Functional Characterization of Phosphorylation of the Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Nucleocapsid Protein

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The nucleocapsid (N) protein of porcine reproductive and respiratory syndrome virus (PRRSV) is a basic multifunctional protein which has been reported to be a serine phosphoprotein with yet-identified functions. As a first step towards understanding the general role of N protein phosphorylation during virus replication, the non-phosphorylated mutant N gene was constructed by mutating all serine residues to alanine. This recombinant N protein was identified to be unphosphorylated, confirming that serine residues truly function as core amino acids responsible for N protein phosphorylation. The PRRSV N protein has been shown to possess the biological features of nuclear localization and N-N homodimerization which individually play critical roles in virus infection. In the present study, therefore, it was attempted to investigate whether these two properties of the N protein are modulated by its phosphorylation status. However, experimental results showed that the non-phosphorylated N protein was still present in the nucleus and nucleolus, and was able to associate with itself by non-covalent interactions. Taken together, the data suggest phosphorylation-independent regulation of N protein nuclear transport or oligomerization, thereby implying the potential involvement of phosphorylation in regulating the activities of the N protein at other levels including RNA-binding capacity.

Key words: PRRSV, nucleocapsid protein, serine phosphoprotein, nuclear localization, homodimerization

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an emerged infectious disease of swine, which continues to cause significant economic losses in the pig industry worldwide [1, 11]. PRRS virus (PRRSV), the causative agent of PRRS, is a small enveloped virus possessing the single-stranded positive-sense RNA genome of approximately 15 kb in size [9, 13, 14]. The virus belongs to the family *Arteriviridae* that were re-grouped into the newly created order *Nidovirales* together with the *Coronaviridae* family [2, 9]. The PRRSV genome consists of the 5' untranslated region (UTR), at least nine open reading frames (ORF1a, ORF1b, ORF2a, ORF2b, and ORFs 3 through 7), and the 3' UTR followed by the poly(A) tail [9, 13, 14].

The nucleocapsid (N) protein of PRRSV encoded in ORF7 is a small basic multifunctional protein with a

molecular weight of 15-kDa [13, 15]. As the sole structural component of viral capsid, the PRRSV N protein associates with itself by both covalent and non-covalent interactions, providing the critical basis for nucleocapsid assembly and virus infectivity [6, 17]. The entire life cycle of PRRSV occurs in the cytoplasm of infected cells, resulting in cytoplasmic and perinuclear distribution of the N protein. The PRRSV N protein, however, has been found to specifically localize in the nucleus and nucleolus of infected cells through an NLS-dependent importin α/β-mediated nuclear transport pathway [12]. Although the precise role of N protein nuclear localization during infection is not well understood, a previous study has shown its important function in viral pathogenesis [8].

In addition, the viral N protein has been demonstrated to be a serine-phosphoprotein [16]. However, the relevance of N protein phosphorylation and its biological function during virus replication is presently unknown. It is well documented that viral protein phosphorylation is an important post-translational modification that is involved in modulating a variety of macromolecular events including

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subcellular localization, nucleic acid binding, or protein-protein interaction [4, 16]. In this study, therefore, it was aimed to investigate whether phosphorylation affects the properties of the PRRSV N protein including nuclear transport and N-N oligomerization.

Materials and Methods

Cells, virus, and antibody

HeLa and Marc-145 cells (a subclone of MA104 cells) [5] were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 8% fetal bovine serum (FBS; Invitrogen), penicillin (100 U/mL), and streptomycin (50 µg/mL). Cells were maintained at 37°C. A stock of vaccinia recombinant expressing T7 RNA polymerase (vTF7-3) [3] was prepared in HeLa cells. A monoclonal antibody (MAb) SDOW17 specific for N is described elsewhere [10].

