Phosphorylation of Bluetongue Virus Nonstructural Protein 2 Is Essential for Formation of Viral Inclusion Bodies

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In bluetongue virus (BTV)-infected cells, large cytoplasmic aggregates are formed, termed viral inclusion bodies (VIBs), which are believed to be the sites of viral replication and morphogenesis. The BTV nonstructural protein NS2 is the major component of VIBs. NS2 undergoes intracellular phosphorylation and possesses a strong single-stranded RNA binding activity. By changing phosphorylated amino acids to alanines and aspartates, we have mapped the phosphorylated sites of NS2 to two serine residues at positions 249 and 259. Since both of these serines are within the context of protein kinase CK2 recognition signals, we have further examined if CK2 is involved in NS2 phosphorylation by both intracellular colocalization and an in vitro phosphorylation assay. In addition, we have utilized the NS2 mutants to determine the role of phosphorylation on NS2 activities. The data obtained demonstrate that NS2 phosphorylation is not necessary either for its RNA binding properties or for its ability to interact with the viral polymerase VP1. However, phosphorylated NS2 exhibited VIB formation while unmodified NS2 failed to assemble as VIBs although smaller oligomeric forms of NS2 were readily formed. Our data reveal that NS2 phosphorylation controls VIBs formation consistent with a model in which NS2 provides the matrix for viral assembly.

Protein phosphorylation is a ubiquitous protein modification that controls a number of intracellular processes. In eukaryotic systems, phosphorylation occurs almost exclusively on serine, threonine, or tyrosine residues (26). Also for RNA viruses, including vesicular stomatitis virus, ebola virus, human immunodeficiency virus type 1 (HIV-1), and rubella virus, protein phosphorylation has been shown to regulate vital processes such as virus transcription and replication, RNA binding activity, and virus assembly (9, 22, 28, 34). The nonstructural protein 2 (NS2) is the only phosphorylated protein of the 10 viral proteins synthesized during a bluetongue virus (BTV) infection (25).

BTV is the prototype of the *Orbivirus* genus in the *Reoviridae* family and hence characteristically possesses a double-stranded RNA genome enclosed by three consecutive capsid layers of multiple proteins. The BTV genome consists of 10 segments, each encoding one protein. There are seven structural proteins (viral polymerase 1 [VP1] through VP7), of which two are outer capsid proteins (VP2 and VP5) and the remaining five are associated with the BTV core. The viral core consists of a double-layered shell composed of VP3 and VP7, and within the core, there are three virus-encoded proteins (VP1, VP4, and VP6), all of which are enzymatically involved in viral transcription and replication. VP4 is the mRNA-capping enzyme, exhibiting guanylttransferase and methyltransferase activities, VP6 is a double-stranded RNA helicase, and the largest protein, VP1, is the viral RNA-dependent RNA polymerase (5, 32, 36, 41). Core particles are transcriptionally

active, producing and releasing mRNA. The remaining three BTV proteins are nonstructural proteins (NS1 through NS3). While NS1 represents the most abundantly synthesized protein during a BTV infection, the secondmost abundant BTV protein in infected cells is NS2. Investigations of NS2 have revealed that NS2 is a multifunctional protein. It has the enzymatic ability of hydrolyzing nucleotide triphosphates to nucleotide monophosphates (23, 42). The protein also binds single-stranded RNA (ssRNA) very efficiently, and BTV RNAs are preferentially bound over nonspecific RNAs (30, 31). An important structural feature of NS2 is its ability to form multimers (45). The exact size of NS2 oligomers is not defined yet but is likely to be between 6 and 11 subunits, with the most recent structural investigation favoring a decameric composition (8, 42). The most remarkable feature of NS2 is that it is the major component of viral inclusion bodies (VIBs) formed in BTV-infected cells (7, 11). These VIBs appear to be the site of viral replication and of early viral assembly. While newly assembled BTV core particles have been identified within VIBs, complete BTV particles were found at the edges of the inclusions. Recombinant NS2, expressed by the baculovirus expression system, has been shown to form intracellular aggregates in insect cells which resemble VIBs of BTV-infected cells (44). These data indicate that NS2 is responsible for the formation of VIBs. A prominent structural feature of NS2 is that it undergoes phosphorylation in BTV-infected cells or when expressed in insect cells (10, 44). Although autophosphorylation of NS2 has been considered by several groups, other data suggest that a cellular kinase is responsible for NS2 phosphorylation (42, 43).

To determine if NS2 phosphorylation plays a role in the structure-function relationships of the protein, in this report, we have mapped the phosphorylated sites within BTV NS2

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using two different expression systems, mammalian and insect cells. Protein kinase CK2 was subsequently identified as the cellular kinase that was responsible for phosphorylation of NS2. NS2 mutants lacking the phosphorylated amino acids were investigated with respect to their RNA binding properties, interaction with BTV VP1, and NS2 oligomerization. The data obtained demonstrated that the ability of NS2 to form intracellular inclusion bodies is dependant on its phosphorylation status and thus indicated that NS2 phosphorylation might regulate VIB formation in BTV-infected cells.

MATERIALS AND METHODS

Cells and viruses. *Spodoptera frugiperda* Sf9 and Sf21 cells were grown, propagated, and infected with recombinant baculovirus and as described by King and Possee (27).

293T cells were cultured in modified Eagle medium (MEM; Gibco) containing 10% fetal calf serum (FCS; Gibco), 100 U of penicillin/ml, and 100 µg of streptomycin/ml (Sigma). BHK-21 and HeLa cells were grown in Dulbecco's MEM (Sigma) containing supplements as described above.

