Identification of Cellular Proteome Modifications in Response to West Nile Virus Infection* [S]

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Flaviviruses are positive-stranded RNA viruses that are a public health problem because of their widespread distribution and their ability to cause a variety of diseases in humans. West Nile virus is a mosquito-borne member of this genus and is the etiologic agent of West Nile encephalitis. Clinical manifestations of West Nile virus infection are diverse, and their pathogenic mechanisms depend on complex virus-cell interactions. In the present work, we used proteomics technology to analyze early Vero cell response to West Nile infection. The differential proteomes were resolved 24 h postinfection using two-dimensional DIGE followed by mass spectrometry identification. Quantitative analysis (at least 2-fold quantitative alteration, FDR < 0.05) revealed 127 differentially expressed proteins with 68 up-regulated proteins and 59 down-regulated proteins of which 93 were successfully identified. The implication for mammalian cellular responses to this neurotropic flavivirus infection was analyzed and made possible more comprehensive characterization of the virus-host interactions involved in pathogenesis. The present study thus provides large scale protein-related information that should be useful for understanding how the host metabolism is modified by West Nile infection and for identifying new potential targets for antiviral therapy. Molecular & Cellular Proteomics 8:1623–1637, 2009.

West Nile virus (WNV) is a mosquito-borne flavivirus belonging to the Japanese encephalitis virus (JEV) serocomplex.

The virus is maintained in nature in enzootic cycles in which it is transmitted between ornithophilic mosquitoes and avian hosts. In mammals, including humans, WNV is an encephalitic flavivirus and can cause natural infections of the central nervous system (CNS) with a neuropathogenesis involving neuroinvasiveness (ability to enter the CNS) and neurovirulence (replication within the CNS) (1). To date, no pharmacological treatment exists for WNV, and a vaccine is only available for horses.

First isolated in 1937, WNV has become endemic in Africa, the Middle East, and parts of Asia and Europe (2, 3). Phylogenetics analysis groups WNV strains into two distinct lineages. Viruses in lineage 2 are found only in Africa, whereas viruses in lineage 1 are present both in Africa and in other areas, particularly Asia and Europe. Since 1999, WNV from lineage 1 (NY99) has reached North America where, in 2002, it caused the largest arboviral meningoencephalitis outbreak ever recorded in this area (4).

It is known that flavivirus replication can cause extensive rearrangement of host cell cytoskeletal and membrane compartments leading to a "cytopathic effect" in various cell cultures of human, primate, rodent, and insect origin (5). Recent studies have revealed specific effects of viruses on cellular processes. It has been demonstrated that flaviviruses can induce cell death directly through viral replication and the production of proapoptotic proteins (6–11), but the mechanism of pathogenesis has not been elucidated.

Although neurons are regarded as the major target of WNV in vivo (2), WNV infection has been shown to induce apoptosis in different cell lines in a similar manner in vitro (12, 13). This includes a wide range of different cell types with, in particular, the African green monkey kidney continuous cell line (Vero) recommended by the World Health Organization Collaborating Center for systematic research and isolation of arboviruses as well as a substrate to develop live attenuated

TUNEL, terminal deoxynucleotidyltransferase dUTP nick-end labeling; BP, band pass; NCBInr, National Center for Biotechnology Information non-redundant; NS, nonstructural protein; Hsp, heat shock protein; hnRNP, heterogeneous nuclear ribonucleoprotein; eEF, eukaryotic translation elongation factor; eIF, eukaryotic translation initiation factor.

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and inactivated vaccines. Acute infection of Vero cells by WNV produces a lytic infection with a characteristic rounding cytopathic effect and the production of a large number of infectious particles in the culture fluid within 3 days postinfection (14). Although this permissive mammalian cell system is widely used for flavivirus isolation, propagation, and titration, to date no studies have focused on identifying Vero cellular proteins whose expression has been altered by WNV infection. We considered that Vero cells could be a good model for in vitro identification of cell protein alterations with possible implication in certain pathogenic mechanisms.

In the present work, fluorescent 2D DIGE technology combined with MS analysis was used to examine the consequences of Vero cell infection by WNV. To evaluate early mammalian cell response after infection and to avoid the effect of cell death and protein degradation, the culture conditions (e.g. infectious dose and incubation time) were optimized. A total of 93 differentially expressed protein spots were identified (over ±2-fold, p < 0.05) and confirmed by fluorescent Western blot analysis. The implication for cellular responses to this flavivirus infection as well as the potential roles of certain altered identified proteins are discussed to characterize the pathophysiologic processes. This study can also provide useful clues for antiviral research.

EXPERIMENTAL PROCEDURES

Reagents—N-Hydroxysuccinimide esters Cy2, Cy3, and Cy5; urea; glycerol; mineral oil; Immobiline DryStrip gels (18 cm, pH 4–7 and 6–11); and IPG buffer solutions (pH 4–7 and 6–11) were purchased from GE Healthcare. Acrylamide, DTT, Tris, glycin, SDS, and SYPRO Ruby gel stain solution were purchased from Bio-Rad. Dimethylformamide, CHAPS, L-lysine, ammonium persulfate, iodoacetamide, agarose, bromphenol blue, and TFA were purchased from Aldrich. Thiourea, TEMED, acetone, ACN, and ethanol were purchased from Fluka (Buchs, Switzerland). Trypsin (sequencing grade) was purchased from Promega (Madison, WI). All buffers were prepared with Milli-Q water (Millipore, Bedford, MA).

Virus Production and Titration—All work with infectious virus was carried out in a biosafety level 3 laboratory. WNV strain New York 99 viral inoculum used in this study were prepared by two passages in C6/36 cells (from Virgentec S.A., Herstal, France) in accordance with published detection assays (19).

Viral RNA Extraction and Real Time RT-PCR Quantification—Cells were harvested and analyzed. Mock Vero cells served as the controls and was added to all wells, and the cultures were incubated at 37 °C. Twenty-four hours after infection, supernatants were collected and frozen at −80 °C in 50 mM Hepes. Viral titers were estimated by titration (VibraCell 732412, Bioblock Scientific, Illkirch, France) five times for 60 s on ice at maximum amplitude. The resulting homogenate was centrifuged for 15 min at 16,000 × g at 4 °C, and the supernatant was further precipitated with 100% acetone. Protein concentrations for each sample were determined in duplicate using the Lowry method (DC Protein Assay kit, Bio-Rad) according to the manufacturer’s instructions and confirmed by SDS-PAGE. Samples were resuspended in standard cell lysis buffer to obtain a protein concentration adjusted to 2.5 μg/μl. CyDye Labeling—Proteins in each sample were minimally labeled with CyDye according to the manufacturer’s recommended protocols.

