Jab1 Mediates Cytoplasmic Localization and Degradation of West Nile Virus Capsid Protein*

Wonkyung Oh†, Mi-Ran Yang‡, Eun-Woo Lee†, Ki-moon Park‡, Suhkneung Pyo†, Joo-sung Yang‡, Han-Woong Lee†, and Jaewhan Song†,‡,§

From the †Department of Food Science and Biotechnology, the ‡Department of Genetic Engineering, and the §College of Pharmacy, Sungkyunkwan University, Suwon 440-746, and the †Department of Biochemistry, Yonsei University, Seoul 120-749, Republic of Korea

The clinical manifestations of West Nile virus (WNV), a member of the Flavivirus family, include febrile illness, sporadic encephalitis, and paralysis. The capsid (Cp) of WNV is thought to participate in these processes by inducing apoptosis through mitochondrial dysfunction and activation of caspase-9 and caspase-3. To further identify the molecular mechanism of the WNV capsid protein (WNVCp), yeast two-hybrid assays were employed using WNV-Cp as bait. Jab1, the fifth subunit of the COP9 signalosome, was subsequently identified as a molecule that interacts with WNVCp. Immuno precipitation and glutathione S-transferase pulldown assays confirmed that direct interaction could occur between WNVCp and Jab1. Immunofluorescence microscopy demonstrated that the overexpressed WNVCp, which localized to the nucleolus, was translocated to the cytoplasm upon its co-expression with Jab1. When treated with leptomycin B, Jab1-facilitated nuclear exclusion of WNVCp was prevented, which indicated that the CRM1 complex is required for Jab1-facilitated nuclear export of WNVCp. Moreover, Jab1 promoted the degradation of WNVCp in a proteasome-dependent way. Consistent with this, WNVCp-mediated cell cycle arrest at the G2 phase in H1299 was prevented by exogenous Jab1. Finally, an analysis of WNVCp deletion mutants indicated that the first 15 amino acids were required for interaction with Jab1. Furthermore, the double-point mutant of the WNVCp, P5A/P8A, was incapable of binding to Jab1. These results indicate that Jab1 has a potential protective effect against pathogenic WNVCp and might provide a novel target site for the treatment of disease caused by WNV.

West Nile virus (WNV) is a member of the Japanese encephalitis virus complex within the genus Flavivirus, family Flaviviridae. Other members of this genus include St. Louis Encephalitis virus, Kunjin virus, Dengue virus, and Murray Valley encephalitis virus (1). WNV was first isolated in 1937 from the West Nile Province of Uganda (1). WNV quickly became one of the most prevalent flaviviruses distributed in Africa, the Middle East, Europe, and the United States. Its clinical symptoms include febrile illness, fatal meningoencephalitis, and acute flaccid paralysis syndrome (2).

WNV contains a single positive-sense RNA genome of ~11 kb in length; this genome encodes polyprotein. This protein is processed by viral and cellular proteases to generate three structural proteins of the capsid (Cp), envelope, and premembrane, as well as seven nonstructural proteins, which are mainly involved in the process of replication. These seven nonstructural proteins include NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (3, 4). The pathway of WNV infection is mediated through a clathrin-dependent endocytic pathway (5). After replication subsequent to inoculation, WNV spreads to the lymph nodes and the bloodstream (6). It then invades the central nervous system in a Toll-like and tumor necrosis factor receptor-dependent manner, which facilitates the infiltration of WNV across the blood-brain barrier (7, 8).

WNV infection is known to trigger cell death through either a necrotic or an apoptotic pathway (5, 9). Infection induces overexpression of Bax, followed by apoptotic cell death in the host cells (9). However, it has also been reported that a high level of viremia in WNV infection could lead to a necrotic pathway (10). Although the detailed mechanism involved in this process has not yet been defined clearly, several reports have suggested that molecules derived from the WNV genome are responsible for apoptosis. For example, the WNVCp protein was shown to mediate the activation of caspase-9; this activation may occur as a result of mitochondrial dysfunction (11). Through the functional comparison of proteins NS2B-NS3 from other flaviviruses, WNV NS2B-NS3 was also shown to induce caspase-8- and caspase-3-mediated apoptosis (12). Although the prevalence of WNV has increased globally and its serious clinical manifestations have prompted extensive research worldwide, the pathogenic mechanism of WNV in the central nervous system is not clearly understood at a molecular level, and further research is required to determine the role of Jab1 in the pathogenesis of WNV.
establish adequate options for both the treatment and prevention of WNV infection.

