JVI Journals.ASM.org

Regulation of Influenza A Virus Nucleoprotein Oligomerization by Phosphorylation

Lauren Turrell, Edward C. Hutchinson, Frank T. Vreede, Ervin Fodor
Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

In the influenza virus ribonucleoprotein complex, the oligomerization of the nucleoprotein is mediated by an interaction between the tail-loop of one molecule and the groove of the neighboring molecule. In this study, we show that phosphorylation of a serine residue (S165) within the groove of influenza A virus nucleoprotein inhibits oligomerization and, consequently, ribonucleoprotein activity and viral growth. We propose that nucleoprotein oligomerization in infected cells is regulated by reversible phosphorylation.

The segmented single-strand negative-sense RNA genome of influenza A virus is contained in viral ribonucleoprotein (vRNP) complexes. Within the vRNP, the genomic RNA termini associate with the viral RNA-dependent RNA polymerase while the rest of the RNA is bound to oligomeric nucleoprotein (NP). The oligomeric NP is arranged in a double-helical conformation within the vRNP (1, 2). The RNA polymerase carries out transcription of the genomic viral RNA (vRNA) into mRNA and replication through a cRNA intermediate that serves as a template for the synthesis of vRNA (reviewed in references 3 and 4). NP is essential for both transcription and replication of full-length RNA genome segments by the viral RNA polymerase. However, it is dispensable for the transcription and replication of short model RNA templates (5).

NP is a crescent-shaped protein with a head and a body domain which are separated by a highly basic region that is involved in RNA binding (6, 7). NP can homo-oligomerize by the insertion of the tail-loop of one NP molecule into a groove in the body domain of a neighboring NP molecule (8). NP homo-oligomerization is essential for RNP complex activity on full-length templates (5, 8, 9). Recent work suggested that NP needs to be maintained in a monomeric conformation prior to assembly into vRNPs (10, 11). In particular, it was found that NP readily forms trimers and tetramers in solution and that, although both the monomeric and trimeric/tetrameric forms can bind RNA, only monomers of NP can form oligomers on RNA that resemble oligomeric NP in vRNPs. However, how NP is maintained in a monomeric form prior to assembly into vRNPs remains unclear.

Mapping of the phosphoproteome of influenza A and B viruses identified multiple phosphorylation sites within NP (12). Three sites, S165, S457, and S402/403, were present within the homo-oligomerization domain of NP, and it was hypothesized that phosphorylation at these sites might prevent NP oligomerization by inhibiting the interaction between the tail-loop and groove of neighboring NP monomers. Ribonucleoprotein reconstitution assays suggested that reversible phosphorylation at positions S402/403 and S457 is not essential for RNP complex activity, whereas reversible phosphorylation at position S165 was required (12). A separate study also detected phosphorylation at NP S165 and showed that mutation of S165 to aspartic acid, which mimics constitutive phosphorylation, resulted in predominantly monomeric NP *in vitro* (10). However, the biological significance of this observation remained unclear.