Isoelectric Focusing—DeStreak buffer containing 1.2% (v/v) IPG buffer (pH 4–7 or 6–11) was used for overnight rehydration of 18-cm IPG strips with different pH gradient ranges (4–7 or 6–11). The samples were applied at the acidic end of the IPG strips using a cup-loading technique. IEF was carried out at 20 °C for a total of 65 kV·h on an Ettan IPGphor II electrophoresis unit (GE Healthcare).

Second Dimension Electrophoresis—Prior to separation in the second dimension, the IPG strips were reduced and alkylated in equilibration buffer containing 50 mM Tris·HCl, pH 8.6 buffer, 6 μl urea, 2% SDS, and 30% glycerol supplemented with 1% (v/v) DTT or 2.5% (v/v) iodoacetamide instead of DTT for 15 min. Equilibrated IPG strips were then transferred onto 10% uniform polyacrylamide gels cast in low fluorescence glass plates using an Ettan-DALT Six
system (GE Healthcare). Strips were overlaid with 0.5% agarose in 1 x running buffer containing bromphenol blue, and electrophoresis was run at 10 watts/gel until the dye front migrated out of the gel at 20 °C.

**Image Analysis**—After electrophoresis, the gels with CyDye-labeled proteins were scanned three times with a Typhoon™ Trio image scanner (GE Healthcare) each time at different excitation wavelengths (Cy3, 580 BP 30/green (532 nm); Cy5, 670 BP 30/red (633 nm); Cy2, 520 BP 40/blue (488 nm)). Prescans were performed to adjust the photomultiplier tube voltage to obtain images with a maximum intensity of 60,000–80,000 U. Images were cropped with ImageQuant™ software (GE Healthcare) and further analyzed using the software package DeCyder v6.5 (GE Healthcare). Intragel spot detection and quantification were performed using the differential in-gel analysis mode, whereas images from different gels were matched using the biological variance analysis mode. Matching between gels was performed using the in-gel standard from each image pair. The paired t test was used for statistical analysis of the data. A false discovery rate (FDR) correction was applied to eliminate false positives. Protein spots that were expressed differentially between two experimental groups (ratio ≥ 2, p < 0.05) were marked. After 2D DIGE imaging and image analysis, the protein spot pattern was visualized by SYPRO Ruby staining (Bio-Rad) according to the manufacturer’s protocol.

**In-gel Digestion**—Based on the DeCyder v6.5 analysis, spots of interest were excised using a manual spot picker from SYPRO Ruby stain gels. Excised plugs were digested overnight at 37 °C by sequencing grade trypsin (12.5 μg/ml; Promega) in 50 mM NH4HCO3. The resulting extracted peptides were then stored at −20 °C before their analysis by MS.

**Mass Spectrometry Analysis**—The samples were analyzed by nanoscale capillary LC-MS/MS. Purification and analysis were performed on a C18 capillary column using a CapLC system (Waters, Milford, MA) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF Ultima, Waters). Chromatographic separations were conducted on a reversed-phase capillary column (AtlantisTM dC18, 3 μm, 75 μm × 150 mm; Nano Ease™, Waters) with a 180–200 nl min −1 flow. The gradient profile consisted of a linear gradient from 95% A (H2O, 0.1% HCOOH) to 60% B (80% acetonitrile, 0.1% HCOOH) in 60 min followed by a linear gradient to 95% B in 10 min. Mass data acquisitions were piloted by MassLynx 4.0 software using automatic switching between MS and MS/MS modes. The internal parameters of Q-TOF were set as follows. The electrospray capillary voltage was set to 3.2 kV, the cone voltage was set to 30V, and the source temperature was set to 80 °C. The MS survey scan was m/z 400–1300 with a scan time of 1 s and an interscan time of 0.1 s. When the intensity of a peak rose above a threshold of 15 counts, tandem mass spectra were acquired. Normalized collision energies for peptide fragmentation were set using the charge state recognition files for +2 and +3 peptide ions. The scan range for MS/MS acquisition was from m/z 50 to 1500 with a scan time of 1 s and an interscan time of 0.1 s. Fragmentation was performed using argon as the collision gas and with the collision energy profile optimized for various mass ranges and charges of precursor ions. Mass data collected during a nanoscale capillary LC-MS/MS analysis were processed using ProteinLynx Global Server 2.2 software (Waters) with the following parameters: no background subtraction, smooth 3/2 Savitzky Golay, and no deisotoping to generate peak lists in the Micromass pkl format. pkl files were then fed into a local search engine, Mascot Daemon v2.2.2 (Matrix Science, London, UK). The data were searched against the primates (295,078 sequences) and viruses (435,150 sequences) National Center for Biotechnology Information non-redundant (NCBI/nr) protein database (December 2, 2007). Search parameters allowed for one missed tryptic cleavage site, the carbamidomethylation of cysteine, and the possible oxidation of methionine; precursor and product ion mass error tolerance was <0.2 Da. All identified proteins have a Mascot score greater than 69, corresponding to a statistically significant (p < 0.05) confident identification. Moreover among the positive matches, only protein identifications based on at least two different non-overlapping peptide sequences of more than 6 amino acids and with a mass tolerance <0.05 Da were accepted. These additional validation criteria are a good compromise to limit the number of false positive matches without missing real proteins of interest.

**SDS-PAGE, Blotting, and Analysis Procedures**—Immunoblotting with fluorescence-based methods including the ECL Plex-CyDye immunoblot detection system (GE Healthcare) was used to detect both the total protein expression profile and the specific immunoreactive proteins. The same protein samples used for 2D DIGE were minimally labeled with Cy3 cysteine dye as described above (see “CyDye labeling”). Labeled samples were separated by 10% SDS-PAGE in a Mini-PROTEAN Cell (Bio-Rad) according to Laemmli (21). Gels were transferred to a nitrocellulose membrane (0.45 μm; GE Healthcare) using a semidry blotting system at 200 mA for 30 min (22). Blots were saturated with 5% nonfat dried milk in PBS containing 0.1% (v/v) Tween 20 for 1 h. Western blot analyses were carried out with anti-tubulin (1:500, mouse IgG isotype FITC conjugate) (clone DM1A, Sigma), anti-vimentin (1:500, mouse IgG isotype) (clone V9, Sigma), anti-enolase (1:1000, rabbit antibody) (Sigma), and anti-transglutaminase type 2 (1:2000, goat antibody) (Sigma) diluted in PBS containing 0.2% (v/v) Tween 20 with 5% nonfat dried milk. After 2 h of incubation at room temperature, anti-vimentin and anti-enolase were revealed with ECL Plex goat anti-mouse IgG Cy2- and goat anti-rabbit IgG Cy5-conjugated secondary antibody (GE Healthcare) (1:2000), respectively. Anti-transglutaminase was revealed with mouse anti-goat IgG FITC conjugate (1:2000) (Jackson ImmunoResearch Laboratories, West Grove, PA). All manipulations were protected from light. The gels, just after the electrophoresis and immunoblots, were scanned using a Typhoon Trio image scanner as described above (see “Image Analysis”). Immunoreactive bands were analyzed using Quantity One software (Bio-Rad). To evaluate the expression level of the different proteins, immunoreactive band intensities were normalized to the intensities of a global protein pattern labeled with Cy3. Band intensities were also corrected for adjacent background. Standard molecular weight markers were loaded in each gel (Bio-Rad).