Jab1 is the fifth of the COP9 signalosome (CSN) complex; the Arabidopsis homologs of the CSN complex are responsible for the photomorphogenic process (13). The CSN complexes also share sequence and structural homologies with the 19 S proteasome lid and the eIF3 translation initiation complex (14). In mammalian cells, Jab1 seems to display a broad spectrum of regulatory activities through its mediation of the stabilization or destabilization of various proteins. It was first identified as a c-Jun-interacting protein that enhances AP-1 transcriptional activities (15). Jab1 also facilitates the 26 S proteasome-dependent degradation of several proteins, including p27Kip1, LHR, p53, estrogen receptor, Smad4, Smad7, Id1, Id3, and topoisomerase IIα (16–23). However, the relationship between Jab1 and the 26 S proteasome complex during protein degradation is not yet clearly understood. It was recently reported that in cooperation with CSN, Jab1 mediates the deneddylation of the cullin component of SCF ubiquitin-protein isopeptide ligase, which may promote cullin-dependent proteolysis (24).

In this study, we performed yeast two-hybrid screening and found that Jab1 was capable of binding to WNVCp. The overexpressed WNVCp, which consistently localized to the nucleolus, was exported to the cytoplasm in the presence of Jab1. Jab1 subsequently induced the degradation of WNVCp in a proteasome-dependent manner. In accordance with these data, the cytotoxic effect of WNVCp, which induced cell cycle arrest at G2 phase in H1299, was prevented in the presence of Jab1.

EXPERIMENTAL PROCEDURES

Plasmids—pGBK-T7-WNVCp, a bait plasmid for yeast two-hybrid screening, was created by cloning an EcoRI-Xhol fragment from pcDNA3-His-WNVCp into pGBK-T7 (Clontech). pGAD-T7-Jab1 was generated by subcloning an EcoRI-Xhol fragment from pGEX-4T-1-Jab1 (provided by K. W. Kim, National University of Seoul) into pGAD-T7 (Clontech). pcDNA3-HA-WNVCp and pET28a-His-WNVCp were prepared by subcloning an EcoRI-Xhol fragment from pcDNA3-his-WNVCp into pcDNA3-HA and pET28a-His (Invitrogen). pcDNA3-HA-WNVCp (P5A/P8A) and pET28a-His-WNVCp (P5A/P8A) were generated by PCR using pcDNA3-HA-WNVCp as a template and the following primer pairs: 5′-CCGAAATTTCGCTTCTAAAAACCGAGTTGGCCC-3′ (forward) and 5′-CCGCTGAGCTACGCGCCACCGCTTGCGATCAG-3′ (reverse). pcDNA3-HA-WNVCp deletion mutants, Δ1–15, Δ1–30, Δ1–60, Δ76–123, Δ91–123, and Δ106–123, were prepared by PCR using pcDNA3-HA-WNVCp as a template and the following primers: 5′-CCGAAATTTCGCTGAGCGCCACCCATTGCTGCTGAGCC-3′ (forward) and 5′-CCGCTGAGCTACGCGCCACCGCTTGCGATCAG-3′ (reverse). pcDNA3-HA-WNVCp deletion mutants were cloned into the corresponding sites of the pcDNA-3-HA or pET28a-His plasmids. pCS3-MT-BX (6xMyc)-Jab1 was generated by subcloning of the EcoRI-Xhol fragment into pCS3-MT-BX (6xMyc) (a generous gift from J. H. Ahn, Sungkyunkwan University). All constructs were confirmed by enzyme digestion and DNA sequence analyses. The levels of endogenous Jab1 were decreased by the use of Jab1 siRNA (5′-GCCUCAGAUGAUCCUGAGAAA-3′) oligonucleotides with 3′-dTdT overhangs, and were synthesized using Qiagen. Control siRNA in experiments refers to a mixture of scrambled siRNA oligonucleotides (Qiagen). The cells were transfected with 200 nM of siRNA by using Oligofectamine according to the instructions of the manufacturer (Invitrogen).

