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Short communication

In vitro inhibition of the replication of classical swine fever virus by capsid-targeted virus inactivation

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ABSTRACT

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), a highly contagious fatal disease of swine. Few effective antiviral drugs are currently available against CSFV infections. To explore the feasibility of using capsid-targeted viral inactivation (CTVI) as an antiviral strategy against CSFV infections, we expressed the CSFV capsid protein (Cap) fused with the nuclease of *Staphylococcus aureus* (SN) in *Escherichia coli* and investigated its effects on the replication of CSFV in PK-15 cells. The results indicated that the fusion protein Cap-SN showed a strong Ca^{2+}-dependent nuclease activity and inhibited the replication of CSFV in a dose-dependent manner, with complete inhibition at a concentration of 15 μg/ml, whereas the Cap fused with an enzymatically inactive SN (Cap-SN*) showed no nuclease activity or antiviral effects. Thus, the CTVI approach might be applicable to CSFV inhibition as a novel antiviral strategy.

Classical swine fever (CSF) is a highly contagious fatal disease of pigs caused by classical swine fever virus (CSFV) belonging to the *Pestvirus* genus within the *Flaviviridae* family (ICTV, 2006). CSF outbreaks often lead to extensive epidemics and severe economic losses (Moennig, 2000), disrupting trade of pigs and swine products. Vaccination with C-strain-based vaccines has been carried out in many developing countries, yet immunization failure occurs occasionally due to improper vaccination schedule. Moreover, vaccination with conventional vaccines may result in difficult differentiation between infected and vaccinated animals (Beer et al., 2007) while few efficacious marker vaccines against CSF are currently commercially available (Beer et al., 2007). Also, few drugs are currently available for the control of CSFV infections (Vrancken et al., 2008, 2009). Thus, seeking a novel control strategy for CSFV infections is urgently necessary.

Capsid-targeted viral inactivation (CTVI) emerges as a powerful antiviral strategy termed "intracellular immunization", which introduces deleterious enzymes, such as nucleases, into outgoing viral particles during assembly by means of capsid fusion protein (Natsoulis and Boeke, 1991). The nuclease chosen as the effector molecule in CTVI is generally a protein enzyme, which is catalytically more efficient than a ribozyme (Qin et al., 2005). The principal feasibility of CTVI has been thoroughly investigated in the experimental system for several viruses, such as murine leukemia virus (Schumann et al., 1996, 1997, 2001; VanBrocklin et al., 1997; VanBrocklin and Federspiel, 2000), hepatitis B virus (Beterams and Nassal, 2001; Liu et al., 2003) and human immunodeficiency virus type 1 (HIV-1) (Okui et al., 2001). These studies showed that CTVI is specific and efficient and could be developed as antiviral drugs. To develop a new antiviral strategy of CTVI against CSFV infections, we fused the *Staphylococcus aureus* nuclease (SN) gene to the capsid protein (Cap) gene of CSFV as the CTVI effector and investigated its effects on the production of infectious virions when introduced into PK-15 cells infected with CSFV.

CSFV Shimen strain was used to amplify the Cap gene, and *S. aureus* HRB strain (a gift of Dr. Si-Guo Liu) was used to amplify the SN gene. A fusion gene Cap-SN was constructed encoding the SN fused to the CSFV Cap with a C-terminal 6× His-tag (Fig. 1A). Additionally, an N-terminal HIV-1 Tat protein transduction domain (PTD) has been introduced to increase transport efficiency, as has been demonstrated for various heterologous proteins or peptides into various tissues *in vitro* and *in vivo* (Schwarze et al., 1999; Wheeler et al., 2003). As a control, Cap-SN* gene encoding a fusion protein of the CSFV Cap and an enzymatically inactive mutant of SN (a double-site E43S and R87G mutant version of SN) (Fig. 1B) was obtained by site-directed mutagenesis of Cap-SN as described previously (Schumann et al., 1996; Weber et al., 1991). The fusion genes Cap-SN and Cap-SN* were cloned into pProEXHTc (Invitrogen), resulting in pProEXHTc-Cap-SN and pProEXHTc-Cap-SN* (Fig. 1). The His-tagged recombinant fusion proteins Cap-SN and Cap-SN* were expressed by induction of the *Escherichia coli* DH5α cells transformed with the resulting plasmids and probed by anti-His-tag antibody by Western blot. As expected, the fusion proteins of approximately 35.6 kDa were detected (data not shown).