**RESULTS**

**Virus Infection Conditions**—Optimal culture conditions (e.g. infectious dose and incubation time) were tested to determine the early host responses of Vero cells following WNV infection at the proteome level. Chu and Ng (12) have previously studied the effect of WNV infectious dose on the process of cell death. The authors demonstrated that a low infectious dose of virus (m.o.i. ≤ 1) induced a delay in the kinetics of virus replication and in apoptotic cell death in Vero cells. To obtain a high percentage of infected cells with minimal cell death in our assays, a m.o.i. of 1 and 24 h postinfection were the conditions chosen for subsequent proteomics analysis. Under these conditions, more than 90% of cells were infected by the virus, and cell death was less than 2%, comparable to the mock control cells. DNA fragmentation by TUNEL assay was controlled with mock- and WNV-infected Vero cells cultivated for 36 h with a m.o.i. of 1. In these conditions, up to 10% of infected cells were labeled with fluorescein-dUTP. A representative result of three independent experiments is shown in

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**Cell Response to West Nile Virus Infection**

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Moreover real time RT-PCR quantification of WNV RNA in the cell culture supernatants indicated a productive virus infection (data not shown).

Analysis of Differentially Expressed Proteins after WNV Infections—To identify the cell proteins involved in response to WNV infection, 2D DIGE experiments were performed. Four independent cultures of mock- and WNV-infected Vero cells cultivated for 24 h with a m.o.i. of 1 were included in this analysis. After 2D DIGE, the Cy2, Cy3, and Cy5 channels of each gel were individually imaged, and the images were analyzed using DeCyder 6.5 software.

For proteins separated in the pH 4–7 range, 1950 protein spots were matched. Of them, 79 spots were significantly modified between the mock- and WNV-infected Vero cells (33 spots were up-regulated, and 46 spots were down-regulated; \( \text{ratio}_{\text{WNV/mock}} \geq 2, p \leq 0.05 \) after FDR correction; spots shown in Fig. 2). For proteins separated in the pH 6–11 range, 1674 protein spots were matched. Of them, 48 spots were significantly modified between the mock- and WNV-infected Vero cells (35 spots were up-regulated, and 13 spots were down-regulated; \( \text{ratio}_{\text{WNV/mock}} \geq 2, p \leq 0.05 \) after FDR correction; spots shown in Fig. 3). A total of 127 protein spots were found to have been modified following Vero cell infection by WNV under our conditions.

Identification of Differentially Expressed Proteins after WNV Infection—One hundred and twenty-seven differentially detected protein spots were excised manually from gels, subjected to in-gel digestion, and analyzed by LC-MS/MS. The resulting fragment ion spectra were searched against primate and viral protein databases (NCBInr). Tables I and II list proteins that were identified by tandem mass spectrometry from gels with pH ranges of pH 4–7 and pH 6–11, respectively. Ninety-three protein spots (73%) were identified with a high degree of confidence (Tables I and II and supplementalTables 1 and 2). As expected, 15 protein spots with a significant average ratio -fold change were identified as WNV proteins such as polyprotein precursor or nonstructural protein 1 (NS1), NS3, and NS5 (Tables I and II).

In addition to these viral proteins, 54 distinct cellular proteins were identified according to their NCBI number (Tables I and II). The main proteins with profile modification were localized in the cytoplasm (60.7%) and nucleus (19.6%) (Fig. 4A). These proteins were grouped into functional categories, such as transcription and translation, cytoskeletal and cell mobility, chaperones, energy pathways, apoptosis, lipid metabolism, ubiquitin-proteasome pathway, regulation and metabolism of nucleotide, binding, and transport (Tables I and II and Fig. 4B). Several proteins (e.g. vimentin and transglutaminase 2) were detected in more than one spot, suggesting different isoforms and/or post-translational modifications. Tumor rejection antigen (gp96) protein (Table I) was detected in seven spots with an up-representation for three of them (that is 436, 437, and 442) and a down-representation for four of them (that is 1187, 1189, 1192, and 1195). These results indicated that following WNV infection of Vero cells a group of host protein isoforms (e.g. 436, 437, and 442) was privileged.
to the detriment of the second group of other isoforms from the same host protein. Thirty-four protein spots were not identified probably because of insufficient amounts of protein or of low MS spectra qualities.

**Protein Validation by Western Blotting Analysis**—To verify the 2D DIGE results, Western blot analyses were performed. Several proteins identified by MS were selected on the basis of their representativity and the availability of the corresponding antibodies. For all Western blots, each protein sample was labeled with Cy3 cyanine dye revealing the variations in sample loading that were taken into account for the calculation of the average band volume ratio. The same samples used for

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**Fig. 2.** 2D DIGE analysis of mock- or WNV-infected Vero cells at 24 h postinfection (m.o.i. = 1). Representative data from a 2D DIGE experiment using a 10% homogenous SDS-polyacrylamide gel with the pH range from 4 to 7 is shown. Proteins from mock-infected Vero cells were conjugated to Cy5, and proteins from WNV-infected Vero cells were conjugated to Cy3. As determined by DeCyder software, protein spots that were differentially expressed between two experimental conditions (ratio ≥ 2, p ≤ 0.05 after an FDR correction) were marked with master numbers. Normal and italic numbers correspond to protein spots significantly up- and down-regulated following Vero cell infection by WNV, respectively. Proteins identified by mass spectrometer are listed in Table I.
2D DIGE analysis were subjected to one-dimensional SDS-PAGE and blotted with the antibodies against tubulin, vimentin, transglutaminase, and enolase. Anti-tubulin FITC-conjugated antibody confirmed that this protein was up-regulated following WNV infection of Vero cells (Table I and Fig. 5). For vimentin, the antibody detected two molecular weight isoforms (Fig. 5A). This is consistent with the results of 2D DIGE analysis that showed two groups of protein spots (1482 and 1483 and 1574, 1575, and 1577) corresponding to vimentin with different molecular weights. Both analyses indicated, for the two groups of protein spots, a down-representation of vimentin following WNV infection (Fig. 5B). Anti-transglutaminase antibodies detected two bands by Western blot that were up-regulated following WNV infection (Fig. 5, A and B). Although proteomics analysis identified two protein spots (741 and 742) corresponding to transglutaminase that were also up-expressed following WNV infection (Fig. 2 and Table I), these two protein spots were transglutaminase isoforms with the same molecular weight. These two protein spots could thus correspond to only one of the two bands detected on the Western blot. It could effectively be hypothesized that the expression level of the highest band and its -fold change detected on the Western blot were not sufficient for the inclusion of this isoform in the list of statistically significant protein spots differently regulated following WNV infection using proteomics analysis. Using anti-enolase antibodies, we confirmed the down-expression of this protein (Fig. 5). Thus, Western blot experiments confirmed the results obtained by 2D DIGE analysis and protein identification by mass spectrometry.