Recombinant baculoviruses containing the BTV-10 NS2 gene or mutated BTV NS2 genes (AcBTV-10 NS2, AcBTV-10 NS2_{2A}, and AcBTV-10 NS2_{2D}) were obtained and propagated as described previously (27, 44).

Bluetongue virus serotype 10 was used for cell infection. Cell monolayers were washed with FCS-free growth medium and then incubated with viruses at the required multiplicity of infection (MOI). Virus adsorptions were carried out for 2 h, followed by incubation in growth medium supplemented with 5% FCS.

Molecular cloning of expression plasmids. As expression vectors for the transient expression of NS2, NS2f, VP1, VP4, and VP6, pCAGGS plasmids (termed pCAG-NS2, pCAG-NS2f, pCAG-VP1, pCAG-VP4, and pCAG-VP6) were constructed by inserting the sequences of NS2, NS2f, VP1, VP4, or VP6 downstream of the chicken β-actin promoter using PCR and standard molecular cloning techniques (38). Expression vectors for NS2 mutants were produced using the QuikChange site-directed mutagenesis kit (Stratagene) with pCAG-NS2 as template. For the production of a recombinant baculovirus expressing wild-type NS2, the NS2 sequence was inserted into the pTriEx-1 vector (Novagen). For generating NS2 substitution mutants, mutations were introduced as described above. In order to generate a plasmid for the T7-dependent expression of NS2 for pull-down experiments that carries an N-terminal S peptide (15 amino acids), the NS2 sequence was inserted into pTriEx-4 using the BamHI and BglII restriction sites (pTriEx4-S-NS2). This was used to express a fusion protein that contained a total N-terminal extension of 62 amino acids. For generating S-tagged NS2 substitution mutants, mutations were introduced as described above. All wild-type sequences and mutants were verified by sequencing.

Protein expression in mammalian cells and metabolic labeling with [³²P]orthophosphate or [³⁵S]methionine. For coprecipitation and phosphorylation state analysis, subconfluent 293T cells (9 × 10⁵ cells in 10-cm² wells) were transfected with the respective plasmids encoding NS2 or NS2 mutants by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 22 h or 40 h posttransfection, cells were starved for 1 h with methionine-deficient Dulbecco's MEM (Sigma) or phosphate-deficient MEM (Sigma). Medium was removed, and cells were labeled for 2 h with 20 µCi of [³⁵S]methionine (ICN) or for 3.5 h with 140 µCi phosphorus-32 (Amersham) in 1 ml starvation medium.

Protein labeling with [³²P]orthophosphate in Sf9 cells. For phosphorylation state analysis, confluent Sf9 cells (1.2 × 10⁶ cells in 10-cm² wells) were infected with recombinant baculovirus expressing NS2, NS2_{2A}, or NS2_{2D}, respectively, at an MOI of 4 and incubated at 28°C. At 28 h postinfection, cells were starved for 1 h using phosphate-deficient MEM whereas the pH of the medium was shifted to pH 6.5 beforehand using HCl. Medium was removed, and cells were labeled for 22 h with 125 µCi phosphorus-32 (Amersham) in 1 ml starvation medium.

Immunoprecipitation. After metabolic labeling, the medium was removed and cells were washed twice with ice-cold phosphate-buffered saline and scraped into 0.5 ml ice-cold immunoprecipitation buffer (20 mM Tris-HCl, [pH 7.6], 100 mM NaCl, 0.4% [wt/vol] deoxycholic acid, 1% [wt/vol] NP-40, 2 mM dithiothreitol, 1:250 protease inhibitor cocktail [Sigma]). For phosphorylation state analysis, the immunoprecipitation buffer contained additionally 10 mM NaF, 200 µM Na₃VO₄, and 8 µg RNase A (Sigma) and immunoprecipitations were performed as described previously (20). Radioactive signals were visualized by exposing dried gels or nitrocellulose membranes to a phosphorimaging screen (Amersham) and quantified using a Storm 840 PhosphorImager (Molecular Dynamics) and ImageQuant 5.0 software (Molecular Dynamics).

Western blot analysis. Cells were transferred onto a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham) as described previously (38). Primary antibodies for the detection of NS2 or NS2 mutants were a guinea pig anti-NS2 serum raised against BTV-10 NS2 and, for the detection of VP1_{his}, a monoclonal antipolyhistidine clone his-1, ascites fluid (Sigma). Secondary antibodies were an alkaline phosphatase-conjugated goat anti-guinea pig immunoglobulin G (IgG, 1:10,000; Sigma) and an alkaline phosphatase-conjugated goat anti-mouse IgG (1:5,000; Sigma), respectively.

In vitro phosphorylation of NS2 by CK2. NS2 and NS2 mutants were expressed as S-tagged NS2 constructs produced with the TNT Quick Coupled Transcription/Translation reaction system (Promega) according to the manufacturer's instructions. Reactions were either analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or used for further experiments. Ten microliters of each cold TNT reaction was incubated in kinase buffer (New England Biolabs) supplemented with 1 µCi of [γ-³²P]ATP, 4 µM cold ATP, and 5 U of CK2 (New England Biolabs). In a control reaction, myricetin was added at 1 µM. The reactions were incubated for 25 min at 30°C and stopped by the addition of 25 mM EDTA. Next, 20 µl of S-agarose protein beads and 5 µg of bovine serum albumin were added to the reactions and samples were incubated for 20 min at room temperature in a rotor. The lysates were washed twice with pull-down buffer (20 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1:200 protease inhibitor cocktail, 10 mM NaF, 200 µM Na₃VO₄). Samples were spun down at 500 × g for 5 min prior to resuspension in 20 µl of standard SDS-PAGE sample buffer and followed by incubation for 5 min at 95°C. Labeled products were identified by SDS-PAGE and autoradiography.