To address the role of phosphorylation at S165 of influenza A virus NP in more detail, we have used a baculovirus system to express recombinant NP. The kinase responsible for the phosphorylation of serine at position 165 has not been identified; therefore, mutations were used to mimic changes in the phosphorylation state. We used the baculovirus system to express NP with an S165A mutation that prevents phosphorylation, or with an S165E mutation or an S165D mutation which mimics constitutive phosphorylation, or wild-type (WT) NP as a control. Recombinant baculoviruses were constructed using a MultiBac system (13) to express influenza A/NT/60/68 (H3N2) virus NP with an N-terminal protein A tag (2 copies) and a tobacco etch virus (TEV) protease cleavage site. Infected lepidopteran Sf9 cells were lysed 36 h postinfection using a lysis buffer containing 150 mM NaCl, 50 mM HEPES (pH 8), 25% glycerol, 0.5% Igepal CA-630 (Sigma), 1 mM β-mercaptoethanol, 1× complete EDTA-free protease inhibitor mixture (Roche), and 1 mM phenylmethylsulfonyl fluoride (PMSF). Recombinant WT and mutant NPs were purified using IgG Sepharose chromatography followed by release performed with TEV protease in a cleavage buffer containing 150 mM NaCl, 1 mM HEPES (pH 8), 10% glycerol, 0.1% Igepal CA-630, 1 mM PMSF, and 1 mM dithiothreitol. To assess the oligomeric state of WT and mutant NPs, the purified material was separated by the use of Superdex S200 chromatography in cleavage buffer (without PMSF) and fractions were analyzed by SDS-PAGE and staining with Coomassie brilliant blue (Fig. 1). WT NP eluted in two peaks. The major peak, eluting at approximately 12 ml, corresponds to higher-order oligomers of about 340 kDa, estimated from the elution profile of size markers. The minor peak, eluting at 17 ml, corresponds to monomeric NP of approximately

Received 12 August 2014 Accepted 26 October 2014
Accepted manuscript posted online 29 October 2014
Citation Turrell L, Hutchinson EC, Vreede FT, Fodor E. 2015. Regulation of influenza A virus nucleoprotein oligomerization by phosphorylation. J Virol 89:1452–1455. doi:10.1128/JVI.02332-14.
Editor: A. García-Sastre
Address correspondence to Frank T. Vreede, frank.vreede@path.ox.ac.uk, or Ervin Fodor, ervin.fodor@path.ox.ac.uk.
Copyright © 2015, American Society for Microbiology. All Rights Reserved.
doi:10.1128/JVI.02332-14

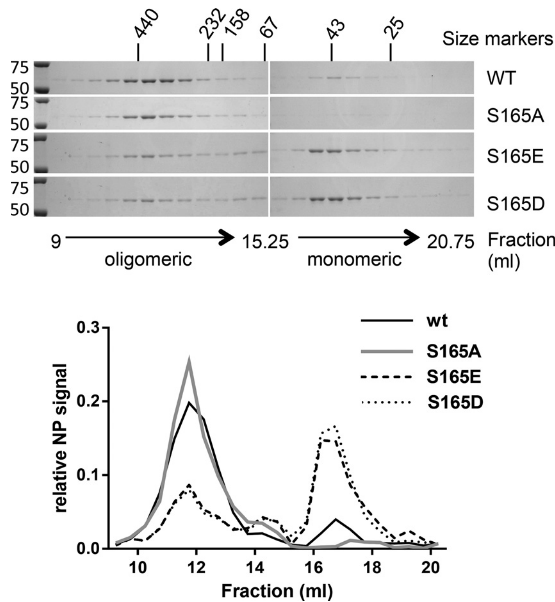

FIG 1 Analysis of WT and mutant NPs by size exclusion chromatography. NP was separated through a Superdex S200 column using approximately equal concentrations for WT and mutant proteins. The fractions were analyzed by SDS-PAGE and Coomassie brilliant blue staining. The positions at which molecular weight marker proteins from low-molecular-weight (LMW) and high-molecular-weight (HMW) gel filtration calibration kits (Amersham Biosciences) were eluted from the column are indicated above the panel (in thousands). NP present in the fractions was quantified by densitometry. The data shown are representative of the results of two independent experiments.

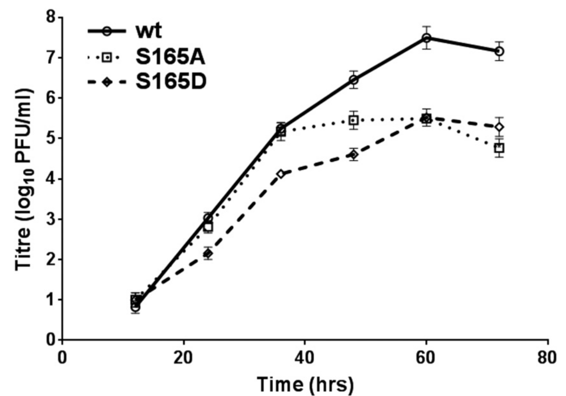

FIG 2 Growth kinetics of recombinant influenza viruses with mutations at position S165 of NP. MDBK cells were infected with WT or mutant viruses at a multiplicity of infection (MOI) of 0.001. The amounts of released virus at the indicated times postinfection were determined by plaque assay on MDBK cells. The means and standard deviations (s.d.) of the results of three independent experiments are shown.