DISCUSSION

A clear understanding of WNV pathogenesis is lacking, so the discovery of new virus-host interactions is necessary to identify novel strategies to treat or prevent this viral infection. Despite the importance of mosquito-borne virus diseases, few studies have examined host metabolism alterations following flavivirus infection. In this way, microarray studies have analyzed changes at the transcription level in response to dengue virus (DENV) or WNV infections (23–28). To date, only two studies have analyzed the interplay between flavivirus and host cells using proteomics analysis (31, 32), and more investigations are needed to understand the mechanism of virus replication and pathogenesis. However, studies combining transcriptomics and proteomics analysis indicated that significant divergence could be observed between protein levels and mRNA abundance (29, 30). In this way, our study provides global information on protein alterations in mammalian cells infected by WNV, an emergent pathogenic flavivirus. Of the 127 spots differentially expressed in Vero cells after
## Table I

**Proteins identified from the differential 2D DIGE (pH 4–7) analysis after WNV infection**

The proteins were identified by mass spectrometry following in-gel trypsin digestion. The spot numbers correspond to the same numbers as indicated on Fig. 2. The identities of the spots, their NCBI accession numbers, and the theoretical molecular masses and pI values as well as the number of peptide sequences, the corresponding percent sequence coverage, and the Mascot score are listed for MS/MS analysis. (Protein scores greater than 69 are considered as significant (p < 0.05).) Paired average volume ratio and p values (t test) between Vero cells infected by WNV versus uninfected cells (WNV/mock) were quantified using DeCyder software. n.i., no identification; TNF, tumor necrosis factor; CTBP, C-terminal-binding protein; CBP, cAMP-response element-binding protein.

| Accession number (NCBI) | Protein name | Spot number | Molecular mass (kDa) | pI | Number of MS/MS peptide sequences | Sequence coverage | Mascot score | p value | Average volume ratio | WNV/mock |
|-------------------------|--------------|-------------|----------------------|----|-----------------------------------|------------------|--------------|---------|----------------------|----------|
| gi90025144              | Polypeptide precursor (West Nile virus) | 732         | 384.48               | 8.72 | 5 | 2 | 219.9 | 2.60 | 0.17 |
| gi158516892             | Nonstructural protein 1 (West Nile virus) | 1072        | 40.40                | 5.73 | 6 | 16 | 274.3 | 6.19 | 0.0059 |
| gi1083                  |                          | 1083        |                      | 6.00 | 6 | 16 | 257.7 | 6.04 | 0.0018 |
| gi1353                  |                          | 1353        |                      | 7.38 | 5 | 14 | 223.6 | 7.43 | 0.00015 |
| gi1370                  |                          | 1370        |                      | 11.38 | 8 | 24 | 385.0 | 18.34 | 0.00015 |
| gi1372                  |                          | 1372        |                      | 3.38 | 8 | 20 | 419.4 | 40.49 | 0.00015 |
| gi158516895             | Nonstructural protein 3 (West Nile virus) | 1492        | 69.29                | 7.32 | 11 | 19 | 661.1 | 23.19 | 0.00059 |
| gi21929235              | Polypeptide precursor (West Nile virus) | 1497        | 384.47               | 8.69 | 8 | 2 | 483.5 | 2.83 | 0.0018 |
| gi109070524             | Predicted: similar to heat shock 70 kDa protein 1-like isoform 2 (Macaca mulatta) | 807         | 70.72                | 5.96 | 5 | 8 | 269.5 | 3.48 | 0.0062 |
| gi109070528             | Predicted: similar to heat shock 70kDa protein 1-like isoform 1 (M. mulatta) | 824         | 63.30                | 6.34 | 4 | 8 | 266.6 | 3.96 | 0.010 |
| gi109071319             | Predicted: heat shock 90-kDa protein 1, beta isoform 2 (M. mulatta) | 1569        | 80.57                | 5.26 | 9 | 13 | 534.7 | 2.39 | 0.010 |
| gi109071321             | Predicted: heat shock 90-kDa protein 1, isoform 1 (M. mulatta) | 1586        | 80.57                | 5.26 | 9 | 13 | 534.7 | 2.39 | 0.010 |
| gi109071323             | Predicted: heat shock protein 90-kDa alpha (cytosolic), class B member 1 isoform 1 (M. mulatta) | 1674        | 68.71                | 4.77 | 7 | 12 | 393.9 | 3.06 | 0.0095 |
| gi109100929             | Predicted: similar to ATP-dependent DNA helicase II (M. mulatta) | 541         | 96.53                | 9.39 | 6 | 6 | 306.8 | 2.20 | 0.019 |
| gi109098491             | Predicted: tumor rejection antigen (gp96) 1 (M. mulatta) | 436         | 92.84                | 4.76 | 24 | 34 | 1473.3 | 4.33 | 0.016 |
| gi109081460             | Predicted: similar to annexin A2 isoform 1 (M. mulatta) | 2628        | 53.79                | 9.49 | 5 | 10 | 253.3 | 2.51 | 0.0095 |
| gi109083355             | Predicted: vimentin (M. mulatta) | 1482        | 53.64                | 5.14 | 15 | 33 | 895.1 | 2.26 | 0.0094 |
| gi109083350             | Predicted: p15.5 (M. mulatta) | 1483        | 35.47                | 5.14 | 15 | 33 | 895.1 | 2.26 | 0.0094 |
| gi109088640             | Predicted: tubulin alpha ubiquitously (M. mulatta) | 1574        | 53.79                | 9.49 | 5 | 10 | 253.3 | 2.51 | 0.0095 |
| gi10910367              | Predicted: gelatin isoform 4 (M. mulatta) | 1575        | 13.38                | 15.38 | 15 | 33 | 895.1 | 2.26 | 0.0094 |
| gi109110401             | Predicted: gelatin isoform 2 (M. mulatta) | 1577        | 29.64                | 15.38 | 15 | 33 | 895.1 | 2.26 | 0.0094 |
| gi109111790             | Predicted: annexin I isoform 1 (M. mulatta) | 1578        | 38.92                | 6.63 | 5 | 16 | 283.4 | 2.23 | 0.016 |
| gi109105736             | Predicted: similar to heterogeneous nuclear ribonucleoprotein U (M. mulatta) | 2319        | 38.92                | 6.63 | 5 | 16 | 283.4 | 2.23 | 0.016 |
| gi109106916             | Predicted: reticulocalbin 1 (M. mulatta) | 2527        | 32.45                | 4.74 | 5 | 20 | 364.2 | 3.33 | 0.013 |