Yeast Two-hybrid Screening—Yeast two-hybrid screening was performed with the pretransformed MATCHMAKER human brain cDNA library (Clontech), used according to the instructions of the manufacturer. In brief, pGBK-T7-WNVCp was transformed into yeast strain AH109. Transformants containing bait plasmid were mated with the pretransformed human brain cDNA library. Candidates for two-hybrid interaction were initially selected on SD medium (−His, −Leu, and −Trp) and further confirmed on SD medium (−Ade, −His, −Leu, and −Trp) containing X-gal. Plasmid DNA was isolated from the positive clones and sequenced according to the instructions of the manufacturer. To further confirm the interaction between WNVCp and Jab1, Y187, and AH109, each pair containing pGAD-T7-Jab1 and pGBK-T7-WNVCp, respectively, were mated and tested using SD medium (−Ade, −His, −Leu, and −Trp) containing X-gal.

Cell Biology—Human cancer cell lines, H1299 (p53 null Lung carcinoma) and 293T (kidney carcinoma), were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin (Invitrogen). Transient transfections were performed using Lipofectamine plus reagent (Invitrogen) or Welfect (Wellgene) according to the recommendations of the manufacturers. Immunoprecipitation and Western blot analysis were carried out as follows. At 48 h after transfection, the cells were harvested in a lysis buffer (150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% Triton X-100, 0.1% sodium deoxycholate, 50 mM Tris-HCl (pH 7.5), 1 mM diethiolethiol, 0.2 mM phenylmethylsulfonyl fluoride, and 100× protease inhibitor mixture). Whole cell extracts were incubated with the target antibody for 2 h at 4 °C. A/G beads (Roche Applied Science) were added to the lysates, incubated for 2 h at 4 °C, precipitated, and washed three times in a lysis buffer. The bound proteins were dissolved in the sample buffer and resolved by SDS-PAGE. The resolved proteins were transferred to a nitrocellulose membrane (Amersham Biosciences), blotted with the primary antibody, and incubated with horseradish peroxidase antirabbit or antimo-mouse IgG (Zymed Laboratories Inc.) for 1 h at 25 °C. Antibody binding was detected using an ECL system (Amersham Biosciences).

Protein Purification and Pulldown Assay—GST and GST-Jab1 were prepared as described previously (25). His-WNVCp and His-P5A/P8A were prepared according to the instructions of the manufacturer (Invitrogen). In brief, BL21 cells, which have been transformed with pET28a-His-WNVCp or pET28a-His-P5A/P8A, were treated with isopropyl 1-thio-β-D-galactopyranoside (1 mM) at 25 °C. The crude extract
was prepared using lysozyme (Sigma). The extract was resolved over a Resource Q (35 ml; Amersham Biosciences), His-bind resin (20 ml; Novagen), and DEAE column (50 ml; Amersham Biosciences). Fractions containing His-WNVCp or His-P5A/P8A were concentrated using a Centriprep-30 (Millipore). The pulldown experiment was carried out as described previously (26).

Immunofluorescence Staining—Cells were plated in 6-well plates with coverslips; transfections were performed using Lipofectamine reagent. After 24 h, cells were fixed with 4% paraformaldehyde solution for 15 min at room temperature, washed with PBS (Invitrogen), and permeabilized with 0.5% Triton X-100 in PBS for 15 min. The cells were then blocked with 5% bovine serum albumin (Santa Cruz Biotechnology) in PBS for 30 min and incubated overnight with the specific primary antibody at room temperature. The samples were incubated with Alexa Fluor 488 anti-mouse or Alexa 594 anti-rabbit antibodies (each diluted at 1:400) for 1 h at room temperature. The cells were stained with 4,6-diamidino-2-phenylindole (Sigma) for 5 min. The slides were analyzed using confocal or immunofluorescence microscopes (Carl Zeiss Vision, LSM510 and 5203 Axiophot, respectively, Oberkochen).