55 kDa. As phosphorylation at serine residues in baculovirus-infected cells is inefficient (14), we assume that the ratio of monomeric NP to oligomeric NP was largely independent of the effects of regulatory phosphorylation. Consistent with this, previous studies of bacterially expressed NP, which should not be phosphorylated, also found most NP present in oligomers but with a small monomeric fraction (7, 11, 15). We next used mutations at S165 to mimic the effects of constitutive phosphorylation or of a lack of phosphorylation. The S165A mutant showed almost no detectable monomers, with most of the protein eluting in the high-molecular-weight fraction (Fig. 1). However, both the S165E and S165D mutants mainly eluted as monomers, with a smaller portion of the protein present in the oligomeric fraction. Comparing the alanine and the phosphomimetic mutants, our results clearly demonstrate that the nature of residue 165 strongly affects the oligomeric state of NP. Both phosphomimetic mutations strongly inhibited oligomer formation, suggesting that phosphorylation would result in predominantly monomeric NP.

To investigate the biological significance of this observation, we attempted to generate recombinant influenza A/WSN/33 viruses carrying mutations at S165 in NP, using a modified 8-plasmid reverse genetics system where the pHW2000-NP plasmid was replaced with either the WT or a mutant pPOLI-NP-RT plasmid and a WT pcDNA-NP plasmid (16–18). Three attempts to generate virus were carried out. In all cases, WT, S165A, and S165D viruses could be generated but not S165E virus. The presence of the desired mutations in the recombinant viruses was confirmed by sequencing. Growth kinetics analysis of the recombinant viruses in Madin-Darby bovine kidney (MDBK) cells revealed that both the S165A and S165D mutants were highly attenuated (Fig. 2). Maximal titers reached by both mutants were reduced by approximately 2 logs, suggesting that neither constitutive phosphorylation nor the absence of phosphorylation at position S165 is compatible with optimal viral growth and that both the nonphosphorylated and phosphorylated forms have a biological role during the viral replication cycle. Interestingly, the two mutants showed differences in the exponential phase of growth; the S165A mutant results resembled those seen with the WT, while the S165D mutant showed a clear reduction. This suggests that the mechanisms of attenuation of these two mutant viruses are different.

To investigate the mechanisms leading to the inhibition of viral growth, we performed RNP reconstitution assays using WT or mutant NPs to address the issue of whether or not these NP mutants can support viral transcription and replication (Fig. 3A and B). We transfected human embryonic kidney 293T cells with expression plasmids to express the three subunits of the RNA polymerase and WT or mutant NP as well as with plasmids to express the PB2, NA, or NS genomic vRNA segments (Fig. 3A). Mutant NPs were shown to be expressed at levels similar to that of the WT (Fig. 3C). Total RNA was isolated at 30 h posttransfection, and the viral mRNA and vRNA levels were analyzed by primer extension (17, 19). We found that mutations S165A, S165E, and S165D all inhibited the accumulation of both mRNA and vRNA (Fig. 3A). These results suggest that reduced transcription and replication are responsible for the attenuated growth of recombinant viruses with the S165A and S165D mutations. The S165E mutation had the most pronounced effect on RNA levels, and, consistently, no infectious virus carrying this mutation could be generated. Interestingly, the inhibitory effect of mutations appeared to be dependent on the length of the template, with the PB2 template showing the strongest inhibition. This suggested that the regulatory effects of phosphorylation could be dependent on the length of the template. In order to investigate this further, we tested the effect of NP mutations on the transcription and replication of short model vRNA templates of 583, 221, and 101 nucleotides (nt) in length that were derived from the NP gene segment by central deletions (Fig. 3B). The 583-nt template was generated by the same method

