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NOTE

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Defect of rabies virus phosphoprotein in its interferon-antagonist activity negatively affects viral replication in muscle cells

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ABSTRACT. Attenuated derivative rabies virus Ni-CE replicates in muscle cells less efficiently than does the parental pathogenic strain Nishigahara. To examine the mechanism underlying the less efficient replication of Ni-CE, we compared the activities of Ni-CE and Nishigahara phosphoproteins, viral interferon (IFN) antagonists, to suppress IFN-β promoter activity in muscle cells and we demonstrated a defect of Ni-CE phosphoprotein in this ability. Treatment with an IFN-β-neutralizing antibody improved the replication efficiency of Ni-CE in muscle cells, indicating that produced IFN inhibits Ni-CE replication. The results indicate the importance of IFN antagonism of rabies virus phosphoprotein for viral replication in muscle cells.

KEY WORDS: interferon antagonist, muscle cells, phosphoprotein, rabies virus

Rabies is a viral zoonotic disease associated with severe neurological symptoms and a high case fatality rate of almost 100%. The etiological agent, rabies virus (RABV), belongs to the genus *Lyssavirus* of the family *Rhabdoviridae*. The genome is an unsegmented negative-sense genomic RNA encoding five structural proteins: nucleoprotein (N protein), phosphoprotein (P protein), matrix (M) protein, glycoprotein (G protein), and large (L) protein [19]. The P protein, together with the N and L proteins and genomic RNA, constitutes a ribonucleoprotein (RNP) complex and is involved in viral RNA synthesis as a cofactor of the RNA-dependent RNA polymerase. The P protein also functions as a type I interferon (IFN) antagonist by inhibiting both IFN induction and response [1–3, 6, 9, 14, 17, 18].

RABV is a highly neurotropic virus that spreads along neural pathways from the bite site: the virus infects peripheral nerves and then spreads to the central nervous system (CNS), where it causes an acute infection. While neurons are targets for viral replication in the CNS, muscle cells are known to be targets in peripheral sites [4, 5, 10, 11, 13]. In fact, muscle cells express the nicotinic acetylcholine receptor, which is the first identified RABV receptor [7]. However, despite the fact that several studies have suggested the importance of RABV replication in muscle cells in the pathogenesis of rabies [4, 5, 10, 11, 13], the detailed mechanism by which RABV stably replicates in muscle cells has been unclear.

Recently, we reported that the highly neuroinvasive RABV strain Nishigahara (Ni) stably replicates in muscle cells, whereas the derivative variant Ni-CE with less neuroinvasiveness does not (Fig. 1a) [20]. We also demonstrated that the chimeric mutant CE(NiP), which has the Ni P gene in the genetic background of the Ni-CE, replicates in muscle cells and infects peripheral nerves more efficiently than does Ni-CE. Importantly, infection with Ni-CE induces the *Ifnb* gene and IFN-stimulated genes (ISGs) in muscle cells more strongly than does infection with CE(NiP) [20]. Taken together, these findings suggest that Ni P protein functions to assist viral replication in muscle cells by suppressing IFN induction and consequently enhances infection of peripheral nerves, whereas Ni-CE P protein has a defect in this function. However, we have not directly compared the IFN antagonist activities of Ni and Ni-CE P proteins in muscle cells and therefore there exists no clear evidence that the defect of Ni-CE P protein in IFN antagonist function is responsible for the low efficiency of viral replication in muscle cells. In this study, we sought to determine whether the functional defect of Ni-CE P protein negatively affects viral replication in muscle cells.

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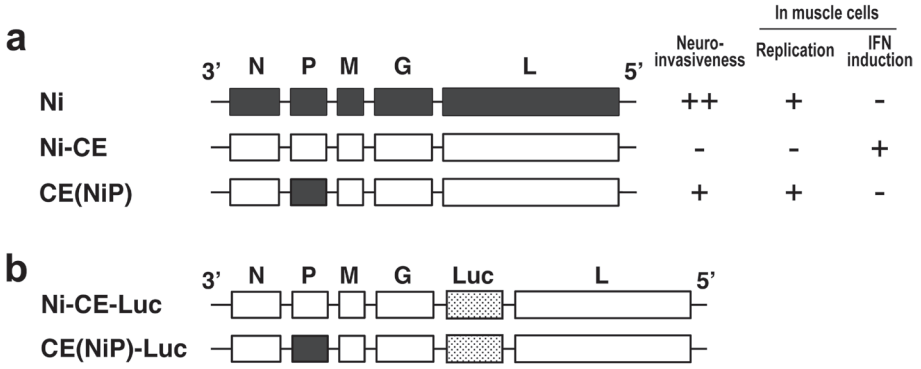