Indirect immunofluorescence analysis. BHK, 293T, or HeLa cells grown on glass coverslips to 90% confluency were transfected with pCAGGS plasmids using 5 µl polyethylenimine and 1.25 µg of DNA per transfection according to the method of Boussif et al. (4). Infection of cells with BTV-10 was carried at an MOI of 0.5 for 24 h posttransfection. At 40 h posttransfection, cells were fixed with 3.5% paraformaldehyde and processed for immunofluorescence assay by using the following primary antibodies for detection: (i) for NS2 and NS2 mutants, a polyclonal anti-NS2 serum (1:100), (ii) for protein kinase CK2, a monoclonal anti-CK2 antibody (5 µg/ml; Biomol), (iii) for VP1, a polyclonal anti-VP1 serum (1:100), (iv) for VP4, a polyclonal anti-VP4 serum (1:100), (v) for VP6, a polyclonal anti-VP6 serum (1:100), and (vi) for Flag-tagged NS2 proteins, a monoclonal anti-FLAG antibody (dilution 1:100; Sigma). Secondary antibodies used for visualization were as follows: (i) for NS2 or NS2 mutants, a fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea pig IgG or a tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-guinea pig IgG as indicated in the legends to Fig. 2, 4, and 6, (ii) for protein kinase CK2 and (iii) VP1, a TRITC-conjugated goat anti-mouse IgG, (iv) for VP4, a TRITC-conjugated goat anti-guinea pig IgG, (v) for VP6, a TRITC-conjugated goat anti-mouse IgG, and (vi) for Flag-tagged NS2 proteins, a FITC-conjugated goat anti-mouse IgG (all 1:100; Sigma) as described previously (21) and examined using a confocal microscope (Axiovert 200 M; Zeiss).

Coimmunoprecipitation of VP1 and NS2. Both NS2 and six-histidine-tagged VP1 (VP1_{his}) were expressed in the baculovirus expression system and purified as described by Horscroft et al. (23) and Boyce et al. (5). Next, 5 µg of purified NS2 and 4 µg of VP1_{his} were incubated in 500 µl coimmunoprecipitation buffer (20 mM Tris-Cl [pH 7.6], 100 mM NaCl, 5 mM EDTA, 5 mM MgCl₂, 1% NP-40, 0.4% deoxycholic acid, 5 mM dithiothreitol, 50 µg RNase A) for 4 h at 4°C. Nonspecific proteins were removed by preincubation with protein A-Sepharose. Supernatants were subsequently incubated for at least 3 h with 20 µl protein A-Sepharose and 2 µl of a polyclonal anti-BTV-10 NS2. After washing with coimmunoprecipitation buffer, the pellet was resuspended in 20 µl of standard SDS-PAGE sample buffer and subjected to SDS-10% PAGE. Either gels were stained with Coomassie blue or proteins were blotted onto a nitrocellulose membrane.

RESULTS

Mapping the phosphorylated sites of NS2. NS2 is the only BTV protein which is phosphorylated. Phosphoamino acid analyses of ³²P-labeled recombinant NS2 has revealed that only serine residues of the protein were phosphate acceptor sites and that neither phosphothreonine nor phosphotyrosine was identified (10, 44). There are 19 serine residues within the BTV-10 NS2, and these are evenly distributed without showing any identifiable clustering (16). In order to narrow down the

FIG. 1. Phosphorylation state analysis of NS2 and NS2 mutants expressed in mammalian cells and insect cells. (A) 293T cells were transfected with plasmids encoding NS2 and NS2 mutants in which serine residue 249 or 259 was substituted by alanine residues as indicated. The mutant NS2_{2A} had both serines exchanged for alanines. At 24 h (lanes 1 through 4) or 40 h (lanes 5 through 9) posttransfection, proteins were metabolically labeled with ³²P_i and immunoprecipitated from the cellular lysate using an anti-NS2 antiserum. Precipitated proteins were visualized by Western blotting (wb) and radioactive signals detected by exposure to a phosphorimaging screen. (B) Quantification of the phosphorylation state of NS2. The ³²P signals of NS2 or NS2 mutants from expression in 293T cells were quantified using ImageQuant 5.0 software and normalized to the expression level of the respective protein (Western blot signals). Each mutant was tested in three independent experiments. (C) Sf9 cells were infected with a recombinant baculovirus carrying the genes for NS2 or NS2 mutants. NS2_{2A} contains serine residues 249 and 259 exchanged for alanines and NS2_{2D}, both serines substituted by aspartates. At 28 h postinfection, proteins were labeled, immunoprecipitated, and visualized as described above.

serine residues representing potential targets for protein kinases, the amino acid sequence of NS2 was analyzed by using the computer prediction programs NetPhos (3) and Scansite (35) and also by the identification of conserved serines within NS2 proteins of different Orbiviruses. Based on these analyses, six serine residues (amino acids 150, 175, 249, 259, 289, and 298) of BTV-10 NS2 were identified as potential phosphorylation sites. To examine whether the selected serine residues were indeed the phosphorylated sites of the protein, NS2 mutants were generated by substituting the respective serine residues with alanine residues and both wild-type NS2 and NS2 mutants were expressed in BTV-susceptible 293T cells. Cells were metabolically labeled with ³²P_i, and after cell lysis, NS2 was immunoprecipitated using a guinea pig polyclonal anti-NS2 serum. Immune complexes were analyzed by SDS-10% PAGE separation followed by autoradiography. Mutants that exhibited reduction of phosphorylation were subsequently used for further analyses. Wild-type NS2 was strongly phosphorylated as expected, while the substitution of the serine residue 249 (NS2_{S249A}) or 259 (NS2_{S259A}) resulted in a reduced level of phosphorylation (Fig. 1A). When both residues were mutated to alanine, the dual NS2 mutant (NS2_{2A}) showed complete loss of a phosphorylation signal (Fig. 1A). For quantification of the phosphorylation frequency on each of the phosphorylated serine residues, Western blot analysis was performed prior to the detection of the respective ³²P signals.