Fluorescence-activated Cell Sorter Analysis—For fluorescence-activated cell sorter analysis, H1299 cells were

**FIGURE 1.** WNVCp interacts with Jab1. **A,** schematics of Jab1 fragments identified as WNVCp-binding proteins. WNVCp binds to Jab1 containing JAMM, Jab1/MPN/Mov34 metalloenzyme motif. B, plasmids expressing HA-WNVCp and FLAG-Jab1 were co-transfected into 293T cells. The cell lysates were immunoprecipitated using anti-HA mouse antibodies. The whole cell extract (WCE) and immunoprecipitates (IP) were detected using anti-HA rabbit and anti-FLAG mouse antibodies. C, purified GST-Jab1, GST, and His-WNVCp were resolved by 12% SDS-PAGE followed by Coomassie Blue staining (lanes 1–3). His-WNVCp (5 μM) was incubated with either GST-Jab1 (1 μM) or GST (1 μM) (lanes 1–3, 5–7). Proteins bound to glutathione-Sepharose beads (lane 7) were pelleted, eluted, and resolved as described above. D, the plasmids expressing HA-WNVCp were transfected into 293T cells. The cell lysates were immunoprecipitated using anti-HA mouse antibodies. The whole cell extract and immunoprecipitates were detected using anti-HA rabbit and anti-Jab1 rabbit antibodies.

**FIGURE 2.** Jab1 promotes nuclear export of WNVCp in a CRM1-dependent manner. **A,** H1299 cells were transfected with plasmids expressing HA-WNVCp. 24 h after transfection, cells were fixed and stained with anti-HA and Alexa 488 anti-mouse antibodies or with anti-nucleolin and Alexa Fluor 594 anti-rabbit antibodies. B, H1299 cells, which were transfected with the plasmids expressing Myc-Jab1 (panels 1–4) or Myc-Jab1 + HA-WNVCp (panels 5–8), were detected using polyclonal anti-HA and Alexa Fluor 488 anti-rabbit antibodies or using monoclonal anti-Myc and Alexa 488 anti-mouse antibodies. The cells were treated with or without LMB (5 μM) for 7 h. The images were captured by immunofluorescence microscopy. The cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei. Diagram represents the percentage (%) of cells displaying only nuclear or cytoplasmic localization of WNVCp. A total of 100 cells was counted for each experiment.
transfected with pcDNA3-HA-WNVcp, pcDNA3-HA-P5A/P8A, or pcDNA3-HA-Δ106–123 with or without pCMV-FLAG-Jab1. At 48 h after transfection, the cells were harvested using trypsin and subsequently fixed with 70% EtOH. After washing three times with PBS, the fixed cells were incubated with RNase A (1 μg/ml) at 37 °C for 1 h, followed by treatment with propidium iodide (50 μg/ml) for 30 min. Stained cells were detected by flow cytometry analysis (BD Biosciences). Data were analyzed using CellQuest Pro software (BD Biosciences).

RESULTS

Jab1 Is Capable of Directly Binding to WNVcp and Promotes Its Nuclear Export—WNVcp is known to induce cell death through mitochondrial dysfunction and activation of caspase-9 in tissue culture (11). When its gene was delivered to mouse brain or skeletal muscle, it caused cell death and inflammation in vivo (11). This observation led us to search for a factor that might be associated with WNVcp. To identify an interacting protein, we performed a yeast two-hybrid assay using the entire region of WNVcp as bait. Among the several proteins identified, yeast two-hybrid screening demonstrated the interaction of two Jab1 fragments with WNVcp (Fig. 1A). Co-immunoprecipitation and GST pulldown assays between the two purified proteins indicated that WNVcp was able to directly interact with Jab1 (Fig. 1, B and C). Interaction of the two proteins was confirmed by immunoprecipitation of ectopic WNVcp and endogenous Jab1 (Fig. 1D).

