane 4*). The cofactor activity observed in both fractions was not due to incomplete removal of unbound C4BP as cleavage of the α′-chain of C4b was only detected in the solution of the first but not second and third washes (Fig. 5*F*). These results suggest that once C4BP attaches to cells via an NS1 bridge, it reaches a steady state between cell-bound and free forms. Analogous to the results of Fig. 5*A*, C4BP that was recruited to the cell surface by NS1 enhanced factor I-mediated cleavage of C4b, as greater amounts of the cleavage fragments α3-C4d and C4d were detected (Fig. 5*E*, *lane 4*). These experiments confirm that soluble NS1 can recruit C4BP to the cells to inactivate C4b on the plasma membrane.

The two mechanisms employed by flavivirus NS1 for downmodulating complement activation are schematically shown in Fig. 6.

## Discussion

The complement system contributes to the host defense against viral pathogens, including flaviviruses. In response to the antiviral effects of complement, viruses have developed strategies to evade complement activation. WNV NS1 attenuates AP activation by recruiting the complement regulator factor H to facilitate C3b inactivation by factor I (47). Additionally, flavivirus NS1 attenuates lectin and CP activation by binding C1s/proC1s and C4 in a complex to efficiently cleave C4 to C4b, thus reducing the amount of native C4 available for complement activation (48). In

this study, we define a second mechanism by which flavivirus NS1 restricts the CP and lectin activation pathways by targeting the plasma complement regulator C4BP. NS1 bound C4BP and recruited its cofactor activity for factor I-mediated cleavage of C4b in solution; furthermore, soluble NS1 recruited C4BP to the surface of cells to inactivate C4b on the plasma membrane.

Sequestration of C4BP as a strategy for complement evasion has been described for several pathogens including Gram-negative and -positive bacteria, fungi, and nematodes (52–54). Binding of C4BP by bacteria renders them resistant to complement-mediated lysis and phagocytosis (53, 55). Soluble flavivirus NS1 is the first described viral protein that targets C4BP for a complement evasion strategy. In contrast to other microorganisms in which recruitment of C4BP is mediated by a surface receptor on the outer membrane of pathogens (56–67), flaviviruses use secreted NS1 for targeting C4BP. Our mapping data using C4BP deletion mutants suggest that NS1 requires multiple CCP domains (CCP2–5 and CCP8) of the C4BP α-chain for optimal interaction. Bacterial and fungal proteins also use various CCP domains for interaction with C4BP (reviewed in Ref. 53). The involvement of multiple CCP domains for complement regulatory functions and binding to C3b/C4b has been demonstrated in several complement proteins including C4BP (7). Besides CCP2–5, NS1 binds to CCP8 of C4BP, which is in close proximity to the C-terminal oligomerization domain and therefore could influence the conformational structure of C4BP. Thus, an absence of CCP8 could affect accessibility of CCP2–5 for NS1. Alternatively, the NS1–C4BP interaction may involve an avidity phenomenon in which two binding sites on C4BP interact with distinct binding sites on NS1. The interaction of NS1 with C4BP appeared to be nonionic in nature, as binding was detected even in the presence of 1.2 M NaCl; this contrasts with C4b binding to C4BP, which readily dissociates under high-salt conditions (8). An analogous hydrophobic interaction between C4BP and pathogen immune evasion proteins has been described (58, 66, 68).

Although the binding sites for C4b and NS1 on C4BP overlap [C4b binds CCP1–3 (7), whereas CCP2–5 are required for NS1 interaction], C4BP retained its cofactor activity in solution and on the cell surface after binding NS1. CCP2 and CCP3 are the most critical domains for the interaction of C4BP with C4b (7). One explanation as to how both NS1 and C4b can interact with C4BP is the oligomeric (seven identical α-chains) nature of C4BP. Thus, a single molecule of C4BP could use binding sites from different subunits to engage multiple ligands concurrently. However, once soluble NS1 attached to the cell surface via sulfated glycosaminoglycan (26), it lost the ability to interact with C4BP. This could be because sulfated glycosaminoglycans occupy the C4BP binding sites on cell-bound NS1. In a likely related observation, mAbs that map to the N-terminal region (aa 1–157) of WNV NS1 also failed to recognize cell-surface forms of NS1 (35).