The amounts of precipitated protein and their corresponding phosphorylation signal were determined in relation to native NS2, which was considered to be 100% phosphorylated. According to three independent experiments, Ser249 was on average phosphorylated to 38% and Ser259 to 59% relative to the native protein (Fig. 1A and B). Since protein phosphorylation is subjected to a permanent turnover mediated by protein phosphatases and protein kinases, the phosphorylation states of wild-type NS2 and the three mutants (NS2_{S249A}, NS2_{S259A}, and NS2_{2A}) were determined at 24 h and 40 h postexpression (Fig. 1A). The results at both time points were essentially the same, revealing that the phosphorylation state of NS2 was stable in the absence of viral infection. No difference could be detected in the migration patterns of phosphorylated and nonphosphorylated NS2 (Fig. 1A). To characterize further the phosphorylation of NS2, we generated recombinant baculoviruses expressing NS2, the dual mutant NS2_{2A}, and an additional mutant in which the two residues Ser249 and Ser259 were substituted by two aspartate residues (NS2_{2D}). Due to the negative charge, an aspartate mimics a phosphoserine residue, i.e., NS2_{2D} mimics the fully phosphorylated form of NS2 (37, 39). Insect cells infected with each of the recombinant viruses were metabolically labeled with ³²P_i as described in Materials and Methods. Recombinant NS2 and NS2 mutants were recovered from lysed cells and immunoprecipitated. The ³²P-labeled proteins were separated by SDS-10% PAGE and an-

FIG. 2. Phosphorylation of NS2 by protein kinase CK2. (A) Amino acid sequence of positions 246 through 262 of NS2 aligned to the consensus recognition sequence of protein kinase CK2. Serine residues 249 and 259 are in bold. (B) Colocalization of NS2 and CK2 in BHK-21 cells 40 h posttransfection. NS2 was visualized using an FITC-conjugated antibody and protein kinase CK2 with a TRITC-conjugated antibody. (C) S-tagged NS2 (lanes 1 and 2) or S-tagged NS2 mutants (lanes 3 through 5) synthesized by in vitro transcription/translation were incubated with purified CK2 and [γ -³²P]ATP, pulled down using S-agarose protein beads, separated on an SDS-PAGE gel, and visualized by autoradiography. Myricetin was added to inhibit CK2 activity when indicated at 1 μ M.

alyzed by autoradiography. The wild-type NS2 was highly phosphorylated, but as expected, the mutant NS2_{2A} gave no detectable ³²P signal (Fig. 1C, lanes 1 and 2). Likewise, the NS2 mutant NS2_{2D} did not exhibit any significant phosphorylation signal. This revealed that the phosphorylation state of NS2 expressed in insect *Sf9* cells was the same as that in mammalian 293T cells, indicating a ubiquitous protein kinase phosphorylating NS2.

NS2 can be phosphorylated by protein kinase CK2 in vitro. The two serine residues, Ser249 and Ser259, that were identified by the in vivo metabolic labeling as being the phosphorylation sites of NS2 have the downstream signature sequences, i.e., SDDD and SDDE, respectively, that are consensus recognition signals for protein kinase CK2 activity (Fig. 2A). In order to investigate if CK2 indeed interacts with NS2, the intracellular distribution of CK2 and recombinant NS2 was

examined. For visualizing intracellular localization, NS2 was transiently expressed in BHK cells and immunofluorescence analysis was carried out using confocal microscopy. Figure 2B shows that NS2 aggregated at defined sites within the cells. When an anti-CK2 antibody was used, colocalization of NS2 and CK2 was clearly visible.

To confirm that CK2 is indeed responsible for the phosphorylation of NS2, in vitro phosphorylation experiments were carried out using NS2 and NS2 mutants that were synthesized by the TnT-coupled transcription/translation system. The in vitro-synthesized proteins were incubated with purified CK2 in the presence of [γ -³²P]ATP. NS2 proteins used in this assay were expressed as fusion proteins with an S-peptide to facilitate the pull-down of the proteins with S-agarose as described in Materials and Methods. As a negative control, an additional reaction was also undertaken in which myricetin, a specific CK2 inhibitor, was added to the NS2 phosphorylation assay. As shown in Fig. 2C, CK2 was able to phosphorylate wild-type NS2 (lane 1) and mutants NS2_{S249A} and NS2_{S259A} but failed to phosphorylate the double mutant NS2_{2A} (lanes 3 through 5). Myricetin was able to inhibit the phosphorylation of wild-type NS2 by CK2 (Fig. 2C, lane 2).

The data from the in vivo metabolic labeling experiments and the in vitro phosphorylation of NS2 by protein kinase CK2, together with the data obtained from the intracellular colocalization of NS2 and CK2, provide evidence that NS2 is exclusively phosphorylated at two serine residues, Ser249 and Ser259, and that both residues are recognized as substrates by the cellular protein kinase CK2.