WNVcp has been shown to localize to the nucleolus (11). As reported previously, the WNVcp, which had been transfected into H1299 (p53-null lung carcinoma) cells, mostly localized to the nucleolus. This was confirmed by its colocalization with nucleolin, the nucleolus protein (Fig. 2A). On the other hand, when WNVcp was co-transfected with Jab1, ~45% of cells displayed WNVcp that had translocated to the cytoplasm (Fig. 2B, panels 5–8, and graph). Jab1 is known to participate in the translocation of various proteins, including p27Kip and Smad, through a CRM1-dependent pathway (16, 20, 21). To identify whether Jab1 could induce the translocation of WNVcp in a similar way, LMB, an inhibitor of CRM1 (27), was administered to cells overexpressing Jab1 and WNVcp. The results of this investigation showed that LMB prevented Jab1-mediated cytoplasmic localization of WNVcp (Fig. 2B, panels 9–12, and graph). This indicates that Jab1 is capable of inducing nuclear exclusion of WNVcp to the cytoplasm, rather than inhibiting the localization of WNVcp to the nucleus from the cytoplasm. Overall, the data indicated that Jab1 directly interacted with WNVcp, which could induce its nuclear exclusion in a CRM1-dependent manner.

The N-terminal Domain of WNVcp Is Responsible for Its Interaction with Jab1—Next, we analyzed the WNVcp domain, which might be responsible for its localization to the nucleolus and interaction with Jab1. We constructed serial deletion mutants of WNVcp, as depicted schematically in Fig. 3. The N-terminal deletion mutants, Δ1–15, Δ1–30, and Δ1–60, and the C-terminal deletion mutant, Δ106–123, were all localized to the nucleolus (Fig. 3, panels 1–10). However, Δ91–123 and Δ76–123 were localized to the cytoplasm, which implies that the nuclear localization signal (NLS) might be located between the amino acid residues 90 and 105 (Fig. 3, panels 11–14, and Fig. 9C).

The effects of Jab1 on the localization of the deletion mutants were further tested using immunofluorescence microscopy. Although the localization of Δ1–15, Δ1–30, and Δ1–60 was not affected in the presence of exogenous Jab1, Δ106–123 was exported to the cytoplasm (Fig. 4, panels 5–12, and data not shown).
**FIGURE 4.** The N terminus of WNVCp is required for Jab-facilitated nuclear exclusion of WNVCp. H1299 cells were transfected with plasmid expressing HA-WNVCp, HA-Δ1–15, and HA-Δ106–123 with Myc-Jab1. The cells were stained as described in Fig. 2B. DAPI, 4,6-diamidino-2-phenylindole.

**FIGURE 5.** Jab1-facilitated nuclear exclusion of WNVCp requires the presence of PXXP sequences at the N terminus of WNVCp. A, alignment of the N-terminal protein sequences of WNVCp with the p53 and p27 motifs necessary for their Jab1 interaction. Dark gray and light gray shading indicate identical and similar residues, respectively. B, H1299 cells were transfected with plasmid expressing HA-P5A/P8A alone or HA-P5A/P8A and HA-WNVCp with Myc-Jab1 and stained as described in Fig. 2B.
**Jab1 Induces the Proteasome-dependent Degradation of WNVCp**—It was reported that Jab1-mediated nuclear export of substrates such as p53 and p27 is correlated with their degradation (28, 29). To test whether WNVCp nuclear export by Jab1 also leads to degradation, we treated cells overexpressing Jab1 and WNVCp with or without MG132 and assessed them using immunofluorescence and degradation assays. In the absence of MG132, we were able to observe the dispersion of WNVCp in the cytoplasm, as well as in the nucleus, in the presence of Jab1 (Fig. 7, panels 1–8). On the other hand, in the absence of Jab1, WNVCp remained in the nucleoli (Fig. 7, panels 9–11). Similar to these results, in the presence of MG132, WNVCp localized to the cytoplasm (Fig. 7, panels 12–19). However, unlike the cytoplasmic WNVCp distribution in the absence of MG132, we observed that the amount of WNVCp that accumulated in the cytoplasm was significantly increased (Fig. 7, panels 12–19). Interestingly, the co-localization of Jab1 with WNVCp was more obvious in the presence of MG132. It seems that the prevention of WNVCp degradation by MG132 led to the formation of more complexes between WNVCp and Jab1. Taken together, these data show that the localization of WNVCp is correlated with the nuclear export pathway.

