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Short communication

Addition of an EGFP-tag to the N-terminal of influenza virus M1 protein impairs its ability to accumulate in ND10

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ABSTRACT

A previous report demonstrated that influenza virus infection induces accumulation of EGFP-tagged M1 protein (EGFP-M1) in the sub-nuclear domain ND10. Here, we show that the transfection of four viral protein (NP, PB2, PB1, PA) expression vectors and eight RNA segment expression vectors induced the formation of nuclear dots of EGFP-M1 as seen in virus infections. Omission of the segment 7 RNA expression vector, however, abolished the nuclear dots of EGFP-M1. This result suggests an essential role for authentic M1 protein and/or M2 protein, both of which are encoded in segment 7, in the formation of nuclear dots of EGFP-M1. Co-expression of M1 protein but not M2 protein with EGFP-M1 induced the formation of nuclear dots of EGFP-M1. The dots co-localized with PML protein, which is an indicator of ND10. When only M1 protein was expressed, immunostaining of M1 protein clearly revealed the nuclear dots and their colocalization with PML protein. These results demonstrate that the accumulation in ND10 is an intrinsic characteristic of M1 protein and EGFP addition abolishes this characteristic. The addition of EGFP to M1 protein induced a defect in M1 protein.

Influenza virus M1 protein composes a matrix layer underneath the envelope of the influenza virion and bridges the viral envelope proteins and the ribonucleoprotein (RNP) complex (Harris et al., 2006; Schulze, 1972; Wakefield and Brownlee, 1989). M1 protein is believed to play an important role in the viral budding process, although virus-like particles of influenza virus can form without M1 protein (Bobone et al., 2017; Chen et al., 2007); and M2 protein, which is produced by alternative splicing of the same RNA segment from which M1 protein is produced, has been shown to play a crucial role in the budding process (Chen et al., 2007; Leser and Lamb, 2017; Wang et al., 2010). The exact role of M1 protein in the budding of influenza virus requires further investigation. M1 protein plays another important role during RNP export from the nucleus (Bui et al., 2000; Noton et al., 2007; Ye et al., 1999). The binding of M1 proteins to the newly formed RNP complexes in the nucleus induces the export of RNP to the cytoplasm with the aid of NS2 (nuclear export protein, NEP), which is associated with M1 protein (Elton et al., 2001; Watanabe et al., 2014; Yasuda et al., 1993). In the nucleus, M1 protein also regulates the transcription of viral genes (Elster et al., 1997; Watanabe et al., 1996).

Previous reports have also suggested another role for M1 protein, involving the colocalization of M1 protein with the promyelocytic leukemia (PML) protein in influenza virus-infected cells (Halder et al., 2013; Sato et al., 2003; Shibata et al., 2009). PML protein is a major component of the sub-nuclear structure ND10 (nuclear domain 10), also called PML oncogenic domains (PODs) or PML nuclear bodies (PML-NBs). In constitutively expressing cells, influenza virus M1 protein tagged with EGFP at its N-terminal (EGFP-M1) is distributed uniformly throughout the cytoplasm and nucleus. When these cells were infected with influenza virus, accumulation of EGFP-M1 protein in ND10 was induced (Sato et al., 2003). The accumulation of authentic M1 proteins in ND10 in influenza virus-infected cells was also confirmed (Shibata et al., 2009). These results suggest that M1 protein localizes to ND10 with the help of other viral proteins and is involved in the function of ND10.