Influence of NS2 phosphorylation on RNA binding of NS2. NS2 binds ssRNA molecules in general, although it exhibits a higher RNA affinity for BTV ssRNA than for the other non-specific RNA molecules (30, 31). It was possible that the phosphorylation status of NS2 may influence its selective RNA binding ability. Several competitive RNA binding assays were performed using a ³²P-labeled BTV ssRNA probe and unlabeled ssRNA competitors (specific or nonspecific). Wild-type NS2 and the two NS2 mutants, NS2_{2A} and NS2_{2D}, were purified to homogeneity (Fig. 3A) from insect cell cultures infected with a recombinant baculovirus expressing the respective protein and were used for the competitive RNA binding assays.

RNA binding assays were essentially carried out as described by Lymeropoulos et al. (30). In a typical binding assay, each type of NS2 at the concentration of 3.7 μ M (75 pmol) was incubated with 8 nM of ³²P-labeled ssRNA probe (1,125 nucleotides [nt]) that was derived from BTV segment 8 (S8). As specific competitor, the same but unlabeled S8 ssRNA was used, while as the nonspecific competitor, a similar-sized ssRNA (1,165 nt) transcript derived from the T7-dependent transcription of pGEM3zf (+) was employed. Both competitors were used at a range of concentrations starting from 80 μ M up to 800 nM.

When [³²P]RNA complexes were analyzed by electrophoretic mobility shift assay, competition was already detectable with the unlabeled S8 RNA at a 1:10 molar excess (Fig. 3B, lane 6) and at a 1:100-fold excess, the unlabeled RNA competed out the radiolabeled RNA from the complex (Fig. 3B, lane 7). Both NS2 and the nonphosphorylated NS2_{2A} behaved in exactly the same manner (Fig. 3B, lanes 6, 7, 12, and 13), indicating that NS2 phosphorylation had no direct influ-

FIG. 3. Influence of NS2 phosphorylation on its RNA binding properties. (A) Coomassie blue-stained SDS-10% PAGE comparison of NS2 and NS2 mutants expressed in *Sf9* cells and purified by column chromatography. (B through D) Electrophoretic mobility shift assays of NS2 or NS2 mutants bound to 32 P-labeled transcripts (8 nM) of BTV segment S8 (1,125 nt) and in the presence of increasing amounts (80 pM to 800 nM) of unlabeled competitor, either BTV S8 (panel B, lanes 3 through 7 and 9 through 13, and panel D, lanes 3 through 7) or pGEM3zf (+)-derived RNA (1,165 nt) (panel C, lanes 3 through 7 and 9 through 13, and panel D, lanes 8 through 12). Competitors were used in 10-fold increments (left to right). (B, C, and D) Lane 1, 32 P-labeled transcript only; all other lanes, 32 P-labeled transcript and NS2 or NS2 mutants. NS2 was used in panels B and C, lanes 2 through 7; NS2_{2A} was used in panels B and C, lanes 8 through 13; and NS2_{2D} was used in panel D, lanes 2 through 12.

ence on the BTV ssRNA binding activity of the protein. When a nonspecific competitor (derived from pGEM vector) was used instead of BTV ssRNA, very little competition was detected in the presence of the 100-fold excess competitor (Fig. 3C, lane 7). Again, the electrophoretic mobility shift was indistinguishable whether wild-type NS2 or the mutant NS2_{2A} was used (Fig. 3C, lanes 7 and 13), indicating that the phosphorylation status of NS2 neither enhanced nor reduced RNA binding activity in comparison to that of a nonphosphorylated NS2. This finding was further supported by performing the RNA binding experiment using the second mutant, NS2_{2D}, which mimics the phosphorylated state of the protein. When the RNA-NS2_{2D} complexes were analyzed (Fig. 3D), the RNA shift pattern was clearly the same as that with the wild-type protein and the NS2_{2A} mutant. This suggested that NS2 phosphorylation does not affect its RNA binding properties.

Influence of NS2 phosphorylation on binding to the BTV inner-core proteins. From ultrastructural analysis, it has been demonstrated that BTV cores are associated with VIBs (7). Since NS2 is the major constituent of VIBs, it is likely that the NS2 is directly responsible for recruiting various core proteins to VIBs prior to core assembly. Thus, we investigated an interaction of NS2 with the core proteins VP1, VP4, and VP6, the components of the viral polymerase complex.

Initially, colocalization experiments of the proteins were performed by confocal microscopy using transiently coexpressed NS2 with VP1, VP4, and VP6 in HeLa cells. All three core proteins colocalized within the aggregates formed by NS2, indicating that the BTV core proteins were interacting with NS2 (Fig. 4A). To further confirm a direct interaction of NS2 with the BTV RNA-dependent RNA polymerase VP1, recombinant NS2 and His-tagged VP1 (VP1_{his}), both purified from insect cells infected with recombinant baculoviruses, were incubated together prior to an immunoprecipitation assay using a rabbit anti-NS2 antiserum. To detect if VP1 was indeed precipitated using the NS2 antibody, the complex was analyzed by Western blot analysis using an antipolyhistidine antibody.