The degradation of WNVCp was further confirmed by measuring the levels of WNVCp in the presence or absence of Jab1. When Jab1 and WNVCp were co-transfected, we observed a 60% decrease in overexpressed WNVCp (Fig. 8A, lanes 1 and 2). The decreased levels of WNVCp in the presence of Jab1 were recovered when cells were treated with a proteasome inhibitor, MG132, which suggested that Jab1 facilitated the proteasome-dependent degradation of WNVCp (Fig. 8A, lanes 3 and 4). To identify whether the degradation of WNVCp correlates with its Jab1-dependent nuclear export, treatment with LMB was used to prevent the nuclear export of WNVCp, as shown in Fig. 2 (Fig. 8A, lanes 3 and 6). The result indicated that LMB treatment completely blocked Jab1-mediated degradation of WNVCp, which suggested that nuclear export and degradation are related processes. Degradation assays using HA-P5A/P8A and HA-D106–123 as substrates showed that both mutants were resistant to Jab1-facilitated proteasome-dependent degradation (Fig. 8B, lanes 1–4). Because P5A/P8A did not bind to Jab1, it appears that Jab1 might function as a mediator that introduces WNVCp bound to Jab1 to the 26 S proteasome complexes (16–23). Overall, Jab1-mediated cytoplasmic localization of WNVCp was correlated with its degradation. Furthermore, the C terminus of WNVCp is responsible for its Jab1- and proteasome-dependent degradation.

**Jab1 Negatively Regulates WNVCp-mediated Cell Cycle Arrest at G2 Phase**—WNVCp has been known to induce apoptosis in HeLa cells and in tissues such as the mouse brain and skeletal muscle (11). Yet, when we transiently transfected H1299, a human lung cancer cell line, with DNA construct expressing WNVCp, cell cycle arrest was induced at the G2

*FIGURE 6. The N terminus of WNVCp is required for its interaction with Jab1. A, 293T cells were transfected with plasmids expressing HA-WNVCp (lanes 1 and 4), HA-WNVCp + Myc-Jab1 (lanes 2 and 5), HA-P5A/P8A + Myc-Jab1 (lane 3), and HA-D106–123 + Myc-Jab1 (lane 6) and were co-transfected into 293T cells. The cells were treated with MG132 for 6 h before harvesting. The cell lysates were immunoprecipitated using anti-Myc mouse antibodies. The whole cell lysates (WCE) and immunoprecipitates (IP) were detected using anti-HA rabbit, anti-Jab1 rabbit, and anti-Myc mouse antibodies. B, purified GST-Jab1, His-WNVCp, and His-P5A/P8A were resolved as described in Fig. 1C (lanes 1–3). His-WNVCp (5 μM) or His-P5A/P8A (5 μM) was incubated with GST-Jab1 (1 μM) (lanes 4 and 6). Proteins bound to glutathione-Sepharose beads (lanes 5 and 7) were pelleted, eluted, and resolved as described in Fig. 1C. WT, wild type.*

shown). These data indicated that the putative Jab1-binding region of WNVCp might reside on the first 15 amino acids at its N terminus (Fig. 9C). Because Jab1-binding proteins are known to contain a conserved motif that is responsible for their interactions with Jab1, we compared the first 15 amino acids of the WNVCp N terminus with the other Jab1-binding motif, which is found in both p53 and p27Kip (18, 28). The comparison clearly indicates the presence of PXPP sequences within the proteins that were being compared (Fig. 5A). This observation prompted us to substitute the prolines on the 5th and 8th residues of the WNVCp with alanines. Interestingly, the point mutant, P5A/P8A, was not translocated into the cytoplasm by Jab1, which suggested that this mutant might be defective in its interaction with Jab1 (Fig. 5B, panels 5–8).