Several proteins from various viruses have been reported to localize to ND10 in virus-infected cells (Blondel et al., 2002; Everett and Maul, 1994; Florin et al., 2002; Herzer et al., 2005; Korioth et al., 1996; Hofmann et al., 2000), and it is widely accepted that ND10 is involved

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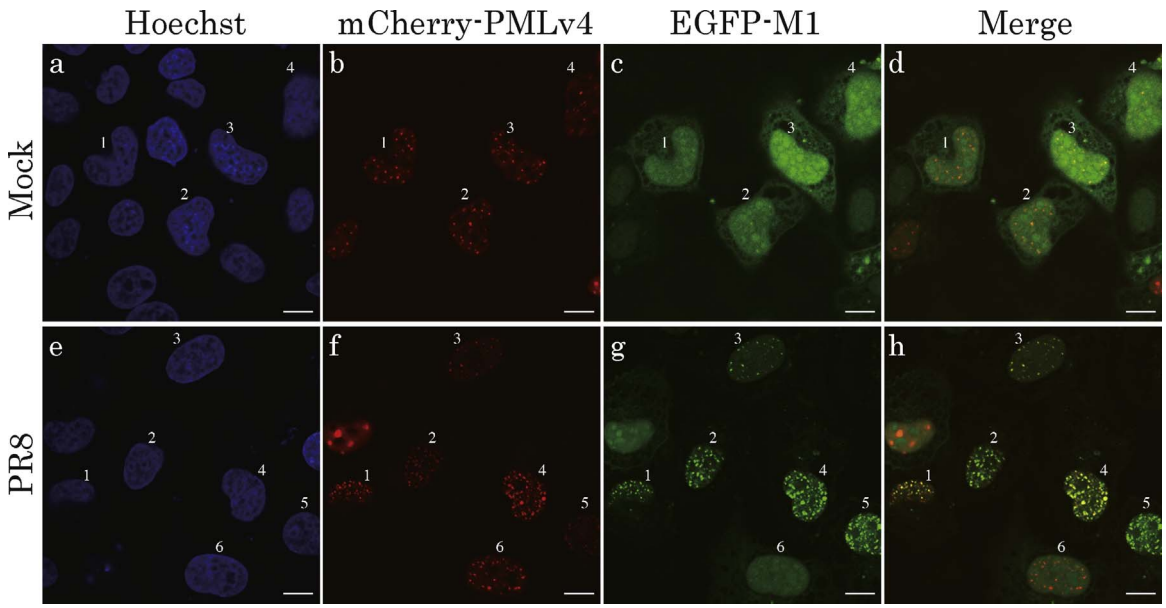

Fig. 1. Induction of the co-localization of EGFP-M1 protein with PML protein by influenza virus infection.
HeLa cells were transfected with 75 ng of pEGFP-C1/M1 and 50 ng of pmCherry-PML plasmids. At 12 h post-transfection, the cells were infected with A/PR/8/34 (H1N1) at an moi of 10 (e, f, g, h) or mock infected (a, b, c, d). At 10 h post-infection, the cells were fixed with 4% paraformaldehyde and stained with Hoechst 33342 (a, e). Fluorescence images were obtained using a FV-1000 confocal microscope (Olympus). Merged images of mCherry-PMLv4 and EGFP-M1 are also shown (d, h). Scale bar = 10 μm. To analyze colocalization of mCherry-PMLv4 and EGFP-M1, Pearson's correlation coefficients of nucleic regions were calculated. Nuclei of infected cells (e, f, g, h): 1: 0.89, 2: 0.886, 3:0.877, 4:0.878, 5: 0.862, 6: 0.367; mock-infected cells (a, b, c, d): 1: 0.331, 2: 0.238, 3: 0.340, 4: 0.271.

in the viral infection process (Sourvinos and Everett, 2002; Xu et al., 2016). When these viral proteins are expressed alone, most of them show the same localization pattern as that in virus-infected cells, accumulating in ND10. These previous studies often used fluorescence protein-tagged proteins. In this report, we show that influenza virus M1 protein also localizes to ND10 without the aid of other viral proteins. This characteristic, however, was impaired by the addition of an EGFP-tag, in contrast to other viral proteins that localize to ND10.