**Viral proteins**

- Polypeptide precursor (West Nile virus)
- Nonstructural protein 1 (West Nile virus)
- Nonstructural protein 3 (West Nile virus)

**Chaperones**

- Predicted: similar to heat shock 70-kDa protein 1-like isoform 2 (Macaca mulatta)
- Predicted: similar to heat shock 70kDa protein 1-like isoform 1 (M. mulatta)
- Predicted: heat shock 90-kDa protein 1, beta isoform 2 (M. mulatta)
- Predicted: heat shock protein 90-kDa alpha (cytosolic), class B member 1 isoform 1 (M. mulatta)

**Cytoskeletal and cell motility**

- Predicted: similar to villin 2 (M. mulatta)
- Predicted: similar to calponin 3 isoform (M. mulatta)
- Predicted: similar to annexin A2 isoform 1 (M. mulatta)
- Predicted: vimentin (M. mulatta)
- Predicted: p15.5 (M. mulatta)

**Transcription and translation**

- Predicted: similar to heterogeneous nuclear ribonucleoprotein U (M. mulatta)
- Predicted: reticulocalbin 1 (M. mulatta)
### Table I—continued

| Accession number (NCBI) | Protein name | Spot number | Molecular mass (kDa) | pI | Number of MS/MS peptide sequences | Sequence coverage (%) | Mascot score | WNV/mock Average volume ratio | p value |
|-------------------------|--------------|-------------|----------------------|----|-------------------------------|------------------------|--------------|-----------------------------|---------|
| gi|109096946 | Predicted: similar to poly(C)-binding protein 2 (α-CBP) (putative heterogeneous nuclear ribonucleoprotein X) (hnRNP X) (CTBP) (CBP) isoform 4 (M. mulatta) | 1677 | 38.06 | 8.80 | 4 | 13 | 205.1 | -2.62 | 0.011 |
| gi|109130455 | Predicted: DEA(D/H) (Asp-Glu-Ala-[Asp/His]) box polypeptide 3 (M. mulatta) | 737 | 100.97 | 9.27 | 3 | 3 | 112.3 | 2.13 | 0.020 |
| gi|109114623 | Predicted: similar to splicing factor, arginine/serine-rich 1 (ASF/SF2) (M. mulatta) | 2073 | 45.03 | 10.41 | 9 | 21 | 527.2 | -2.31 | 0.012 |
| gi|109084872 | Predicted: tryptophanyl-tRNA synthetase isoform 7 (M. mulatta) | 1072 | 53.90 | 5.67 | 6 | 14 | 277.6 | 6.19 | 0.00059 |
| gi|109123410 | Predicted: eukaryotic translation initiation factor 3, subunit 4 (M. mulatta) | 1670 | 46.15 | 8.64 | 3 | 7 | 188.0 | -2.62 | 0.013 |

**Ubiquitin-proteasome pathway**

| Accession number (NCBI) | Protein name | Spot number | Molecular mass (kDa) | pI | Number of MS/MS peptide sequences | Sequence coverage (%) | Mascot score | WNV/mock Average volume ratio | p value |
|-------------------------|--------------|-------------|----------------------|----|-------------------------------|------------------------|--------------|-----------------------------|---------|
| gi|109107255 | Predicted: similar to proteasome subunit α type 1 (proteasome component C2) (macropain subunit C2) (multicatalytic endopeptidase complex subunit C2) (proteasome ν chain) (30-kDa prosomal protein) (PROS-30) (M. mulatta) | 2278 | 41.68 | 8.57 | 11 | 29 | 518.9 | -2.64 | 0.018 |
| gi|109114262 | Predicted: similar to prohibitin isoform 3 (M. mulatta) | 2276 | 29.13 | 5.39 | 13 | 57 | 784.2 | -2.23 | 0.0090 |
| gi|6005942 | Valosin-containing protein (H. sapiens) | 473 | 89.95 | 5.14 | 25 | 35 | 1367.8 | 2.01 | 0.017 |
| gi|109081748 | Predicted: pyruvate kinase 3 isoform 9 (M. mulatta) | 1370 | 65.04 | 8.20 | 3 | 5 | 130.3 | 18.34 | 0.00015 |
| gi|109085770 | Predicted: vacuolar H⁺-ATPase B2 (M. mulatta) | 1090 | 59.85 | 6.15 | 7 | 14 | 379.6 | 6.54 | 0.0060 |
| gi|109114106 | Predicted: similar to karyopherin β1 (M. mulatta) | 433 | 116.04 | 5.19 | 5 | 5 | 278.4 | 2.57 | 0.012 |
| gi|109130173 | Predicted: similar to spermine synthase (M. mulatta) | 1681 | 46.43 | 4.98 | 6 | 12 | 256.4 | -2.86 | 0.0098 |
| gi|109082685 | Predicted: purine-nucleoside phosphorylase (M. mulatta) | 2304 | 32.26 | 6.08 | 6 | 22 | 369.6 | -2.44 | 0.016 |
| gi|109127465 | Predicted: TNF receptor-associated protein 1 (M. mulatta) | 773 | 78.20 | 6.77 | 12 | 19 | 641.1 | -2.37 | 0.020 |
| gi|109092109 | Predicted: transglutaminase 2 (M. mulatta) | 741 | 75.22 | 5.31 | 5 | 6 | 199.3 | 2.21 | 0.016 |
| gi|109123684 | Predicted: regulator of G-protein signaling 19-interacting protein 1 isoform 2 (M. mulatta) | 1849 | 36.30 | 5.77 | 4 | 15 | 224.5 | -3.91 | 0.010 |
| gi|92110045 | Ubiquinol-cytochrome c reductase core protein 1 (M. mulatta) | 1386 | 53.40 | 5.75 | 10 | 25 | 453.3 | -2.23 | 0.023 |

**Proteins not identified**

| Accession number (NCBI) | Protein name | Spot number | Molecular mass (kDa) | pI | Number of MS/MS peptide sequences | Sequence coverage (%) | Mascot score | WNV/mock Average volume ratio | p value |
|-------------------------|--------------|-------------|----------------------|----|-------------------------------|------------------------|--------------|-----------------------------|---------|
| n.i. | 235 | -2.29 | 0.0079 |
| n.i. | 408 | 2.11 | 0.011 |
| n.i. | 472 | 2.16 | 0.020 |
| n.i. | 739 | 2.59 | 0.012 |
| n.i. | 949 | 2.05 | 0.00070 |
| n.i. | 978 | 2.04 | 0.011 |
| n.i. | 1001 | 2.07 | 0.0067 |
| n.i. | 1358 | -2.21 | 0.013 |
| n.i. | 1628 | -2.23 | 0.0098 |
| n.i. | 1668 | -2.24 | 0.012 |
WNV infection according to 2D DIGE technology, 93 protein spots were identified. The identified cellular proteins function in diverse biological processes, and some functional groups are formed through cluster and pathway analysis. The potential roles of some of these altered proteins belonging to major clusters in response to WNV infection are discussed below in relation with pathogenesis and early host antiviral response.

