Phosphorylation of Marburg Virus NP Region II Modulates Viral RNA Synthesis

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Phosphorylation of the Marburg virus nucleoprotein NP is distributed over 7 regions (I—VII) in its C-terminus. The exact localization of phosphorylated amino acids and function of NP phosphorylation are unknown. Here, we show that the major phosphate acceptor sites in NP region II are serine 446 and serines 453–455; the latter are located in a cluster of 6 serine residues (aa 450–455). The function of phosphorylation in region II was tested using an infectious virus-like particle assay. Phosphorylation influenced reporter gene activity that reflects viral transcription and replication. An NP mutant mimicking 3 phosphorylated serine residues at position 453–455 supported reporter gene activity better than wild-type NP. Negative charges at positions 450–452 and when the serine cluster was completely substituted by alanine inhibited reporter gene activity significantly. These data support the idea that phosphorylation of NP region II modulates viral RNA synthesis in transcription and/or replication.
of NP into MARV particles [3]. Although most phosphorylated nucleoproteins of *Mononegavirales* have only a few phosphorylation sites, MARV NP has 7 regions that are phosphorylated, and only a few of the phosphorylated amino acids have been mapped [15]. The phosphorylated serine and threonine residues are distributed over the variable C-terminus, whereas the conserved N-terminus of NP up to amino acid (aa) 389 is not phosphorylated [15]. Previously, 7 phosphorylated regions have been determined in the C-terminus of NP; one of which, region II, contains a characteristic cluster of 6 consecutive serine residues and several negatively charged amino acids, which together make this region a prime candidate to be phosphorylated by cellular protein kinases. Here, we show that major phosphate acceptor sites in region II are serines 446 and 453–455. Mutation of these serine residues to either aspartate or alanine influenced the function of NP in transcription/replication. NP activity was prominently down-regulated in the early stages of infection, when all serines of the cluster were replaced by aspartate. Negative effects were also observed when the last 3 serines of the cluster were replaced by alanine.

**METHODS**

**Cell Culture and Virus**

HUH7, HEK293, and HeLa cells were cultured in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum, 5 mM of glutamine, and antibiotics at 37°C and 5% CO2. The vaccinia virus MVA-T7 was grown as described elsewhere [8].

**Construction of NP Mutants**

NPregII (aa 439 to 475 of MARV NP) (EMBL nucleotide sequence database accession no. Z12132) was amplified by polymerase chain reaction and subcloned into pGEM-NP, which had been pretreated with the restriction endonuclease PstI. The resulting construct encoded the nonphosphorylated N-terminus of NP fused to region II (pGEM-NPregII) [15]. Substitutions of single amino acids in pGEM-NPregII (pGEM-NPregII458A, pGEM-NPregII458/475A, NPregIIAS, and NPregIIAA) were obtained by site-directed mutagenesis using pGEM-NPregII as template (Stratagene; Clontech). All clones encoding full-length NP containing mutations in the serine cluster of region II (NPAA, NPDD, NPAD, and NPDA) were generated by using pT-NP as template and site-directed mutagenesis [8]. Resulting NP-encoding DNA was subcloned into the expression vector pCAGGS [12]. Full-length NP constructs contained an N-terminal HA-tag that did not affect NP function or localization (authors’ unpublished data). Detailed cloning strategy and primer sequences are available on request. All constructs have been verified by sequencing.

**MVA-T7 Expression System**

Expression and metabolic labeling of NP in HeLa cells (70% confluent) were performed as described by Lötfering et al [15], except that MVA-T7 was used instead of the vaccinia virus vTF7/3.

**Indirect Immunofluorescence Analysis (IFA)**

HUH7 cells were transfected with 500 ng of pCAGGS NP or NP mutants, VP35 or VP30, respectively, using TransIT (Mirrus). IFA was performed as described elsewhere [16]. NP was stained using guinea pig anti-NP (dilution, 1:50) and TexasRed-labeled goat anti-guinea pig or a monoclonal anti-NP antibody (dilution, 1:50), followed by a rhodamine-labeled goat antimouse antibody. VP35 was stained using a guinea pig anti-VP35 antiserum (dilution, 1:50) and an fluorescein isothiocyanate (FITC)-labeled goat anti-guinea pig antibody. VP30 was detected using a monoclonal anti-VP30 mouse antibody (dilution, 1:100) and a secondary FITC-labeled goat anti-mouse antibody. All secondary antibodies were diluted to 1:100. Nuclei of cells were stained with DAPI (dilution, 1:10 000).

**Metabolic Labeling of NP and Immunoprecipitation**

Labeling of NP with [35S] methionine or [32P] and subsequent immunoprecipitation of radio-labeled proteins was performed as described by Modrof et al [13].