The coimmunoprecipitation of VP1_{his} with NS2 is shown in Fig. 4B. Precipitated NS2 was visible in the Coomassie blue-stained gel (Fig. 4B, lane 1), and VP1 was coprecipitated only if NS2 was present in the assay (Fig. 4B, lanes 1, 2, 5, and 6). These results were not affected by the presence of RNase A. This is particularly important since both NS2 and VP1 are known to bind to RNA and thus, coprecipitation of both proteins could have occurred via RNA binding activities rather than direct protein-protein interaction. A comparison of coprecipitated amounts of VP1_{his} in the presence or absence of RNase A showed that equivalent amounts of VP1_{his} were coprecipitated in both cases (data not shown) and thus confirmed that RNA was not involved in the formation of VP1-NS2 complexes.

However, it was possible that the phosphorylation of NS2 might have some effect on binding to VP1. To investigate this, a coprecipitation assay was performed as described above using the substitution mutants NS2_{2A} and NS2_{2D}. In both cases, the ability of the NS2 mutants to coprecipitate VP1_{his} was comparable to that of the wild-type protein (Fig. 4B, right panel). The VP1_{his} signals after coprecipitation with the NS2 mutants appear to be weaker, but also the precipitated amounts of NS2 mutants were weaker in the Coomassie blue-stained gel shown in Fig. 4B than the wild-type NS2. These results revealed that the phosphorylation of NS2 is not needed for the interaction with VP1.

Phosphorylation of NS2 and its oligomerization state. To investigate the influence of phosphorylation of NS2 on its oligomerization state, two additional expression vectors were constructed, each expressing a NS2 protein fused with a FLAG epitope (DYKDDDDK) at its C terminus. One contained the wild-type NS2 sequence (NS2f) and the other the NS2_{2A} sequence (NS2_{2A}f). When the NS2f fusion protein was coexpressed in the presence of [35 S]methionine together with the untagged NS2 in 293T cells, the fusion protein had an altered migration pattern in SDS-10% PAGE compared to that of untagged NS2 (Fig. 5A, lane 2). Using the anti-FLAG anti-

FIG. 4. Interaction of NS2 with BTV core proteins. (A) Colocalization of NS2 with VP1, VP4, and VP6 in transfected HeLa cells at 40 h posttransfection. VP1, VP4, and VP6 were visualized by using a TRITC-conjugated antibody and NS2 by using a FITC-conjugated antibody. (B) Coimmunoprecipitation of VP1_{his} with NS2 or NS2 mutants. Proteins were subjected to SDS-10% PAGE and detected by Coomassie blue staining (left panel) or by Western blotting (right panel) using an antipolyhistidine antibody. In a control reaction, no NS2 (–) or NS2 mutant was added in the assay. Numbers on the right are in kilodaltons.

body, the untagged NS2 was not recognized, but when NS2f and NS2 were coexpressed, not only was NS2f sedimented but also the untagged NS2, indicating the formation of complexes consisting of NS2 and NS2f (Fig. 5A, lanes 5 and 6). When the dual NS2 mutant NS2_{2A} was used in the assay together with the fusion protein NS2_{2A}f, the amount of coprecipitated NS2_{2A} (Fig. 5A, lane 7) was considerably lower than that coprecipitated NS2 in lane 6 in Fig. 5A. To gain insight on the level of NS2-NS2 interaction with or without the phosphorylation modification, we quantified the ³⁵S signals of precipitated proteins by using the computer program ImageQuant 5.0. The data obtained scored a ratio NS2_{2A}/NS2_{2A}f of 0.35 and, for NS2/NS2f, a ratio of 0.65. This indicated that the sedimented complexes for wild-type NS2 contained almost twice as much untagged protein as those formed with NS2_{2A}f, suggesting that the wild-type complexes were either of a bigger size or more stable than the mutant complexes (Fig. 5B).

FIG. 5. Immunoprecipitation of NS2 complexes. (A) 293T cells were transfected with plasmids encoding NS2 and NS2f and labeled with [³⁵S]methionine. Proteins were immunoprecipitated with an anti-NS2 serum (lanes 1 through 4) or an anti-FLAG antibody (lanes 5 through 12), separated by SDS-PAGE, and visualized by phosphorimager analysis. RNase A was omitted during immunoprecipitation in lanes 1 through 8 and used in lanes 9 through 12. Transfections of plasmids encoding NS2 (lanes 1, 5, and 9), NS2 plus NS2f (lanes 2, 6, and 10), or NS2_{2A} plus NS2_{2A}f (lanes 3, 7, and 11) or without any insertion (lanes 4, 8, and 12) are shown. (B) Quantification of ³⁵S signals of NS2 and NS2 mutants from lanes 6, 7, 10, and 11 of panel A. Signals were quantified using ImageQuant 5.0 software.

However, in the presence of RNase A in the assay, the ratio of untagged to FLAG-tagged protein was low in both pairs, when either NS2/NS2f or NS2_{2A}/NS2_{2A}f was coexpressed (Fig. 5A and B). Moreover, there was no significant difference in total amounts of coprecipitated proteins between the two pairs. Since the phosphorylated state of NS2 did not affect its RNA binding capability, these results imply that RNA could trigger NS2 oligomerization and that phosphorylation of NS2 probably stabilizes NS2 complexes.