We performed co-immunoprecipitation or GST pulldown assays to elucidate whether P5A/P8A and D106–123 were able to directly interact with Jab1. In accordance with the immunofluorescence data depicted in Figs. 4 and 5, the immunoprecipitation assays showed that D106–123, but not P5A/P8A, was able to interact with Jab1 (Fig. 6A). When GST pulldown assays were performed using purified His-P5A/P8A, His-WNVCp, and GST-Jab1, only His-WNVCp bound to Jab1 (Fig. 6B, lanes 4–7). This finding suggests that this point mutant is deficient in its ability to bind to Jab1. Taken together, through deletion and point mutant analyses, we found that the N terminus of WNVCp is responsible for its Jab1 binding. Furthermore, the NLS seems to localize between amino acids 90 and 105.

**Jab1 Is a Nuclear Exporter for WNV Capsid Protein**
This demonstrated that WNVCp could cause various cytotoxic effects depending on the genetic backgrounds of the cells in which it is expressed. The population rate of cells transfected with mock vector was 50% at G₁ phase, although it decreased to ~24% when transfected with WNVCp (Fig. 9A). Accordingly, the cell population rate at G₂ phase was increased from 10 to 40%. The co-transfection of Jab1 with WNVCp reduced cell cycle arrest at G₂ phase from 40 to 20%. At the same time, the proportion of cells at G₁ phase was increased from 24 to 45%. We observed similar results in the 293T cell line, a human kidney cell line (data not shown). As expected, the cytotoxic effects of P5A/P8A were not inhibited by exogenous Jab1 (Fig. 9A). Interestingly, it seems that P5A/P8A might have slightly more of a cytotoxic effect than WNVCp itself. This could be due the fact that P5A/P8A was not affected by direct interaction with endogenous Jab1.

The effect of endogenous Jab1 was studied further by the depletion of endogenous Jab1 (Fig. 9A). Using Jab1 siRNA as reported previously, we were able to decrease the endogenous levels of Jab1 by up to 90% (Fig. 9B) (29). Data showed that the depletion of Jab1 led to cell death in the presence of WNVCp, which indicated that endogenous Jab1 plays a certain role in protecting cells against the cytotoxic effects of ectopic WNVCp. As reported previously, the C-terminal deletion mutant, Δ106–123, did not induce cell cycle arrest (Fig. 9A) (11).

Taken together, the results indicate that Jab1 was able to induce the degradation of WNVCp, which arrested H1299 cells at G₂ phase. Thus, the cytotoxic effects of WNVCp were inhibited in the presence of exogenous Jab1. Conversely, when
endogenous Jab1 was depleted, the cytotoxic effects of WNVCp became more lethal. Finally, the C terminus of WNVCp is responsible for the cytotoxic effect of WNVCp on host cells (Fig. 8B).

DISCUSSION

In this study, we suggest a novel pathway that negatively regulates the cytotoxic effects of WNVCp. Yang et al. (11) previously identified that WNVCp was capable of activating caspase-9 and caspase-3 and was also able to induce mitochondria-mediated apoptosis in the HeLa cancer cell line. Importantly, it was shown that the direct delivery of WNVCp into the striatum of mouse brain or skeletal muscle induced inflammation and cell death, further confirming that the capsid itself is an apoptotic agonist in vivo. We observed that WNVCp expressed in the H1299 cell line was able to induce cell cycle arrest at the G2 phase (Fig. 9). Our data imply that, depending on the genomic backgrounds of the cells, WNVCp could have various toxic effects on the fates of cells infected with WNV. However, the detailed mechanism of the induction of apoptosis or cell cycle arrest at the G2 phase by WNVCp and the factors that determine the regulatory pathways of WNVCp remain as questions that must be answered in the future.

Interestingly, the depletion of Jab1 in the presence of WNVCp led to cell death (Fig. 9B). This might be the result of two factors. Because Jab1 is involved in the protection of cells by degrading various factors that are involved in the apoptotic process, including p27, Smad, and p53, WNVCp seems to force stronger cytotoxic effects on cells depleted of Jab1 and thus depleted of its protective roles (16, 20, 29). Another possibility could be that endogenous Jab1 might reduce the apoptotic effects of WNVCp by exporting and inducing the degradation of WNVCp. Thus, when the endogenous levels of Jab1 were reduced, the cytotoxic effect of WNVCp might have increased more strongly.