**Alteration of Cytoskeleton Networks**—The altered microtubule-associated protein identified in the present work, i.e. tubulin α, was up-regulated, whereas the intermediate filament protein vimentin was down-regulated in WNV-infected Vero cells. Like many viruses (33), flaviviruses have to manipulate and utilize the host cytoskeleton to promote viral infection, but this mechanism is still unclear.

Microtubules and microtubule-associated proteins are known to play an important role in the intracellular trafficking of viral components as well as virions in the infected host cell (34). The actin filaments were shown to be involved in mediating the internalization of WNV particles into mammalian cells by clathrin-dependent endocytosis (35). Moreover it has been demonstrated that actin filaments also play a key role in the release of West Nile (Sarafend) virions (36). Microtubules are cytoskeleton polar structures constituting α- and β-tubulin subunits that perform general functions including organelle movement and cargo transport in all kinds of cells. A previous study has shown that JEV infection induced ultrastructural changes in Vero cells with microtubule rearrangement and redistribution (37). This phenomenon could facilitate the transport of viral proteins from the rough endoplasmic reticulum (ER) and Golgi apparatus to the convoluted membrane, which may serve as a reservoir for viral proteins during JEV multiplication. Furthermore as tubulin exhibits the functions of a chaperone (38), overexpression of its cellular component may also help viral proteins to maintain their conformation in the convoluted membrane.

Vimentin is a major component of the type III intermediate filaments found in many cell lines (39). This 57-kDa protein serves to maintain cell shape and to anchor and position the nucleus within the cell. Major changes in the distribution of vimentin are observed when cells move or divide (40, 41), but this protein is also redistributed in cells expressing misfolded proteins and during virus infection (42, 43). The vimentin re-arrangements with the formation of a cage structure often observed could represent a protective response by the cell during virus infection (44). In fact, the vimentin cage may have a cytoprotective role by preventing the diffusion of viral components into the cytoplasm. Several studies have shown that human immunodeficiency virus type 1 protease cleaves the intermediate filament vimentin and induces the collapse of vimentin in infected cells (45, 46). Further elucidation is in progress to determine whether the flaviviral NS2B-NS3 protease can cleave vimentin, resulting in strongly decreased levels and the collapse of the vimentin network. On the other hand, vimentin is also implicated in the regulation of cell death via its caspase-mediated cleavage (47, 48), and the observed down-regulation of the protein could also be related to the induction of apoptosis in WNV-infected Vero cells (49).

**Stress Response Proteins**—The quantities of two specific cellular proteins related to oxidative stress were found to have been modified in WNV-infected Vero cells. The 70-kDa heat shock protein (Hsp70) was highly expressed, whereas Hsp90 was down-regulated. Eukaryotic Hsp70s are highly abundant cytosolic and nuclear molecular chaperones that play essential roles in various aspects of protein homeostasis (50). Moreover the antiviral activity of some drugs has been associated with the induction of the specific Hsp70 (51, 52). Additionally it has been shown that Hsp70 was able to prevent the cytotoxic effects of the WNV capsid protein, suggesting a protective cell function for this molecular chaperone against WNV infection (53). However, the specific overexpression of Hsp70 by flavivirus infection has not been described until now. Thus, the present study provides the first demonstration of the up-regulation of Hsp70 after WNV infection, highlighting the possible role of this specific chaperone as a protector of the cell from apoptosis (54).

Hsp90 is a molecular chaperone that is highly expressed in most cell types in various organisms (55). It displays ATP-dependent folding capacity (56) and appears to have a specific set of client proteins in vivo unlike its promiscuous cousin chaperone Hsp70 (57, 58). Hsp90 involvement in viral replication has been reported for many viruses, and it has been demonstrated that Hsp90 inhibition blocks viral replication
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The proteins were identified by mass spectrometry following in-gel trypsin digestion. The spot numbers correspond to the same numbers as indicated in Fig. 3. The identities of the spots, their NCBI accession numbers, and the theoretical molecular masses and pI values as well as the number of peptide sequences, their corresponding percent sequence coverage, and the Mascot score are listed for MS/MS analysis. (Protein scores greater than 69 are considered as significant (p < 0.05.)) Paired average volume ratio and p values (t test) between Vero cells infected by WNV versus uninfected cells (WNV/mock) were quantified using DeCyder software. n.i., no identification.