Fig. 1. Schematic diagrams of the genome organizations of Ni, Ni-CE, CE(NiP), Ni-CE-Luc, and CE(NiP)-Luc. The black and white squares represent the viral genes that originate from Ni and Ni-CE, respectively. (a) The abilities of Ni, Ni-CE and CE(NiP) to infect peripheral nerves (neuroinvasiveness), as well as their abilities to replicate and to induce IFN in muscle cells are shown by ++, + or − (see [20] for the detail). (b) Luciferase-expressing RABV used in this study are shown. The dot squares represent the firefly luciferase gene (Luc).

To compare activities of Ni and Ni-CE P proteins to suppress IFN induction, we examined IFN-β promoter activities in cultured muscle cells solely expressing Ni or Ni-CE P protein, using a luciferase-based reporter assay. Mouse muscle myoblast G-8 cells (American Type Culture Collection [ATCC] no. CRL-1456) [20] were transfected with 0.25 μg of pCAGGS-NiP or -CEP plasmid expressing Ni or Ni-CE P protein [8], respectively, together with 0.25 μg of IFNB-pGL3 plasmid encoding a firefly luciferase gene downstream of the IFN-β promoter (kindly provided by Rongtuan Lin) and 0.04 μg of pRL-TK plasmid (Promega, Madison, WI, U.S.A.), which expresses the *Renilla* luciferase. At 24 hr post-transfection, the IFN-β promoter was stimulated for 6 hr by transfection of 0.5 μg of a double-stranded RNA homolog, poly (I:C), which is known as a type I IFN inducer. One-way analysis of variance (ANOVA) with Tukey's multiple-comparison test was used to determine statistical significance. It was shown that luciferase activity was clearly increased by poly (I:C) stimulation in cells transfected with an empty vector (pCAGGS/MCS [8]) but the activity was significantly lower in cells expressing Ni P protein (P<0.05) (Fig. 2a). Expression of Ni-CE P protein also inhibited IFN-β promoter-controlled luciferase expression. However, we found that the inhibition in cells expressing Ni-CE P protein was significantly weaker than that observed in cells expressing Ni P protein (P<0.05). Western blotting of cell lysates prepared for the reporter assay demonstrated that the expression level of Ni-CE P protein was comparable to that of Ni P protein (Fig. 2b). These results indicate that, compared to Ni P protein, the function of Ni-CE P protein to suppress IFN induction is diminished in muscle cells.

Next, to know whether the IFN produced by muscle cells infected with Ni-CE inhibits its replication, we check the effect of neutralization of IFN on the replication of Ni-CE in muscle cells. Muscle G-8 cells were infected with a recombinant Ni-CE or CE(NiP) expressing firefly luciferase (Ni-CE-Luc or CE(NiP)-Luc, respectively [20]) (Fig. 1b) at a multiplicity of infection (MOI) of 1. Then the