Influence of phosphorylation of NS2 on formation of BTV inclusion bodies. To address the question of whether phosphorylation of NS2 is responsible for the formation of VIBs, we expressed the dual alanine mutant of NS2 (NS2_{2A}) transiently in BHK cells and performed immunofluorescence analysis using confocal microscopy. As a control, the wild-type NS2 was similarly expressed and examined. The expression of NS2 in mammalian cells resulted in the formation of NS2 aggregates similar to the VIBs in infected cells (Fig. 6A). However, the expression of NS2_{2A} did not show this distinct aggregation of the wild-type protein. Instead, NS2_{2A} was predominantly dispersed throughout the cytoplasm. The investigation of NS2 mutants with only one serine residue substitution resulted in intermediate distribution patterns. When just Ser249 was substituted, the NS2_{S249A} mutant predominantly formed aggregates at particular sites but was also partially dispersed throughout the cell. In contrast, when only Ser259 was substituted, the mutant protein was mainly distributed throughout the cytoplasm (Fig. 6A), indicating that the phosphorylation of

FIG. 6. The formation of inclusion bodies by NS2 is impaired when NS2 is not phosphorylated. (A) BHK-21 cells were transfected with plasmids encoding NS2 or NS2 mutants as indicated or infected with BTV-10. Expressed recombinant NS2 and NS2 mutants were visualized using an FITC-conjugated antibody (green). For BTV-10, cells were infected with BTV-10 and NS2 was visualized using a TRITC-conjugated antibody (red). BHK-21 cells were cotransfected with plasmids encoding Flag-tagged NS2 and untagged NS2 (B) or Flag-tagged NS2_{2A} and untagged NS2 (C). BHK-21 cells were transfected with Flag-tagged NS2 (D) or Flag-tagged NS2_{2A} (E). (D and E) At 24 h posttransfection, cells were infected with BTV-10. (B through E) Flag-tagged NS2 was visualized using an FITC-conjugated antibody (green). NS2 or NS2 mutants were visualized using a TRITC-conjugated antibody (red).

Ser259 was particularly critical for the formation of NS2 inclusion bodies. We also analyzed the mutant NS2_{2D}, which showed the formation of intracellular VIBs similar to that of the wild-type protein, indicating that the negative charges on

the phosphoserine residues rather than the serine residues itself were crucial for the formation of VIBs. These results were subsequently verified by performing the same experiments using 293T and HeLa cells. The results were exactly the same as those obtained from BHK cells (data not shown).

It has been reported previously that nonphosphorylated NS2 could oligomerize (8, 42). Further, by coimmunoprecipitation experiments, we have confirmed this (Fig. 5). However, no data are available to date on whether nonphosphorylated NS2 could also assemble into VIBs. To assess this, we have used the Flag-tagged NS2 protein. To establish first that the FLAG epitope itself does not interfere with inclusion body formation, we coexpressed NS2f and NS2 in BHK cells. When visualized by confocal microscopy, both proteins showed a perfect colocalization with NS2-induced inclusions (Fig. 6B). Subsequently, we coexpressed the NS2_{2A}f, the mutant that was no longer phosphorylated, with wild-type NS2 and examined their intracellular distribution. The two expressed proteins were, although predominantly colocalized, almost homogeneously distributed throughout the cytoplasm, indicating that nonphosphorylated NS2 was able to prevent the wild-type NS2 from forming the intracellular inclusions (Fig. 6C).

To investigate further, NS2f or NS2_{2A}f was expressed in BHK cells for 24 h and then the cells were infected with BTV-10. After an additional incubation of 16 h, cells were examined by confocal microscopy. NS2f was found to be colocalized with BTV inclusion bodies (Fig. 6D). Figure 6D shows a transfected as well as infected cell which is surrounded by three cells that are infected only. Neither the size nor the shape of the inclusion bodies was altered by the coexpression of NS2f compared to VIBs found in cells that were infected only. This suggests that the FLAG epitope fused C terminally to NS2 did not have an inhibitory effect on the formation of VIBs. However, when the nonphosphorylated form of NS2, i.e., NS2_{2A}f was expressed prior to a BTV infection, the formation of VIBs was strongly disrupted (Fig. 6E). These results demonstrated that VIBs induced by BTV infection could be perturbed by NS2_{2A}f, the nonphosphorylated form of NS2, substantiating the importance of the phosphorylation of NS2 for VIB formation and, consequently, virus assembly and replication.

DISCUSSION

We have mapped the phosphorylation sites within BTV-10 NS2 to serine residues 249 and 259. In contrast to wild-type NS2, both NS2 mutants, NS2_{2A} and NS2_{2D}, did not act as a substrate for protein kinases. This reveals that NS2 does not undergo hierarchical phosphorylation or alternate phosphorylation as this has been shown for Sendai virus P protein where the abrogation of initial phosphorylation caused phosphorylation on alternate sites (24).

Protein phosphorylation has often been reported as affecting interactions with nucleic acids or other proteins. This has also been shown for proteins of other RNA viruses (13, 18, 19). For NS2, which exhibits a preferential RNA binding activity for BTV plus-strand RNAs over non-BTV RNAs, a correlation of its phosphorylated state and RNA binding properties has been assumed (30). This was based on the fact that NS2 used in experiments that failed to identify an RNA binding specificity was expressed in *Escherichia coli* and consequently not phos-

phorylated (15, 42). However, in this report, we have demonstrated that nonphosphorylated NS2 shows specific RNA binding equivalent to wild-type NS2.