**Phosphoamino Acid Analysis (2-TLC)**

Phosphoamino acid content was determined as described elsewhere [15]. Quantification was done with Raytest Basreader and TINA 2.1 software.

**In Vitro Kinase Assay for Protein Kinase CKII**

HeLa cells were infected with MVA-T7 and transfected with plasmids encoding for NP or NP mutants. At 16 hours p.i., cells were lysed in ChIP buffer [17] (20 mM of Tris-HCl, pH 7.6; 100 mM of NaCl, 5 mM of EDTA, 1% [w/v] NP40, 0.4% [w/v] deoxycholate) in presence of 100 μM of sodium orthovanadate, 4 mg/mL soybean trypsin inhibitor, 10 μg/mL leupeptin, 2.8 mg/mL aprotinin and 500 μg/mL pepstatin on ice for 30 minutes. Lysates were cleared by centrifugation at 13 000 g for 15 minutes, and the supernatants were used for NP-specific immunoprecipitation. Immunoblotted NP or mutants of NP were dephosphorylated at 37°C for 45 minutes using 20 U calf intestinal phosphatase (Roche) in 50 μL of CIP buffer (Roche). Dephosphorylated immobilized proteins were washed 3 times with ice-cold PBS and used for in vitro phosphorylation with recombinant protein kinase CKII (Roche) as described elsewhere [18].

**MARV-Specific Infectious Virus-Like Particle (iVLP) Assay**

Functional analysis of mutated full-length NP was performed using a MARV-specific iVLP assay, as described by Wenigenrath et al, [12]. Producer cells were lysed at 72 hours p.t., and Renilla reporter activity was measured using the Dual Luciferase Assay (Promega). Supernatants of cells containing infectious virus like particles were collected and used for infection of indicator cells that had previously transfected with plasmids encoding the nucleocapsid proteins NP, VP35, VP30, and L. When NP had
Marburg virus nucleocapsid proteins (NP) were expressed in HeLa cells in the presence of [32P] or [35S]methionine. Immunoprecipitation of NP was performed using a polyclonal rabbit antiserum against nonphosphorylated N-terminus of NP (aa 1–389) fused to region II of NP (aa 439–475). The amino acid sequence is shown in 1 letter code. Metabolic labeling of NP and NPregII. HeLa cells were infected with MVA-T7 and transfected at 1 h p.i. with plasmids encoding NP and NPregII. Cells were radiolabeled with [32P] and [35S]methionine. Immunoprecipitation of NP was performed using a polyclonal rabbit antiserum against Marburg virus nucleocapsid proteins (aNC; dilution, 1:500). The radiolabeled proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and detected by bioimage analyzer BAS-1000. Lane 3 shows MVA-T7 infected cell lysate, and lane 4 shows mock-infected cell lysate. Phosphoamino acid analysis of NPregII. HeLa cells were treated as described in B. The radiolabeled proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to Polyvinylidendifluorid membrane and phosphoamino acid analysis (2D-TLC) was performed. Radioactive phosphoamino acids were detected by bioimage analyzer BAS-1000.

RESULTS AND DISCUSSION

Identification and Localization of Phosphate Acceptor Sites in NP Region II

To determine the localization of phosphorylated amino acids in region II, the nonphosphorylated N-terminus of NP was fused to the phosphorylated region II (NPregII) (Figure 1). NPregII was expressed in HeLa cells in the presence of [32P] or [35S]methionine and subsequently immunoprecipitated using NP-specific antibodies and analyzed. NPregII was phosphorylated, supporting previously published results (Figure 1) [15]. Phosphoamino acid analysis of NPregII showed that mainly serine residues were phosphorylated, and a minor signal of phosphothreonine was detected (Figure 1C). Because several protein kinase CKII recognition sites were present in region II (S/TxxD/E), it was tested whether this kinase contributed to phosphorylation of region II. To this end, aspartate 458 and aspartate 475, which are essential residues of the conserved protein kinase CKII recognition sites (455SFVD458 and 472TLDD475 [Figure 2A]), were substituted by alanine and the phosphorylation state of the resulting constructs, NPregII458A and NPregII458/475A, was analyzed. Neither NP mutant showed a significantly weaker phosphorylation signal than NPregII (Figure 2D, lanes 3 and 6). To confirm these results, we investigated whether NPregII could be phosphorylated in vitro by protein kinase CKII (Figure 2C). In contrast to the full-length NP, which is readily phosphorylated by protein kinase CKII, NPregII is not (Figure 2D, lanes 3 and 6). This data indicated that 2 additional protein CKII recognition sites outside region II located in other previously-determined phosphorylated regions of NP (S549 and T417) might serve as phosphate acceptor sites for protein kinase CKII.