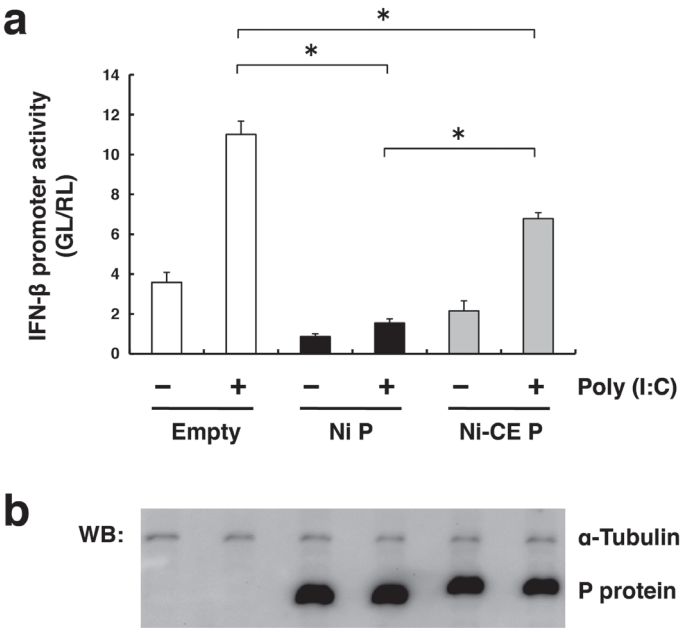

Fig. 2. IFN-β promoter activities in muscle cells solely expressing Ni or Ni-CE P protein. (a) G-8 cells in a 24-well plate were cotransfected with an IFN-β reporter plasmid (IFNB-pGL3) and a control plasmid (pRL-TK) and each plasmid driving the expression of the indicated viral protein or an empty vector. At 24 hr post-transfection, the cells were also transfected with poly (I:C). Six hr later, the cell lysates were subjected to a dual luciferase assay (Promega). The data represent firefly luciferase activity (GL) normalized to *Renilla* luciferase activity (RL). All assays were carried out in triplicate, and the values in the graph are means ± standard errors of the means. *, Significant difference (P<0.05). (b) Western blot (WB) analysis of the cell lysates used for the luciferase assay was carried out using an anti-P protein rabbit serum (kindly provided by Akihiko Kawai) and anti-monoclonal anti-tubulin antibody (Sigma, St. Louis, MO, U.S.A.).

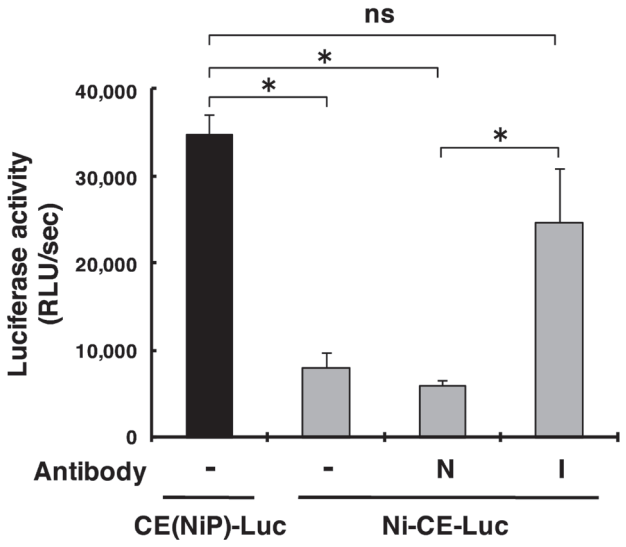

Fig. 3. The replication of Ni-CE in IFN-neutralized muscle cells. G-8 cells in a 24-well plate were infected with Ni-CE-Luc or CE(NiP)-Luc at an MOI of 1 and then cells infected with Ni-CE-Luc were treated with 10 μg of normal rat IgG or rat anti-IFN antibody. The cells were lysed at 3 dpi by using 100 μl of cell culture lysis reagent (Promega), and 10 μl of each was used for analyses of luciferase activities (calculated as RLU/sec) by the Luciferase assay system (Promega). -, no treatment; N, normal rat IgG; I, rat anti-IFN antibody. All assays were carried out in triplicate, and the values in the graph are means ± standard errors of the means. *, Significant difference (P<0.05); ns, Not significant (P≥0.05).

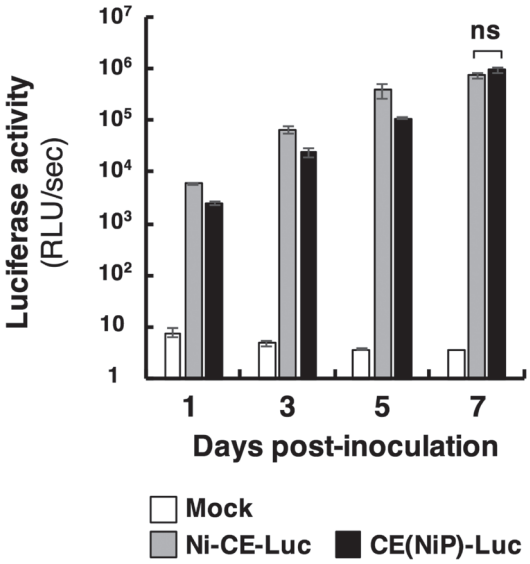

Fig. 4. The replication of Ni-CE in IFN-deficient BHK cells. BHK cells in a 24-well plate were infected with Ni-CE-Luc or CE(NiP)-Luc at an MOI of 0.1. The cells were lysed at 1, 3, 5 and 7 dpi by using 100 μl of cell culture lysis reagent (Promega), and 10 μl of each was used for analyses of luciferase activities (calculated as RLU/sec) by the Luciferase assay system (Promega). All assays were carried out in triplicate, and the values in the graph are means ± standard errors of the means. ns, Not significant (P≥0.05).