PCR-based site-directed mutagenesis

The cDNA cloning of the N gene from the PRRSV strain PA-8 and generation of pCITE-N are described elsewhere [15]. Site-directed mutagenesis was conducted to substitute each serine of N to alanine using the pCITE-N with the mutagenic primer pairs. PCR-directed mutagenesis and screening of mutants were performed as described previously [15].

Protein expression, radiolabeling, and immunoprecipitation

The PRRSV N protein and its serine mutant derivatives were expressed in HeLa cells using the T7-based vaccinia virus vTF7-3. HeLa cells grown to 90% confluency were infected for 1 h at 37°C with vTF7-3 at a multiplicity of infection of 10. Following infection, fresh medium was added and incubation continued for an additional 1 h. The cells were washed twice in OPTI-MEM (Invitrogen) and transfected for 16 h using Lipofectin (Invitrogen) according to the manufacturer's instruction. For radiolabeling, the transfected cells were starved for 30 min in methionine- or phosphate-deficient medium (Invitrogen) and were meta-bolically labeled for 5 h with 50 µCi of [^{35}S]methionine/mL or 300 µCi of [^{32}P]orthophosphate/mL (Perkin-Elmer). At the end of the labeling period, cells were harvested, washed twice with cold PBS, and lysed with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 150 mM

NaCl, 50 mM Tris-HCl [pH 7.4], 10 mM EDTA, 0.1% SDS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). After incubation on ice for 20 min, the cell lysates were centrifuged at 14,000 rpm for 30 min in a microcentrifuge (model 5415; Eppendorf), and supernatants were recovered.

For immunoprecipitation, cell lysates equivalent to 1/15 of a 100 mm-diameter dish were adjusted with RIPA buffer to a final volume of 100 µL and incubated for 2 h at room temperature (RT) with 1 µL of N-specific MAb SDOW17. The immune complexes were adsorbed to 7 mg of protein-A Sepharose CL-4B beads (Amersham Biosciences) for 16 h at 4°C. The beads were collected by centrifugation at 6,000 rpm for 5 min, washed twice with RIPA buffer and once with wash buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl). The beads were resuspended in 20 µL of SDS-PAGE sample buffer (10 mM Tris-HCl [pH 6.8], 25% glycerol, 10% SDS, 0.12% [wt/vol] bromophenol blue) with 10% β-mercaptoethanol (β-ME), boiled for 5 min, and analyzed by 12% SDS polyacrylamide gel electrophoresis (PAGE). The gels were dried on filter paper and radio-graphic images were obtained using a phosphorimager (model PhosphorImager SI; Molecular Dynamics).

Immunofluorescence assay

Marc-145 cells were seeded on microscope coverslips in 35 mm-diameter dishes and grown overnight to a con-fluence of 70%. The cells were transfected with 2 µg of plasmid DNA using Lipofectin according to the manufac-turer's instruction. At 48 h post-transfection, cell monolayers were washed twice in PBS and fixed immediately with cold methanol for 10 min. For immunofluorescence, cells on microscope coverslips were blocked using 1% BSA in PBS for 30 min at RT. The cells were then incubated with N-specific MAb SDOW17 for 2 h. After washing five times in PBS, the cells were incubated for 1 h at RT with goat anti-mouse secondary antibody conjugated with Alexa green dye (Molecular Probes). The coverslips were washed five times in PBS and mounted on microscope glass slides in mounting buffer (60% glycerol and 0.1% sodium azide in PBS). Cell staining was visualized using a fluorescent microscope (model AX70, Olympus).