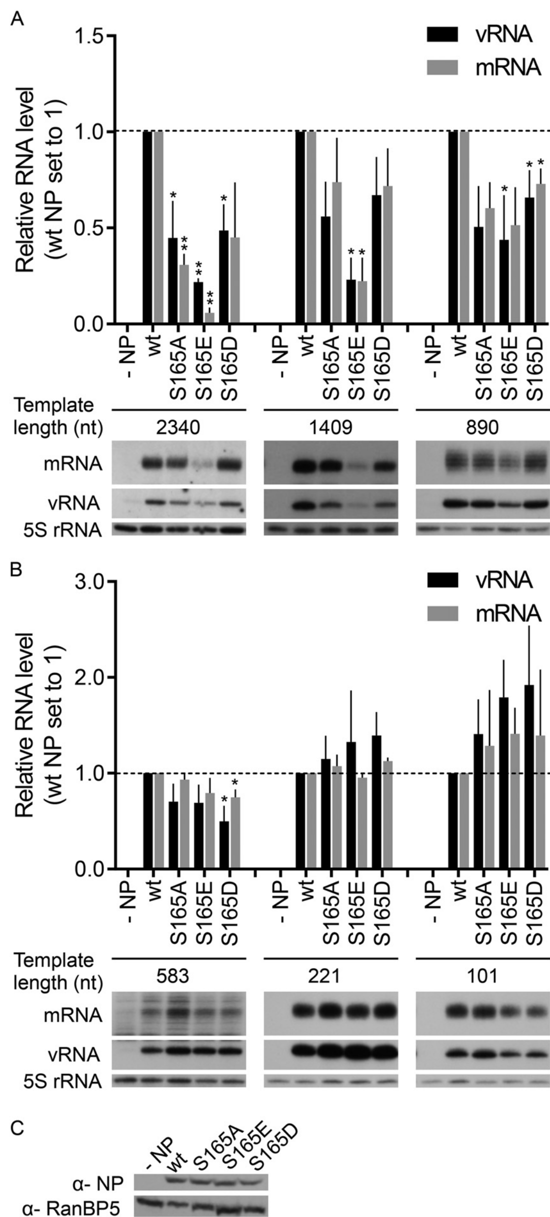

FIG 3 Effect of mutations at S165 of NP on transcription and replication by the viral RNA polymerase. RNP reconstitutions were carried out in 293T cells by transfecting plasmids to express the viral polymerase, a WT or mutant nucleoprotein, and a plasmid to express full-length genomic vRNA segments (PB2, 2,340 nt long; NA, 1,409 nt long; NS, 890 nt long) (A) or model vRNA templates of the indicated length (B). A negative control without NP was included for each template. RNA harvested 30 h posttransfection was analyzed by primer extension as described previously (5, 19). The mRNA and vRNA were quantified by phosphorimage analysis using 5S rRNA as a loading control. The mRNA and vRNA levels were set to 0 in the negative control and to 1 in the presence of WT NP. The means and s.d. of the results of three independent experiments are shown; for each condition, differences from WT were tested using a one-sample *t* test (*, $P < 0.05$; **, $P < 0.01$). (C) To measure the expression of NP in the RNP reconstitutions, cell lysates were analyzed by Western blotting using a rabbit polyclonal anti-NP antibody. RanBP5, detected by a rabbit polyclonal antibody (Santa Cruz), was used as a loading control.

as that previously described for the 221- and 101-nt templates (5). Although transcription and replication of the 583-nt-long template were inhibited in the presence of the S165D mutant compared to WT NP, no inhibition due to NP mutations was observed on the shorter templates (Fig. 3B). Therefore, the S165E and S165D mutants showed the same phenotype as the well-characterized NP oligomerization mutants E339A and R416A, which were able to support the transcription and replication of intermediate-length but not full-length templates (5). The S165A mutant also showed a similar phenotype, although this mutant could form oligomers. We speculate that the unregulated premature oligomerization of this mutant might prevent its efficient recruitment into vRNPs, providing a potential explanation for the observed phenotype.