Ni-CE-Luc-infected cells were treated for 3 days with 10 μg of a rat monoclonal antibody against mouse IFN-β, RMMB-1 (PBL Assay Science, Piscataway, NJ, U.S.A.), to neutralize IFN in the culture medium of G-8 cells. As expected, the luciferase activity in cells infected with Ni-CE-Luc was significantly lower than that in cells infected with CE(NiP)-Luc in the absence of antibody, as determined by ANOVA with Tukey's multiple-comparison test (P<0.05) (Fig. 3). In contrast, the activity in cells infected with Ni-CE-Luc was increased by treatment with the anti-IFN antibody to a level similar to that in cells infected with CE(NiP)-Luc (P≥0.05), which was not observed by treatment with normal rat IgG (Santa Cruz Biotechnology, Dallas, TX, U.S.A.). These results clearly indicate that the host IFN system inhibits the replication of Ni-CE in muscle cells. In order to further evaluate the effect of the IFN system on replication efficiency of Ni-CE, we checked the replication of Ni-CE in baby hamster kidney (BHK) cells, which are known to have a deficit in IFN-α/β production [16]. We inoculated BHK cells with Ni-CE-Luc or CE(NiP)-Luc at an MOI of 0.1 and examined the luciferase activity in cells at 1, 3, 5 and 7 days post-inoculation (dpi). The luciferase activities in both BHK cells infected with Ni-CE-Luc and CE(NiP)-Luc similarly increased over time (Fig. 4). Notably, there was no significant difference in the activities between Ni-CE-Luc and CE(NiP)-Luc-infected cells at 7 dpi, as determined by ANOVA with Tukey's multiple-comparison test (P≥0.05). This result indicates that Ni-CE grows similarly to CE(NiP) in the absence of an intact IFN system. Based on the findings described above, it is concluded that the defect of Ni-CE P protein in its activity to suppress IFN induction is responsible for the low replication efficiency of Ni-CE in muscle cells.

In this study, we showed that the inhibition of IFN-β promoter in muscle cells expressing Ni-CE P protein was significantly weaker than that observed in cells expressing Ni P protein. In contrast, it was shown in our previous study that in neuroblastoma SYM-I cells, Ni and Ni-CE P proteins equally inhibited the activation of IFN-β promoter induced by infection with Newcastle disease virus [8]. These findings suggest that the functional deficiency of the IFN antagonism of Ni-CE P protein is cell type-dependent. This interference of the IFN-antagonistic function of Ni-CE P protein in muscle cells might be due to the interaction of Ni-CE P protein with cellular protein(s) that is expressed specifically in muscle cells.

We previously reported that Ni-CE P protein has five amino acid substitutions at positions 56, 58, 66, 81 and 226 [15], indicating that these substitutions in Ni-CE P protein cause the defect in its IFN antagonist function. Interestingly, we demonstrated very recently that, in addition to full-length Ni P protein, the N-terminally truncated P protein isoforms (P2, P3, P4 and P5), which are expressed from in-flame start codons encoding methionine residues at positions 20, 53, 69 and 83, respectively, have activities to antagonize IFN induction in muscle cells [12]. Since at least one amino acid substitution is included in all the isoforms of Ni-CE, it will be valuable to examine whether the substitutions diminish their IFN antagonist activities in muscle cells.

Although we only showed the inhibitory effect of P protein on IFN induction in this study, previous studies have demonstrated

that RABV P protein also functions to inhibit cellular signaling pathways for IFN response [3, 6, 17]. Moreover, we previously reported that Ni P protein blocked IFN response in neuroblastoma SK-N-SH cells more efficiently than did Ni-CE P protein [6]. Therefore, it is possible that Ni P protein also antagonizes IFN response more efficiently than does Ni-CE P protein in muscle cells. Further experiments are needed to evaluate P protein's function to inhibit IFN response in muscle cells.

In the present study, we clearly showed that the defect of RABV P protein in its activity to suppress IFN induction impairs viral replication in muscle cells, indicating the importance of IFN antagonism of RABV P protein for viral replication in muscle cells. Moreover, the results of this study, in addition to our previous findings [20], strongly suggest that the above process involving P protein ultimately enhances the infection of peripheral nerves. It will be important for future studies to use IFN-α/β receptor (IFNAR) knockout (KO) mice, in order to test whether Ni-CE intramuscularly inoculated into these mice shows the ability to infect peripheral nerves followed by an efficient replication in muscle cells. We believe that the information obtained in this study will be useful for the establishment of a live rabies vaccine strain with a high level of safety and also for the development of novel prophylactic approaches for rabies.

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