The amino acid analysis showed that mainly serine residues were phosphorylated in region II. We replaced all serine residues in the region and analyzed whether the phosphorylation state of the respective mutants was changed. We started with serine 446, which is located close to the serine cluster, and replaced it by alanine (NPregII446A) (Figures 3A and 3B). We found phosphorylation of NPregII446A to be reduced by 40%, compared with NPregII (Figures 3B and 3C). Then, the first 3 serine residues of the cluster were also substituted by alanine (NPregII446AS), which did not reduce phosphorylation significantly in comparison to NPregII446A suggesting that the first 3 serines of the cluster were not phosphorylated (Figure 3). Next, all serines in the cluster except serine 454 were substituted by alanine. Serine 454 was substituted by aspartate (NPregIIAA) to avoid a putative instability of the mutant when all of the anticipated negative charges in the cluster were removed. NPregIIAA showed a phosphorylation signal, which was reduced, compared with NPregII, by >90% (Figure 3C). These data indicated that serines at position 453–455 together with the adjacent serine at position 446 represent the major phosphorylation acceptor sites of NP region II.

Phosphorylation of NP Region II Does Not Influence Inclusion Body Formation and Interaction With VP30 and VP35

NP is the central nucleocapsid protein of MARV. It interacts with itself and the other nucleocapsid proteins, VP35 and VP30, in the process of RNA synthesis and nucleocapsid morphogenesis. To analyze whether phosphorylation of NP region II influenced interaction between nucleocapsid proteins, we constructed full-length NP mutants containing substitutions in region II (Figure 4A). All NP constructs contained a serine-to-alanine substitution at position 446. In addition, either the first 3 serine residues of the cluster were substituted by aspartate...
and the last 3 serine residues by alanine or vice versa to mimic phosphorylated and nonphosphorylated serine residues in the cluster (NP_{AD}, NP_{DA}). In addition, we substituted all serines in the cluster with either alanine or aspartate (NP_{AA}, NP_{DD}). The resultant mutants were coexpressed together with VP35 or VP30, and the intracellular localization of the proteins was analyzed. As expected from previous publications, NP formed intracellular inclusions into which VP35 and VP30 were re-
cruited [2]. All tested mutants of NP behaved like wild-type NP, indicating that phosphorylation of region II is neither required in the formation of NP inclusions nor essential for the re-
cruitment of VP35 and VP30 into inclusions, which is con-
sidered a key step in the morphogenesis of nucleocapsids (data not shown). To confirm these results, we performed coimmunoprecipitation analyses, which also showed that NP self-
interaction and interaction of NP with VP35 was not influenced by phosphorylation of region II (authors’ unpublished data).

Phosphorylation of NP Region II Modulates Viral Transcription of Replication
NP and the mutants of NP were then used in a MARV-specific infectious virus like particle assay to test their function in MARV-specific transcription and replication (Figure 4) [12]. When the mutants of NP were used in this assay, we found that all of them were able to replace NP in viral transcription/re-
lication. Moreover, mutant NP_{AD} significantly increased viral RNA synthesis in comparison with wild-type NP (Figure 4). Because of the expression of all viral proteins in the infectious
virus like particle assay, mininucleocapsids are incorporated into virus like particles that are released into the supernatant of the producer cells [12]. We therefore analyzed whether mutations in region II of NP had impact on the ability of nucleocapsids to be recruited into virus like particles. We found that all mutants were incorporated into virus like particles as efficiently as NP wild-type (Figure 4). The situation in the producer cells reflects late stages of MARV infection, because the intracellular amount of viral proteins and template is high and allows transcription and replication of viral RNA, as well as morphogenesis and budding of virus like particles [12]. Our data indicated that, at these stages of MARV infection, phosphorylation of the serine cluster in the phosphorylated region II is not critical for interaction of NP with itself, the other nucleocapsid proteins, transcription, and replication. Moreover, phosphorylation of region II was not involved in the phosphorylation-dependent recruitment of NP into viral particles that had been described elsewhere [3].