In summary, we addressed the role of phosphorylation of amino acid residue S165 of influenza A virus NP in this study. Mutation to glutamic or aspartic acid, to mimic phosphorylation, resulted in monomeric NP with a reduced ability to oligomerize. RNP activity was consistently reduced, in a template-length-dependent manner, though it was not completely eliminated. Thus, mutations mimicking a known posttranslational modification change NP activity in a manner characteristic of well-defined oligomerization mutants (5). Considering the effects on viral growth, the S165E mutation was incompatible with infectious virus formation, but S165D, though severely attenuated, could support some viral growth. Taken together, these data suggest that phosphorylation at S165 reduces but may not entirely prevent NP oligomerization. Consistent with the idea that at least a small amount of phosphorylated NP can be incorporated into oligomers, the S165D and S165E mutants could still form some oligomers in spite of being predominantly monomeric (see Fig. 1) and phosphorylation at S165 was originally identified in purified virions and RNP complexes isolated from infected cell lysates (12). As expected, a mutation to alanine which prevented phosphorylation resulted in predominantly oligomeric NP. However, RNP activity and viral growth were still affected, and we hypothesize that uncontrolled premature oligomerization of NP might hamper the efficient recruitment of NP into vRNPs. Indeed, this would be consistent with recent reports that monomeric NP can bind RNA and then form oligomers resembling those in vRNPs but that trimers of NP cannot oligomerize even though they readily bind RNA (10, 11). Taking the data together, we propose that reversible phosphorylation of S165 of influenza A virus NP could be involved in the regulation of NP oligomerization prior to RNP assembly.

ACKNOWLEDGMENTS

We thank Paul Digard for the NP antibody.

This work was supported by Medical Research Council (MRC) grants MR/K000241/1 (to E.F.) and G1100138 (to F.T.V.) and a BBSRC studentship (to L.T.).

REFERENCES

1. **Arranz R, Coloma R, Chichon FJ, Conesa JJ, Carrascosa JL, Valpuesta JM, Ortin J, Martin-Benito J.** 2012. The structure of native influenza virion ribonucleoproteins. Science **338:**1634–1637. http://dx.doi.org/10.1126/science.1228172.
2. **Moeller A, Kirchdoerfer RN, Potter CS, Carragher B, Wilson IA.** 2012. Organization of the influenza virus replication machinery. Science **338:** 1631–1634. http://dx.doi.org/10.1126/science.1227270.
3. **Fodor E.** 2013. The RNA polymerase of influenza a virus: mechanisms of viral transcription and replication. Acta Virol **57:**113–122. http://dx.doi.org/10.4149/av_2013_02_113.
4. **Resa-Infante P, Jorba N, Coloma R, Ortin J.** 2011. The influenza virus RNA synthesis machine: advances in its structure and function. RNA Biol **8:**207–215. http://dx.doi.org/10.4161/rna.8.2.14513.