| Accession number (NCBI) | Protein name | Spot number | Molecular mass (kDa) | pI | Number of MS/MS peptide sequences | Sequence coverage | Significance (Mascot score) | Average volume ratio | WNV/mock p value |
|-------------------------|--------------|-------------|---------------------|----|----------------------------------|-------------------|--------------------------|---------------------|------------------|
| Viral proteins          |              |             |                     |    |                                  |                   |                          |                     |                  |
| gi|110645015 | Polyprotein precursor (West Nile virus) | 650 | 384.55 | 8.70 | 18 | 5 | 1057.1 | 21.53 | 0.00088 |
| gi|158516895 | Nonstructural protein 3 (West Nile virus) | 649 | 69.29 | 7.32 | 17 | 8 | 409.3 | 6.27 | 0.0021 |
| gi|158516898 | Nonstructural protein 5 (West Nile virus) | 652 | 104.52 | 6.63 | 9 | 11 | 487.3 | 23.78 | 0.0032 |
| gi|55669122 | Polyprotein (West Nile virus) | 301 | 384.38 | 7.70 | 6 | 1 | 322.01 | 34.02 | 0.0044 |
| gi|90025152 | Polyprotein precursor (West Nile virus) | 303 | 384.40 | 7.00 | 13 | 4 | 518.43 | 34.62 | 0.0068 |
| Transcription and translation |              |             |                     |    |                                  |                   |                          |                     |                  |
| gi|109019116 | Predicted: DEAH (Asp-Glu-Ala-His) box polypeptide 9 isoform 1 (M. mulatta) | 120 | 142.53 | 6.41 | 9 | 7 | 493.51 | 2.82 | 0.0038 |
| gi|109094275 | Predicted: similar to DEAD box polypeptide 17 isoform p82 (M. mulatta) | 502 | 93.16 | 9.33 | 8 | 11 | 385.42 | 2.25 | 0.014 |
| gi|109101818 | Predicted: similar to nucleolin (M. mulatta) | 257 | 101.04 | 5.05 | 11 | 12 | 494.4 | 4.57 | 0.0063 |
| gi|109122950 | Predicted: eukaryotic translation elongation factor 2 (M. mulatta) | 320 | 157.37 | 9.40 | 4 | 4 | 126.39 | 4.62 | 0.012 |
| gi|109124618 | Predicted: similar to heterogeneous nuclear ribonucleoprotein L isoform 3 (M. mulatta) | 733 | 60.82 | 6.65 | 11 | 24 | 540.15 | 4.78 | 0.011 |
| gi|119613715 | Glutamyl-prolyl-tRNA synthetase, isoform CRA_a (H. sapiens) | 44 | 172.03 | 7.02 | 11 | 7 | 593.79 | 2.99 | 0.0021 |
| gi|825664 | Glutaminyl-tRNA synthetase (H. sapiens) | 46 | 90.33 | 8.61 | 9 | 11 | 486.5 | 3.16 | 0.0076 |
| Lipid metabolism |              |             |                     |    |                                  |                   |                          |                     |                  |
| gi|109102275 | Predicted: similar to hydroxyacyl dehydrogenase, subunit A isoform 3 (M. mulatta) | 583 | 83.68 | 9.19 | 2 | 2 | 91.1 | 4.45 | 0.021 |
| gi|109115441 | Predicted: similar to ATP-citrate lyase isoform 1 isoform 2 (M. mulatta) | 195 | 125.27 | 8.41 | 19 | 16 | 864.18 | 2.67 | 0.015 |
| gi|109115443 | Predicted: similar to ATP-citrate lyase isoform 2 isoform 1 (M. mulatta) | 191 | 118.83 | 7.37 | 9 | 8 | 378.9 | 2.99 | 0.0079 |
| Energy pathways |              |             |                     |    |                                  |                   |                          |                     |                  |
| gi|108996402 | Predicted: enolase 1 isoform 5 (M. mulatta) | 1350 | 47.52 | 7.01 | 9 | 26 | 526.17 | –2.46 | 0.024 |
| gi|109089486 | Predicted: similar to hexokinase domain-containing 1 (M. mulatta) | 240 | 103.66 | 6.95 | 9 | 9 | 414.6 | 8.34 | 0.0037 |
| gi|109095230 | Predicted: glyceraldehyde-3-phosphate dehydrogenase (M. mulatta) | 1704 | 36.13 | 8.57 | 6 | 20 | 284.13 | –3.38 | 0.0081 |
| Apoptosis |              |             |                     |    |                                  |                   |                          |                     |                  |
| gi|109132217 | Predicted: programmed cell death 8 isoform 3 (M. mulatta) | 647 | 67.16 | 9.12 | 2 | 3 | 114.05 | 11.61 | 0.00040 |
| gi|109132219 | Predicted: programmed cell death 8 (apoptosis-inducing factor) isoform 2 (M. mulatta) | 657 | 66.52 | 9.11 | 11 | 19 | 583.54 | 17.34 | 0.00088 |
| Cytoskeletal and cell motility |              |             |                     |    |                                  |                   |                          |                     |                  |
| gi|109081460 | Predicted: similar to annexin A2 isoform 1 (M. mulatta) | 1738 | 53.79 | 9.49 | 13 | 30 | 674.19 | –2.74 | 0.0046 |

TABLE II

Proteins identified from the differential 2D DIGE (pH 6–11) analysis after WNV infection
Several viruses require viral and host molecular chaperones for entry, replication, and assembly as well as other steps in viral production (60, 61). Recently a role for Hsp90 in the control of hepatitis C, flock house, and influenza virus polymerase function has been shown (62–65), and it has been proposed that Hsp90 is a major host factor that is of central importance for viral replication for a wide spectrum of RNA viruses (66). The chaperone Hsp90 has also been identified as an essential factor in the folding and maturation of picornavirus capsid proteins (67). The importance of Hsp90 for the replication of multiple viruses opens up an interesting possibility for developing new antiviral therapies that have not yielded drug-resistant viruses (68). Although the down-regulation of Hsp90 observed in WNV-infected Vero cells was not expected, this result may still be an innate cellular response reflecting an early cellular survival mechanism against WNV infection.

**RNA Processing Machinery and Host Translation Pathway**—Among the differentially expressed host proteins, many of them are known to participate in viral replication and translation (Tables I and II). Positive-strand RNA viruses must recruit normal components of host cellular RNA processing or

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### TABLE II—continued

| Accession number (NCBI) | Protein name | Spot number | Molecular mass (kDa) | pI | Number of MS/MS peptide sequences | Sequence coverage | Significance (Mascot score) | WNV/mock Average volume ratio | p value |
|-------------------------|--------------|-------------|---------------------|----|-------------------------------|-----------------|-----------------------------|--------------------------------|--------|
| Accession number (NCBI) | Protein name | Spot number | Molecular mass (kDa) | pI | Number of MS/MS peptide sequences | Sequence coverage | Significance (Mascot score) | WNV/mock Average volume ratio | p value |
| Others                   | Predicted: similar to serine hydroxymethyltransferase 2 (mitochondrial) isoform 9 (M. mulatta) | 1043 | 55.63 | 8.53 | 11 | 25 | 560.92 | 4.03 | 0.0079 |
| Others                   | gi109097406  |             |                      |    |                               |                 |                             |                                |        |
| Proteins not identified  | n.i.         | 47          |                      |    |                               |                 |                             |                                |        |
| Proteins not identified  | n.i.         | 49          |                      |    |                               |                 |                             |                                |        |
| Proteins not identified  | n.i.         | 110         |                      |    |                               |                 |                             |                                |        |
| Proteins not identified  | n.i.         | 113         |                      |    |                               |                 |                             |                                |        |
| Proteins not identified  | n.i.         | 584         |                      |    |                               |                 |                             |                                |        |
| Proteins not identified  | n.i.         | 644         |                      |    |                               |                 |                             |                                |        |
| Proteins not identified  | n.i.         | 1048        |                      |    |                               |                 |                             |                                |        |
| Proteins not identified  | n.i.         | 1069        |                      |    |                               |                 |                             |                                |        |
| Proteins not identified  | n.i.         | 1102        |                      |    |                               |                 |                             |                                |        |
| Proteins not identified  | n.i.         | 1214        |                      |    |                               |                 |                             |                                |        |
| Proteins not identified  | n.i.         | 1692        |                      |    |                               |                 |                             |                                |        |
| Proteins not identified  | n.i.         | 1697        |                      |    |                               |                 |                             |                                |        |
| Proteins not identified  | n.i.         | 1736        |                      |    |                               |                 |                             |                                |        |
| Proteins not identified  | n.i.         | 1771        |                      |    |                               |                 |                             |                                |        |
| Proteins not identified  | n.i.         | 1870        |                      |    |                               |                 |                             |                                |        |

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**Fig. 4.** Pie chart representing up- and down-regulated proteins identified by mass spectrometry following Vero cell infection by WNV. Proteins have been classified according to their subcellular location (A) and biological function (B). The percentages of proteins associated with each category are indicated in parentheses.
translation machineries for viral RNA synthesis and protein synthesis (69).