Glutathione S-transfererase (GST) pull-down assay

GST pull-down assays were performed as described pre-viously [7]. In brief, radiolabeled recombinant N proteins were prepared using T7-based vaccinia virus expression

system as described above. GST fusion proteins were expressed in *E. coli* strain BL21 followed by incubation with a 50% slurry of glutathione-Sepharose 4B beads (Amersham Pharmacia), resulting in protein-bead complexes in a 20% slurry. For GST-binding assays, approximately equal amounts, as judged by Coomassie blue staining, of GST or the various GST fusion proteins complexed to beads in a 20% slurry, were incubated with [^{35}S]-methionine labeled N proteins in a binding buffer (20 mM Tris-HCl [pH 7.5], 100 mM KCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, 5 mM dithiothreitol, 0.5% NP40, 1 mM PMSF, 5% glycerol) in a final volume of 400 μL overnight at 4°C with constant agitation. The beads were rinsed 4 times with binding buffer, boiled for 5 min in reducing sample buffer, and analyzed by electrophoresis on 12% polyacrylamide gels. The gels were dried and exposed to a phosphorimager to obtain radiographic images.

Results and Discussion

Construction and expression of serine-mutant N proteins

Mutational experiments on individual serine residues of the PRRSV N protein previously revealed that all single and double combination serine mutant N proteins were still phosphorylated, indicating involvement of more than two residues in phosphorylation [16]. In order to delineate the location of the phosphorylated serine residues, therefore, my approach was extended to the conserved serines, since critical phosphorylation sites should be well maintained among different virus isolates. Based on amino acid sequence alignments of European and North American isolates, four highly conserved serine residues at positions 36, 78, 93, and 98 in N were identified. In addition, I found two more serine residues at positions 78 and 99 that are well conserved among European or North American isolates. The highly conserved serine residues were then substituted to alanine in order to construct triple, quadruple, or more multiple serine combination mutant N genes and were examined for their phosphorylation properties. The serine mutant N proteins were individually expressed in HeLa cells using the T-7 based vaccinia virus expression system (Fig. 1). The expression levels and phosphorylation statuses of N gene mutants were assessed by ^{35}S and ^{32}P$_i$ metabolic labeling, respectively. A 15-kDa phosphorylated N protein was still identified in cells transfected with each

mutant N gene, indicating that alanine substitutions for the highly conserved serine residues in the N protein did not affect phosphorylation (Fig. 1A). Consequently, these results suggested the possibility that non-conserved residues may contribute to N protein phosphorylation (Fig. 1A). Thus, mutational analysis was extended to the non-conserved serines that were individually changed to alanine residue to construct more N gene mutants including the serine-null N gene substituting all 10 serine residues. As shown in Fig. 1B, further multiple serine mutants were specifically labeled by ^{32}P$_i$. In contrast, a 15-kD ^{32}P$_i$-labeled protein was not precipitated from cell lysates transfected with the serine-null N gene (Fig. 1A, lane 18). This serine-null protein expression was verified using ^{35}S metabolic labeling, confirming that the PRRSV N protein is truly a serine-phosphoprotein. The data further suggested that a serine residue at amino acid position 120 appears to be important. Since the serine-null mutant N gene was found to be non-phosphorylated, I used this mutant construct in subsequent experiments.

### Analysis of subcellular localization in the serine-null mutant protein

In PRRSV-infected cells, the N protein is mainly found not only in the cytoplasm but also specifically in the nucleus and nucleolus. In order to know whether N protein phosphorylation functions in the regulation of N protein transport, immunofluorescence assay was performed to investigate the subcellular localization of the serine-null mutant N protein. The wild-type N protein was distributed in the perinuclear region and nucleolus as well as the cytoplasm of N gene-transfected cells (Fig. 2A). Likewise, the same intense cytoplasmic and nucleolar staining of the non-phosphorylated N protein was consistently observed in the serine-null mutant N gene-transfected cells (Fig. 2B). The data, therefore, indicated that phosphorylation has no obvious effect on cellular compartmentalization of N, suggesting that N protein nucleolar localization does not appear to be modulated by phosphorylation.