5. **Turrell L, Lyall JW, Tiley LS, Fodor E, Vreede FT.** 2013. The role and assembly mechanism of nucleoprotein in influenza A virus ribonucleoprotein complexes. Nat Commun **4:**1591. http://dx.doi.org/10.1038/ncomms2589.
6. **Ng AK, Zhang H, Tan K, Li Z, Liu JH, Chan PK, Li SM, Chan WY, Au SW, Joachimiak A, Walz T, Wang JH, Shaw PC.** 2008. Structure of the influenza virus A H5N1 nucleoprotein: implications for RNA binding, oligomerization, and vaccine design. FASEB J **22:**3638–3647. http://dx.doi.org/10.1096/fj.08-112110.
7. **Ye Q, Krug RM, Tao YJ.** 2006. The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA. Nature **444:**1078–1082. http://dx.doi.org/10.1038/nature05379.
8. **Chan WH, Ng AK, Robb NC, Lam MK, Chan PK, Au SW, Wang JH, Fodor E, Shaw PC.** 2010. Functional analysis of the influenza virus H5N1 nucleoprotein tail loop reveals amino acids that are crucial for oligomerization and ribonucleoprotein activities. J Virol **84:**7337–7345. http://dx.doi.org/10.1128/JVI.02474-09.
9. **Elton D, Medcalf E, Bishop K, Digard P.** 1999. Oligomerization of the influenza virus nucleoprotein: identification of positive and negative sequence elements. Virology **260:**190–200. http://dx.doi.org/10.1006/viro.1999.9818.
10. **Chenavas S, Estrozi LF, Slama-Schwok A, Delmas B, Di Primo C, Baudin F, Li X, Crepin T, Ruigrok RW.** 2013. Monomeric nucleoprotein of influenza A virus. PLoS Pathog **9:**e1003275. http://dx.doi.org/10.1371/journal.ppat.1003275.
11. **Tarus B, Bakowiez O, Chenavas S, Duchemin L, Estrozi LF, Bourdieu C, Lejal N, Bernard J, Moudjou M, Chevalier C, Delmas B, Ruigrok RW, Di Primo C, Slama-Schwok A.** 2012. Oligomerization paths of the nucleoprotein of influenza A virus. Biochimie **94:**776–785. http://dx.doi.org/10.1016/j.biochi.2011.11.009.
12. **Hutchinson EC, Denham EM, Thomas B, Trudgian DC, Hester SS, Ridlova G, York A, Turrell L, Fodor E.** 2012. Mapping the phosphoproteome of influenza A and B viruses by mass spectrometry. PLoS Pathog **8:**e1002993. http://dx.doi.org/10.1371/journal.ppat.1002993.
13. **Bieniossek C, Imasaki T, Takagi Y, Berger I.** 2012. MultiBac: expanding the research toolbox for multiprotein complexes. Trends Biochem Sci **37:**49–57. http://dx.doi.org/10.1016/j.tibs.2011.10.005.
14. **O'Reilly DR, Miller LK, Luckow VA.** 1994. Post-translational modification, p 216–236. *In* Baculovirus expression vectors: a laboratory manual. Oxford University Press, Oxford, United Kingdom.
15. **Ye Q, Guu TS, Mata DA, Kuo RL, Smith B, Krug RM, Tao YJ.** 2012. Biochemical and structural evidence in support of a coherent model for the formation of the double-helical influenza A virus ribonucleoprotein. mBio **4:**e00467–12. http://dx.doi.org/10.1128/mBio.00467-12.
16. **Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG.** 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci U S A **97:**6108–6113. http://dx.doi.org/10.1073/pnas.100133697.
17. **Fodor E, Crow M, Mingay LJ, Deng T, Sharps J, Fechter P, Brownlee GG.** 2002. A single amino acid mutation in the PA subunit of the influenza virus RNA polymerase inhibits endonucleolytic cleavage of capped RNAs. J Virol **76:**8989–9001. http://dx.doi.org/10.1128/JVI.76.18.8989-9001.2002.
18. **Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A.** 1999. Rescue of influenza A virus from recombinant DNA. J Virol **73:**9679–9682.
19. **Robb NC, Smith M, Vreede FT, Fodor E.** 2009. NS2/NEP protein regulates transcription and replication of the influenza virus RNA genome. J Gen Virol **90:**1398–1407. http://dx.doi.org/10.1099/vir.0.009639-0.