Human hnRNP C1/C2 are members of the heterogeneous nuclear ribonucleoprotein family, which consists of 20 major hnRNP proteins (designated hnRNP A1 through U) with molecular sizes of 36–120 kDa (70). These proteins are involved in mRNA biogenesis and contain important conserved motifs essential for RNA binding, protein-protein interaction, and nuclear localization. Several ribonucleoproteins, hnRNP Q, A1, A2/B, H, and YB-1 have been found to bind specifically to DENV 3’-untranslated region, suggesting that these molecules may play a biologically significant role in the DENV life cycle (71). Moreover YB-1 is directly involved in repression of DENV translation and can participate in early innate immune responses during DENV infection. A specific association has also been demonstrated between hnRNP C1/C2 and dengue virus NS1 proteins that may be favorable for virus survival in host cells (72).

In the present work, hnRNP L and hnRNP U were identified for the first time as being significantly up-regulated in Vero WNV-infected cells, suggesting that these two specific host cellular proteins play a key role in WNV replication. Interestingly we also observed a depletion of the poly(rC)-binding protein (CP-2) after WNV infection. This protein has multiple functions in post-transcriptional control of host and viral gene expression. With regard to the hepatitis A virus, it has been demonstrated that αCP-2 is cleaved by the viral protease 3C, which could down-regulate viral protein synthesis, giving way to viral RNA synthesis (73). Because of several differences between picornavirus and flavivirus replication strategies (genome cyclization and cap-dependent translation), the role of αCP-2 in the flavivirus regulation life cycle remains to be elucidated. In this study, another important regulating protein, nucleolin, was found to be more
abundant in WNV-infected cells. Nucleolin is one of the most abundant nonribosomal proteins in the nucleolus, and it shuttles between nucleoli, nucleoplasm, cytoplasm, and the cell surface (74). Previous works have reported that nucleolin plays a role in virus replication with direct interaction with the hepatitis C viral RNA-dependent RNA polymerase or overexpression in herpes simplex virus-infected cells (75, 76). Based on these data, an up-regulation of nucleolin in WNV-infected Vero cells suggests that the host protein plays an important part in flavivirus biology, making possible future drug target development.

Translation factors have been well documented as playing crucial roles in viral RNA and protein synthesis (77). Moreover it is known that flaviviruses do not shut off host protein synthesis and must compete with the cellular translation machinery for limiting factors (78). In this way, the eukaryotic translation initiation factors 1A (eEF1A) have been identified as up-regulated proteins or important host cell factors for viral RNA replication during DENV or WNV infection (32, 79). In the present study, an increase in the eEF2 level was observed in the WNV-infected Vero cells suggesting that this protein may also play an important role in the translation of WNV viral RNA. Additionally several tRNA synthetases (glutamyl-prolyl/glutamyl/tryptophanyl-tRNA synthetases) were shown to be overexpressed during WNV infection, suggesting that their overall up-regulation is required for virus propagation in Vero cells. Because flaviviruses prevent the shutoff of host cell translation, the decrease in the eukaryotic translation initiation factor 3 (eIF3) was not expected in WNV-infected Vero cells. eIF3 is one of the 11 or more initiation factors that are involved in the first stage of protein synthesis in eukaryotes. This initiation factor plays an important role by bridging the 43 S preinitiation complex to mRNA via the cap-binding complex eIF4F (80). In coronavirus-infected cells, no obvious inhibition of host protein synthesis was observed at least at early stages of the virus infection cycle. However, eIF3h has been identified as a regulator of the translation of virus-induced genes at late stages of the virus infection cycle (81). Although proteases of many picornaviruses and caliciviruses are known to cleave several translation factors (82, 83), this phenomenon had not yet been reported during flavivirus infection. Thus, the eIF3 down-expression observed in WNV-infected Vero cells may be the reflection of translation regulation mechanisms exploited by flavivirus as a means of controlling viral pathogenesis that have not been described yet.

Apoptotic Pathways—Apoptosis has opposing effects on virus pathogenesis by either preventing viral dissemination or promoting viral spread by release of the progeny virus. However, in the case of neurotropic viruses such WNV, neuron cell death has serious consequences for the host, and elucidation of virus-triggered apoptotic pathways may be critical for the understanding of the pathogenicity of flavivirus (49).

The programmed cell death 8 protein or apoptosis-inducing factor (PDCD8/AIF) is a flavoprotein localized in the mitochondrial intermembrane space, and its translocation to the nucleus induces chromosome condensation and fragmentation (84). Up-regulation of this protein in WNV-infected Vero cells reflects the induction of virus-mediated apoptosis.

Flavivirus replication is associated with extensive proliferation of intracellular membranes, which are, in part, derived from the ER. Translation of the flavivirus polyproteins is associated with the ER membranes, and the ER is also the site of viral encapsidation and envelopment. Therefore, it is likely that flavivirus infection triggers not only perturbation of ER homeostasis but also the unfolded protein response as evidenced by substantial induction of the expression of several chaperones (11). During ER stress, tumor rejection antigen, also known as gp96, is one of the important molecular chaperones that has been involved in unfolded protein response and apoptosis (85, 86). In our study, we observed a decrease in protein levels of gp96 in WNV-infected Vero cells. As already described for JEV infection (87), this phenomenon accelerates ER stress-induced apoptosis and may represent a host defense mechanism to limit viral replication.

Conclusion—Several Vero cell proteins that were modulated early in WNV infection were identified in the present study. To our knowledge, this work made it possible to identify the largest number of cellular proteins regulated differently in response to a flavivirus infection. Most of these proteins were involved in transcription/translation processes, alteration of the cytoskeleton networks, stress cellular response, and apoptotic pathways. Although the roles of some of these alterations could be related to cellular antiviral response or pathogenic mechanisms, functional significance of other altered proteins remains unclear and needs further investigation. However, the present study provides large scale protein-related information that should be useful for understanding the modulation of host metabolism in West Nile infection and for identifying new potential targets for antiviral therapy.

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