As previously shown in constitutively expressing MDCK cells (Sato et al., 2003; Shibata et al., 2009), influenza virus infection induced the redistribution of EGFP-M1 protein and its accumulation in ND10 in cells transiently expressing EGFP-M1 protein (Fig. 1). When an EGFP-M1 protein expression plasmid (pEGFP-C1/M1) was transfected into HeLa cells, EGFP-M1 protein was found to be distributed in both the cytoplasm and nucleus, similar to the patterns in cells constitutively expressing EGFP-M1 protein (Fig. 1c) and influenza infection induced the nuclear punctate fluorescence (Fig. 1g). The redistribution of transient expressed EGFP-M1 protein in influenza virus-infected cells was confirmed in MDCK, HeLa, and 293T cells. All strains of influenza virus examined thus far, including A/PR/8/34 (H1N1), A/WSN/33 (H1N1), and A/Udorn/307/72 (H3N2), induced accumulation of EGFP-M1 protein in ND10. It should be noted that the number and size of ND10 slightly differed depending on the infected virus strain. However, colocalization of EGFP-M1 protein and PML protein was always observed. The morphological difference of ND10 might be caused by differences in intracellular immunity against various influenza strains, considering the change in ND10 morphology induced by interferon treatment (Everett and Chelbi-Alix, 2007).

Influenza virus infection caused punctate distribution of EGFP-M1 protein in the nucleus (Fig. 1g). The change in the distribution pattern of transiently expressed EGFP-M1 protein was associated with progression of the infection. In the early stage of infection, which is indicated by restricted presence of NP protein in the nucleus, EGFP-M1 protein did not show a punctate distribution (Fig. 2, cell 1), similar to that in non-infected cells, as indicated by the absence of NP protein throughout the cytoplasm and nucleus (Fig. 2, cell 3). It is well known that NP protein is located in the nucleus at the early stage of influenza virus infection and is then located both in the cytoplasm and nucleus in the late stage of infection (Martin and Helenius, 1991). In contrast, in the cells at the late stage indicated by the distribution of NP protein both in the cytoplasm and nucleus, nuclear punctate distribution of EGFP-M1 protein was observed (Fig. 2, cell 4). In cells showing a faint presence of NP protein in the cytoplasm, only a few nuclear dots of EGFP-M1 protein were observed (Fig. 2, cell 2). To confirm that the punctate fluorescence of the EGFP-M1 protein corresponded to ND10, HeLa cells were cotransfected with a pEGFP-C1/M1 plasmid and an mCherry-tagged PML variant 4 protein (mCherry-PMLv4) expression plasmid (pmCherry-PML). As expected, mCherry-PMLv4 protein showed a punctate distribution in the nucleus regardless of influenza virus infection (Fig. 1b, f), but infection induced a change in EGFP-M1 protein distribution, from a uniform distribution to nuclear dots of EGFP-M1 that colocalized with mCherry-PMLv4 (Fig. 1h). Pearson's correlation coefficients between mCherry-PMLv4 and EGFP-M1 proteins in nuclear regions were calculated for mock-infected cells and infected cells (legend of Fig. 1). The average value for infected cells was 0.793 and the value for mock-infected cells was 0.295, which clearly showed the induction of colocalization by the influenza virus infection.

On the basis of these results, we hypothesized that M1 protein accumulated in ND10 with the help of viral proteins other than M1. To test this hypothesis, we investigated the influence of viral proteins on the distribution of EGFP-M1 protein, using a reverse genetics system. When 293T cells were transfected with eight plasmids (pPol I-seg1-8), each of which produces one of eight segments of the influenza virus RNA genome, together with five expression plasmids for EGFP-M1 protein and three subunits (PB1, PB2, PA) of RNA polymerase and NP protein, punctate fluorescence of EGFP-M1 protein in the nucleus was observed (Fig. 3a). In contrast, no nuclear dots of EGFP-M1 protein were observed in 293T cells transfected only with the five expression plasmids for EGFP-M1, PB1, PB2, PA, and NP proteins (Fig. 3b). This result suggests that the capsid protein of NP and RNA polymerase comprised of PB1, PB2, and PA did not affect the distribution of the EGFP-M1 protein.

We hypothesized that the proteins coded in segments 4, 6, 7, and 8 would contribute to the redistribution of EGFP-M1 protein. We focused