Our results showing that soluble NS1 recruits C4BP to cells to inactivate C4b on the plasma membrane establish another potential mechanism by which viruses are protected from complement attack. Soluble NS1 binds to a variety of cell types that are possible targets for infection, including hepatocytes and endothelial cells (26); recruitment of C4BP to infected cells by NS1 would make complement lysis more difficult and allow sustained virion production. NS1 also has been suggested to bind to the envelope protein of flaviviruses (69); thus, it is possible that NS1 binding to C4BP could attenuate complement activation on the virion surface. In human DENV infection, a high level of circulating NS1 (up to 50 μg/ml) is detected during acute infection (27, 28, 70). Soluble flavivirus NS1 antagonizes C4 directly by forming a complex with C1s/proC1s to promote C4 cleavage to C4b (48). By recruiting C4BP to the surface of infected cells or virions so that C4b is cleaved, NS1 further antagonizes CP and lectin pathway

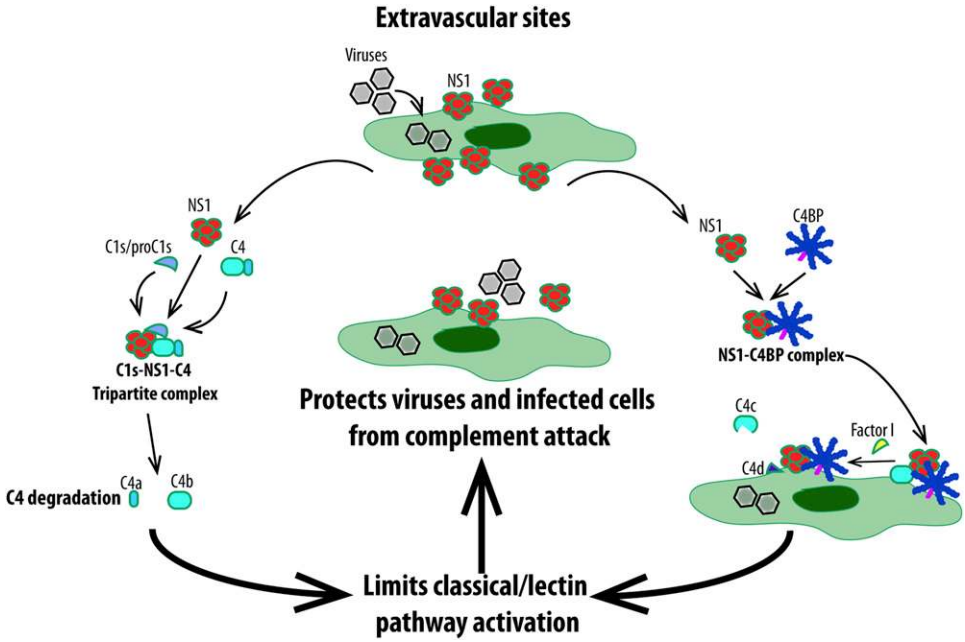


**FIGURE 6.** Model of complement antagonism of the CP by flavivirus NS1. At parenchymal sites of infection, virus-infected cells synthesize and secrete soluble NS1. High local levels of NS1 coupled with low levels of extravascular complement favor attenuation of the CP of complement activation by two mechanisms: 1) flavivirus NS1 binds C1s or proC1s produced by the infected cell or neighboring cells to inactivate C4 in the fluid phase. By forming a complex with C1s, oligomeric NS1 promotes cleavage of C4 to C4b (48). In solution, nascently generated C4b is inactivated by hydrolysis in microseconds (74) and thereby limits the supply of native C4. 2) C4b might still successfully deposit on targets. The interaction of soluble NS1 with C4BP would inactivate C4b on the cell surfaces and restrict the CP and lectin pathway C3 and C5 convertases by enhancing the cofactor activity of C4BP. The end result is protection of viruses and infected cells from complement attack.

activation. Overall, these data point to the importance of the CP/lectin pathway in controlling flavivirus infection. Indeed, mice lacking CP/lectin pathway components are more susceptible to lethal WNV infection (71, 72), and recognition of N-linked glycans on the envelope protein by mannose-binding lectin accelerates the clearance of DENV from plasma (73).

In summary, our studies define a second mechanism by which NS1 inhibits the activation of the CP and lectin pathways. In peripheral tissues where the majority of flavivirus infection occurs, higher local concentrations of NS1 coupled with lower levels of extravascular complement could efficiently attenuate complement activation (Fig. 6). Flaviviruses may use NS1 to bind proC1s produced from infected or neighboring cells to inactivate C4 in the fluid phase. By forming a complex with C1s, oligomeric NS1 promotes cleavage of C4 to C4b. In solution, nascently generated C4b is inactivated rapidly by hydrolysis. However, once C4b is deposited on the surface of targets (e.g., virions or infected cells), the interaction of NS1 with C4BP inactivates C4b and restricts classical and lectin C3 and C5 convertase activities. In this manner, NS1 enables flavivirus to evade complement control and facilitate dissemination.

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## Disclosures

The authors have no financial conflicts of interest.

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