Homotypic interaction of the non-phosphorylated N protein

In order to assess whether the serine-null N protein still retains the ability to interact with itself in vitro, the GST-pull down assay was performed (Fig. 3). The N protein expressed as a GST fusion product (GST-N) in *E. coli* was
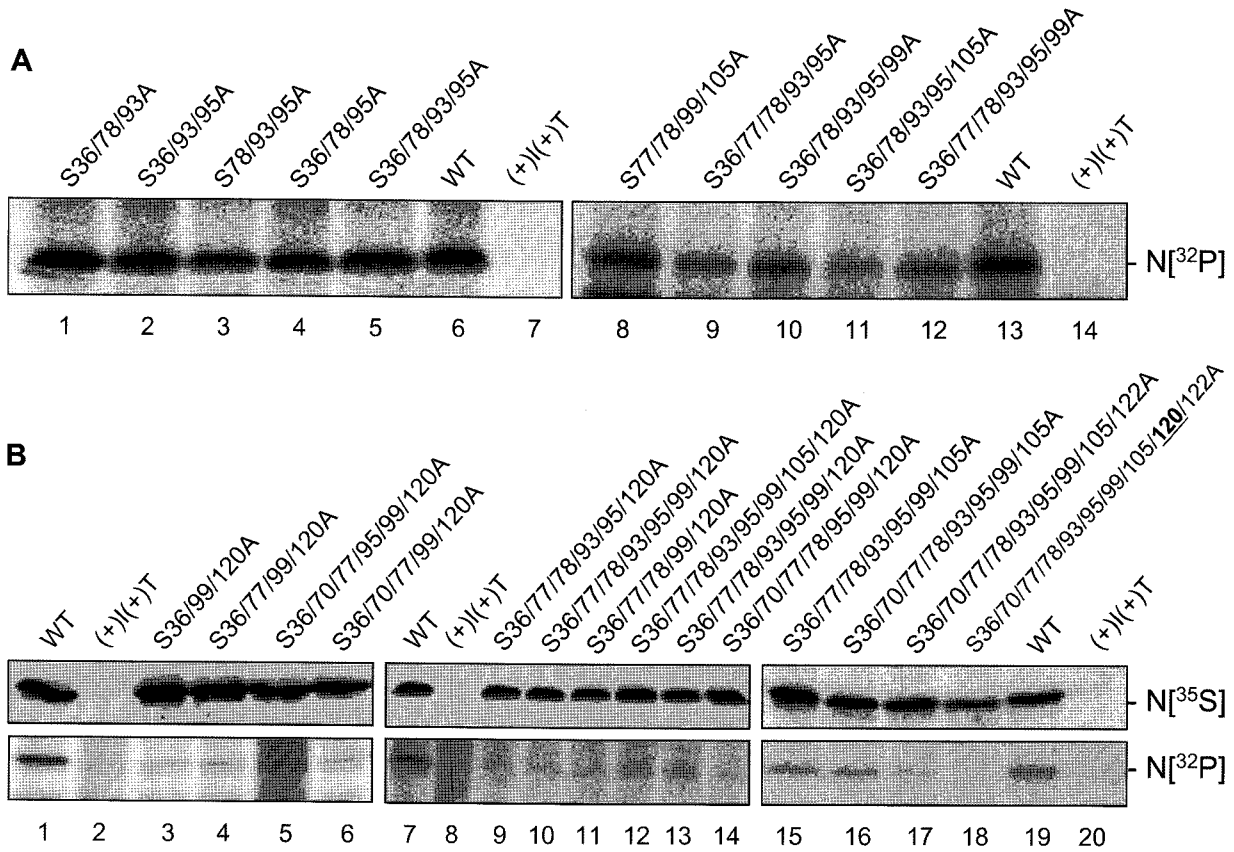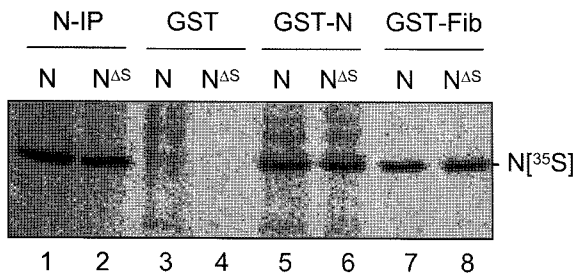
Fig. 1. Phosphorylation properties of the recombinant mutant N proteins. The serine mutants were individually expressed in HeLa cells using the vTF7-3 vaccinia virus. Cells were radiolabeled with [^{32}P$_i$]orthophosphate or [^{35}S]methionine and lysed with RIPA buffer. Cell lysates were immunoprecipitated by N-specific MAb SDOW17 and the immune complexes were disassociated in sample buffer in the presence of β-mercaptoethanol. Dissociated proteins were resolved by SDS-12% PAGE and radiographic images were visualized by a phosphorimager. WT, wild-type N gene transfected; (+)I(+)T, vTF7-3 infected and pCITE vector transfected; N[^{32}P], [^{32}P]phosphate-labeled nucleocapsid protein; N[^{35}S], [^{35}S]methionine-labeled nucleocapsid protein.