As shown previously, when WNVCp was introduced into host cells, it was localized into the nucleolus as well as the cytoplasm (11). The localization of viral protein to the nucleolus has been strategically employed by many viruses for its propagation. For example, the N protein of the coronavirus capsid of human hepatitis B virus are located in the nucleolus and disrupt cytokinesis (30, 31). Thus, it is likely that localization of WNVCp into the nucleolus could be used for the same purpose.

Furthermore, through an analysis of the WNVCp domain, we observed that the deletion mutant, WNVCp (Δ106–123), was not able to induce cytotoxic effects on H1299 cells. However, it localized to the nucleolus and translocated into the cytoplasm in the presence of Jab1 (Figs. 3 and 4). This indicates that the C terminus is likely to participate in the induction of cell cycle arrest and that a putative ligand might be binding to this region. Supporting the data, Yang et al. (11) reported that the C terminus of WNVCp is responsible for the induction of apoptosis in HeLa cells.

The sequence comparisons of the WNVCp N terminus with p27 and p53, which are also known as Jab1-binding proteins, revealed a specific motif at the N terminus of WNVCp (Fig. 5A). Among the three proteins compared, the sequence motif of PXXP was consistently conserved. When both prolines were mutated to alanines, we found that the mutated WNVCp was no longer able to bind to Jab1 (Fig. 6). Consistent with these findings, the nucleolar localization and cytotoxic effect of this mutant were not affected in the presence of Jab1 (Figs. 5B and 8). This observation suggests that this conserved region of WNVCp might have been evolutionarily acquired by WNV; the conserved region could be used as a nuclear exporting motif via the Jab1-mediated shuttle system. This is based on the hypothesis that Jab1 might be used as a mediator that shuttles WNVCp from the nucleus to the cytoplasm. Because nucleocapsid particles of WNV mature in the cytoplasm and plasma membrane, it is necessary for the capsid proteins of WNV to exist in the
cytoplasm, where they can be associated with the RNA genome of WNV (31, 32). Thus, it is likely that WNV employs the export and import machinery of host cells to regulate the localization of WNVCp. By utilizing the host shuttle system, WNVCp could be used for the maturation of the virus and also as the regulatory tool that controls host cell cytokinesis. However, it seems that WNV might need to employ another regulatory pathway that could prevent the 26 S proteasome-dependent degradation of WNVCp facilitated by Jab1 (Fig. 7).

Jab1 is the fifth subunit of the CSN complexes and has been known to function as a member of the CSN complexes, in small complex forms, or in a free form. One of the distinguished features of Jab1 existing as a small complex or in the free form is that it is able to mediate the nuclear export and degradation of several nuclear proteins, including p27, Smad, estrogen receptor, and p53 (16–23). Here we showed that Jab1 also displayed a similar effect on WNVCp. Immunofluorescence assays indicated that Jab1 could mediate the nuclear export of WNVCp through a CRM1-dependent pathway (Fig. 7).

Furthermore, the export process was accompanied by the proteasome-dependent degradation of WNVCp (Figs. 7 and 8A). Thus, it is likely that WNVCp is controlled by Jab1 in a similar way as the other substrates of Jab1 (16–23, 29). Notably, WNVCp (∆106–123), the C-terminal deletion mutant that interacted with and translocated in the presence of Jab1, was protected from Jab1-facilitated degradation (Fig. 7). Because no lysine is present at the region between amino acids 106 and 123, which would allow the ubiquitination process to occur, it is possible that a putative protein such as ubiquitin-protein isopeptide ligase is likely to bind specifically at the C-terminal end of WNVCp and help to degrade WNVCp in coordination with Jab1. However, further studies should be carried out to establish this regulator mechanism.

In summary, this is the first report to elucidate how proteins expressed by WNV could be negatively regulated by host cells. Jab1 was able to prevent G2 phase cell cycle arrest, which is normally induced by WNVCp through the promotion of nuclear exclusion and its subsequent degradation. This novel pathway might shed a new light on how WNV is able to propagate in host cells and could provide a new therapeutic target for WNV infection.

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