We were then interested in the role of phosphorylation of NP region II during early stages of infection which are characterized by limited amounts of template. To this end, we produced infectious virus like particles that had NP replaced by the phosphorylation mutants of NP. Infectious virus like particles were purified from the supernatant of producer cells and aliquots were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot to confirm that equivalent amounts of particles were produced (data not shown). The infectious virus like particles were then used to infect HUH7 cells (indicator cells) that had been pretransfected with plasmids encoding VP35, L, and NP or the same mutant of NP that was used to produce the respective infectious virus like particles. At 60 hours after infection, cells were monitored for reporter gene activity. In contrast to the results gained with the producer cells, in the indicator cells, all NP mutants (except NPAD) were less effective in supporting minigenome activity (ie, viral RNA synthesis than wild-type NP) (Figure 4). Neither mutants that mimic complete dephosphorylation (NPAA) nor mutants mimicking complete phosphorylation of the serine cluster (NPDD) were able to fully support RNA synthesis like wild–type NP. Infectious virus like particles containing NPAD, however, produced ~1.9 times more reporter gene activity than did infectious virus like particles containing wild-type NP. The effect of NPAD seems to be more pronounced in the indicator cells than in the producer cells, suggesting that the positive effect of NPAD on viral RNA synthesis is additive. This could happen if viral transcription and replication are both influenced by NPAD. Increased replication led to higher amounts of templates in the producer cells and can be recruited by NP into the infectious virus like particles. These particles provide initially more templates for transcription and replication in the indicator cells and produce a higher reporter gene signal. These results are similar to those published for the rabies virus nucleoprotein (N), for which it was shown that changes in phosphorylation had a similar influence on both viral transcription and replication [19].

Figure 3. Localization of phosphorylation acceptor sites within region II. A, Schematic presentation of NPregII substitution mutants NPregII 446A, NPregII AS, or NPregII AA. B and C, Metabolic labeling of NP. HeLa cells were infected with MVA-T7, transfected, and radio-labelled as described under Figure 1. NP and mutants of NP were immunoprecipitated and radioactive signals quantified (n = 4). Phosphosignals were normalized by [35S] methionine signals. Phosphorylation of NPregII was set to 100%.
Figure 4. Function of region II phosphorylation for viral RNA synthesis and recruitment of nucleocapsids. A, Schematic presentation of full-length NP mutants NP<sub>AA</sub>, NP<sub>DD</sub>, NP<sub>AD</sub>, and NP<sub>DA</sub>. Full-length NP carrying alanine and/or aspartate substitutions at the phosphorylation site. B, Scheme of Marburg virus (MARV)-specific iVLP assay using mutants of NP. Plasmids encoding for all viral proteins together with a MARV-specific minigenome (MARV-MG) were transfected into producer cells. NP was replaced by the different phosphorylation mutants, as indicated. Infectious virus-like particles (iVLPs) containing either wild-type NP or the respective NP mutant were used for infection of indicator cells that had been previously transfected with plasmids encoding the viral nucleocapsid proteins and the NP mutant, as indicated. C, Analysis of MARV-specific transcription in producer cells. A MARV-specific iVLP assay was performed. When indicated, wild-type NP was replaced by mutants of NP. At 72 p.t., cells were lysed, and Renilla reporter activity reflecting MARV-specific transcription was measured using Dual Luciferase Assay (Promega). Renilla luciferase activity was normalized against firefly luciferase activity reflecting cellular transcription. Activity of wild-type NP was set to 100 % (n = 13). D, Incorporation of NP mutants in infectious virus-like particles. Generated MARV-specific iVLPs were purified from supernatant of producer cells and submitted to a protease protection assay. NP signals were detected and quantified by Odyssey Infrared Imager (LiCor). Cell lysate was analyzed as expression control. E, Analysis of MARV-specific transcription in indicator cells. A MARV-specific iVLP assay was performed replacing wild-type NP with mutants of NP. Purified iVLPs were used for infection of indicator cells that had been previously transfected with plasmids encoding the nucleocapsid proteins VP35, VP30, and L. NP was again replaced by NP mutants, as indicated in producer cells. At 60 p.i., cells were lysed, and Renilla reporter activity was measured reflecting MARV-specific transcription. Activity of wild-type NP was set to 100 % (n = 3).
Phosphorylation of rabies virus N has been shown to influence the encapsidation of viral leader RNA [20]. The authors showed that non-phosphorylated rabies nucleoprotein binds strongly to viral RNA and simultaneously the polymerase function seems to be inhibited. It is suggested that the strong binding of NP to RNA inhibited access of the polymerase complex to the template. Upon phosphorylation, the binding of NP to the RNA is weakened, probably as a result of repulsion of the negatively charged RNA and the negative phosphate groups; thus, polymerase might access the RNA template to transcribe and replicate the RNA [20]. This hypothesis is strengthened by the fact that the phosphorylated serine residue of rabies virus N (serine 389) is located close to the RNA binding site (aa 289–352). A similar mechanism can be envisaged to explain the role of phosphorylation of region II of MARV NP.

Taken together, we localized the major phosphorylation acceptor sites in region II of MARV NP and showed that phosphorylation of a characteristic serine cluster in the region II modulates efficiency of viral RNA synthesis, especially during early stages of viral life cycle, when the availability of templates is limited.

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**References**