Wild-type N   Non-phosphorylated N
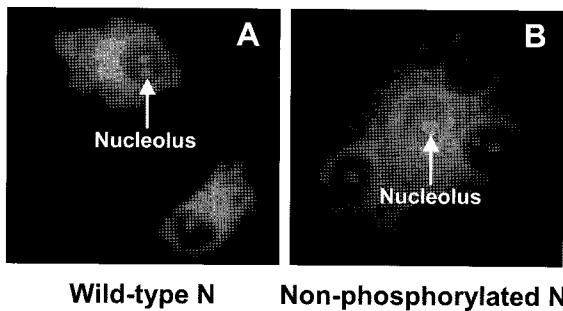
Fig. 2. Subcellular localization of the recombinant serine-null N protein. The wild-type or serine-null N gene was transfected into Marc-145 cells. Gene-transfected cells were incubated for 2 days and reacted with N-specific MAb SDOW17 followed by staining with Alexa-green conjugated goat anti-mouse antibody. Intracellular localization of the serine-null mutant N protein (non-phosphorylated N) shows the presence of N protein in the nuclei and nucleoli.

coupled to glutathione-Sepharose beads and incubated with either the radiolabeled wild-type or serine-null mutant N

protein independently expressed in HeLa cells by vTF-7-3 vaccinia virus. After extensive washing of the beads, bead-bound proteins were dissociated and resolved by SDS-PAGE under reducing conditions followed by autoradiography. In all binding experiments, approximately equal amounts of protein were used, as verified by Coomassie blue staining (data not shown). Wild-type and mutant N proteins immunoprecipitated with N-specific MAb were individually used as a marker (lanes 1, 2). As a negative control, no N protein was found to be precipitated in association with GST alone (lanes 3, 4). In contrast, the GST-N fusion protein efficiently precipitated both wild-type and serine-null mutant N proteins (lanes 5, 6), indicating the interaction between N and GST-N was specific. These results suggest that the phosphorylation status of N is relevant to its homotypic association. In addition, it was further determined if phosphorylation would compromise a specific interaction of N with fibrillarin, since the PRRSV

N-IP   GST   GST-N   GST-Fib

N   N$^{\Delta S}$ N   N$^{\Delta S}$ N   N$^{\Delta S}$ N   N$^{\Delta S}$

N[^{35}S]

1   2   3   4   5   6   7   8

Fig. 3. Interaction of the serine-null N protein (N$^{\Delta S}$) with wild-type N or fibrillarin in vitro measured by the GST pull-down assay. Bacterially expressed GST (lanes 3 and 4), GST-N (lanes 5 and 6), or GST-Fib (lanes 7 and 8) was individually bound to glutathione-Sepharose beads and incubated with the recombinant wild-type N or serine-null N$^{\Delta S}$ protein expressed and radiolabeled with [^{35}S]methionine in HeLa cells. The beads were washed five times, and the bound proteins were eluted in reducing sample buffer followed by SDS-PAGE and autoradiography. N-IP, input N or N$^{\Delta S}$ protein as a marker radioimmunoprecipitated using N-specific MAb SDOW17; GST-N, PRRSV N protein fused with GST; GST-Fib, fibrillarin protein fused with GST.