Regarding protein-protein interactions, we showed that NS2 colocalizes intracellularly with the BTV core proteins that are components of the polymerase complex. Further, a direct interaction between VP1 polymerase and NS2 proteins was confirmed by the coimmunoprecipitation of VP1 with NS2. Since both proteins bind ssRNA, the use of high amounts of RNase A ruled out that RNA acted as a link for the coprecipitation of VP1. This was also confirmed by similar amounts of coprecipitated VP1 whether RNA was present or not. However, phosphorylation of NS2 had no influence on the interaction with VP1. In contrast, although phosphorylation had no impact on the interaction between NS2 and VP1, it did affect oligomerization of the protein. The size of NS2 oligomeric complexes made up by NS2 has been estimated to consist of 6 to 11 subunits according to sedimentation experiments, while recently, by using scattering experiments, NS2 expressed in *E. coli* was determined to exist as decamers (8, 42). These bacterially expressed NS2 oligomers were not likely to be phosphorylated. In this report, we found that in the presence of RNA, more wild-type NS2 was coimmunoprecipitated by Flag-tagged NS2 than NS2 mutants, in which phosphorylation was blocked. However, when RNA was degraded, the coimmunoprecipitation of wild-type and mutant NS2 was similar. The reason for these findings is not yet understood. Since we found that the phosphorylation of NS2 did not have any influence on its RNA binding activity, it can be ruled out that a higher level of cosedimentation of wild-type NS2 in the presence of RNA is connected with a more efficient binding of RNA. It is therefore possible that RNA could trigger NS2 oligomerization and that phosphorylation of NS2 could stabilize NS2 complexes. It has been shown that the C terminus of NS2 is not necessary for the oligomerization of the protein and therefore been suggested that the N terminus of NS2 alone drives the self-association of NS2 (8, 48). The basis for both investigations was the use of NS2 deletion mutants lacking the C-terminal domain of NS2. Although up to half of the NS2 was not present in these experiments, including the phosphorylated sites, multimerization of NS2 was clearly demonstrated. Combined with our data, this suggests that the C-terminal half of the protein not only is dispensable for NS2 multimerization but also could perturb the formation of NS2 multimers. From these observations, it can be hypothesized that the phosphorylation of NS2 is involved in the global protein folding in order to stabilize the multimerization of the protein.

Previously, it has been shown that NS2 proteins of other orbiviruses (African horse sickness virus and epizootic hemorrhagic disease virus) are phosphorylated (10, 43). The mapping of the phosphorylation of an orbivirus NS2 is reported here for the first time, with the result that BTV-10 NS2 is phosphorylated within its C terminus on two serine residues. We have shown that NS2 can be phosphorylated by protein kinase CK2 in vitro and additionally that both proteins colocalize intracellularly upon recombinant expression of NS2. The confirmation of NS2 being phosphorylated by CK2 does not permit the conclusion that NS2 is involved in regulated intracellular processes, such as signal transduction pathways, since the parameters of CK2 regulation are still controversial. Although there

are already more than 300 substrates known for CK2, regulation of the kinase has been indicated, but on the other hand, CK2 has been reported to be constitutively active and not regulated (29, 33). An investigation by Zetina, who has analyzed sequence-dependent helix unfolding in proteins, suggested that phosphorylation of the conserved motif (LS/SL)(D/E)(D/E)(D/E)X(D/E) could stabilize helix unfolding (47). The sequence context of phosphorylated serine residue 249 (²⁴⁸LS DDDQ₂₅₄) in NS2 conforms to this conserved sequence except for the last residue. This indicates that phosphorylation of NS2 could be involved in stabilizing protein folding within its C-terminal domain.

Recently it has been shown that another protein from the *Reoviridae* family, rotavirus NSP5, is also phosphorylated within its C terminus and also within CK2 recognition sites (12). Phosphorylation and especially the hyperphosphorylation of NSP5 results in widely variable migration patterns in SDS-PAGE (1), but this is not the case for BTV NS2. By using NS2 expressed from different sources (mammalian cells, insect cells, or bacteria) or NS2 alanine mutants which are not phosphorylated, the migration velocity of the proteins remained the same in denaturing PAGE. NS2 shares a striking similarity with NSP5 in its localization to VIBs (7, 46). NSP5 contributes to the formation of VIBs independent of virus infection, but only when rotavirus NSP2 is present (14). By contrast, the formation of VIBs has been demonstrated by the single expression of NS2 in insect cells infected with a recombinant baculovirus without the necessity of making available other BTV components (44). Here, we found that intracellular inclusions are also formed by the single expression of NS2 in mammalian cells (BHK, 293T, and HeLa).

The question whether NSP5 phosphorylation plays a role in its intracellular localization is still unclear. An influence of NSP5 phosphorylation on localization to VIBs had been suggested but has been contradicted by recent investigations which showed that the deletion of the phosphorylated sites within the protein did not prevent NSP5 from localizing with VIBs in infected cells (12, 17). Also for reoviruses, single proteins (μ NS and σ NS) have been suggested to be responsible for the formation of VIBs (2, 6). However, these proteins are not known to be posttranslationally modified.

We investigated the role of BTV NS2 phosphorylation in VIB formation, and the data presented here conclusively demonstrate that phosphorylation of NS2 is essential for the formation of intracellular aggregates. To confirm further, we generated additional data demonstrating the disruption of VIBs in infected cells when nonphosphorylated NS2 was coexpressed. As the formation of VIBs, the centers for viral replication and early assembly, requires phosphorylated NS2, it is reasonable to assume that newly synthesized, not yet phosphorylated, NS2 might take on other functions prior to its association to VIBs. In this scenario, NS2 binds VP1 and other BTV core proteins in the cytoplasm and upon phosphorylation NS2 recruits these components to the arising VIBs. This hypothesis is consistent with the recent model proposed for rotavirus VIBs (40). The role of NS2 preferential binding of BTV mRNA over nonspecific RNA would be to retain BTV mRNA within VIBs and make them available for viral replication and assembly. In conclusion, it could be hypothesized that NS2 phosphorylation is the driving force for virus assembly.

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