N protein has been shown to interact with this host cellular nucleolar protein. Both the radiolabeled wild-type N and the serine-null mutant were separately used as a prey in the GST-pull down assay and bound to the GST-fibrillarin fusion protein with approximately equal affinity (lanes 7, 8). Altogether, my data revealed that the protein-protein interactions of N with itself or fibrillarin were independent of N protein phosphorylation.

Phosphorylation of the viral protein plays a critical role in the regulation of protein activities such as stability, subcellular localization or interaction with binding partners like nucleic acid or protein [4]. The PRRSV N protein is known to possess the binding affinity to the viral genomic RNA and itself for nucleocapsid assembly. Furthermore, the N protein is phosphorylated on serine residues and notably translocated to the nucleus and nucleolus of virus-infected cells. It has therefore been tempting to speculate that phosphorylation may affect such properties of the N protein. However, it is unlikely possible for the PRRSV N protein, since my experimental data indicate that the unphosphorylated N protein is still able to interact with itself and to be distributed in all cellular compartments. These results suggest that phosphorylation does not appear to regulate oligomerization and subcellular localization of N. Although I have not addressed the biological function of N protein phosphorylation, the consequences imply a potential involvement of phosphorylation in modulating the macromolecular events of the N protein during the PRRSV

life cycle at other levels including the RNA-binding activity.

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국문요약

PRRS 바이러스 Nucleocapsid 단백질 인산화의 기능학적 연구

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돼지생식기호흡기증후군 바이러스를 구성하고 있는 뉴클레오캡시드(N) 단백질은 다양한 기능을 가지고 있는 basic 단백질로써 또한 아직까지 밝혀지지 않은 역할을 하는 serine 인산화 단백질로 알려져 있다. 먼저 바이러스가 복제되는 동안 뉴클레오캡시드 단백질 인산화가 어떤 생물학적 역할을 하는지에 대한 이해를 하기 위하여 mutagenesis 방법으로 단백질 내 모든 serine 잔기들을 alanine으로 대체하여 변이 뉴클레오캡시드 단백질을 구축하였다. 이 재조합 뉴클레오캡시드 단백질은 비인산화 단백질로 확인되었고 이는 뉴클레오캡시드 단백질 인산화에 serine 잔기들이 중요한 역할을 한다는 것을 증명하였다. 돼지 생식기호흡기증후군 바이러스 뉴클레오캡시드 단백질은 세포핵 내 이동과 N-N dimer 형성 등의 특이적인 생물학적 특성들을 보유하고 있으며 이들 각각은 바이러스 감염 시 중요한 역할들을 하는 것으로 알려져 있다. 따라서 본 연구에서는 이 두 가지 뉴클레오캡시드 단백질의 특성들이 인산화 여부에 의해 조절되는지 살펴보았다. 하지만 본 연구의 결과들은 비인산화된 뉴클레오캡시드 단백질이 여전히 transfection된 세포의 핵 또는 핵인에서 발현 되었고 더욱이 뉴클레오캡시드 자신과 dimer 형성을 할 수 있었다는 것을 보여주었다. 결론적으로 돼지 생식기호흡기증후군 바이러스 뉴클레오캡시드 단백질의 세포핵 내 수송 및 oligomerization 특성들은 인산화 비의존성으로 조절되는 것으로 보여 진다. 아마도 이 인산화 작용은 뉴클레오캡시드 단백질의 RNA-binding 특성등과 같은 다른 수준의 조절과 관련이 있는 것으로 추측되어 진다.