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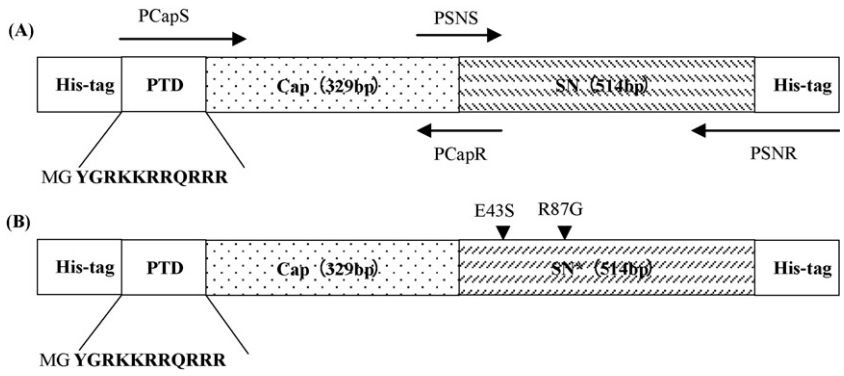

Fig. 1. Schematic diagram of the constructs encoding Cap-SN (A) and Cap-SN* (B). PCapS/PCapR, primers for amplifying Cap gene; PSNS/PSNR, primers for amplifying SN gene; PTD, protein transduction domain of HIV-1 Tat; Cap, capsid protein of CSFV; SN, staphylococcal nuclease; His-tag, 6× His-tag. Double mutations (E43S and R87G) are indicated as arrowheads.

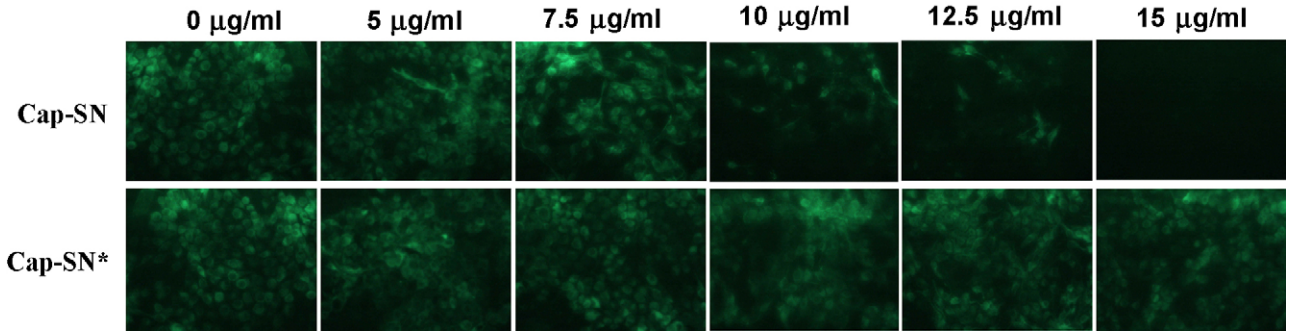

Fig. 2. Inhibition of CSFV replication in PK-15 cells by the fusion proteins detected by immunofluorescence assay. The PK-15 cell cultures in a 96-well microplate were infected with 100 $CCID_{50}$ of CSFV Shimen strain for 1 h followed by replacement with fresh medium. The fusion proteins of indicated final concentrations were added to the wells. After 60 h incubation, the cells were harvested and subjected to detection of viral antigens by immunofluorescence assay as described elsewhere (OIE, 2004).

The antiviral effect of CTVI strategy is dependent on nuclease degradation of viral nucleic acids. To verify the nuclease activity of the fusion proteins, we used an *in vitro* DNA digestion assay. Moreover, the Ca^{2+}-dependence of their nuclease activity was assayed in the presence of various Ca^{2+} concentrations. The results demonstrated that the fusion protein Cap-SN exhibited a strong nuclease activity in a dose-dependent manner, with peak activity at the concentration of 0.2 mg/ml, whereas the mutant Cap-SN* did not show any nuclease activity. The nuclease activity of Cap-SN was shown to be Ca^{2+}-dependent, with an optimal concentration of 2.5 mM (data not shown). The extracellular activity and intracellular inactivity of the fusion protein are crucial for its antiviral properties, in view of efficacy and safety.

To verify the antiviral potential of the fusion proteins, we tested their inhibitory effects on CSFV infection in PK-15 cells using immunofluorescence assay (IFA) (OIE, 2004) and real-time RT-PCR (Zhao et al., 2008). The results showed that Cap-SN inhibited the viral replication of CSFV in PK-15 cells in a dose-dependent manner. Interestingly, complete inhibition was achieved at a concentration of 15 μg/ml, as demonstrated by IFA (Fig. 2) and real-time RT-PCR (Fig. 3). In contrast, no significant reduction in viral antigens and RNA copies was found with the addition of Cap-SN* ($p > 0.05$). To clarify whether Cap-SN can be co-assembled within the progeny virions, we examined the purified progeny virions by Western blot using rabbit antisera directed against SN. The results showed that the Cap-SN-fusion protein was detected in Cap-SN or Cap-SN*-treated PK-15 cells, but not in mock-treated cells (Fig. 4). Cytotoxicity of the fusion protein has been evaluated by observation of the protein-induced cytopathogenic effects and by means of the MTT assay (Qin et al., 2005). The results demonstrated the

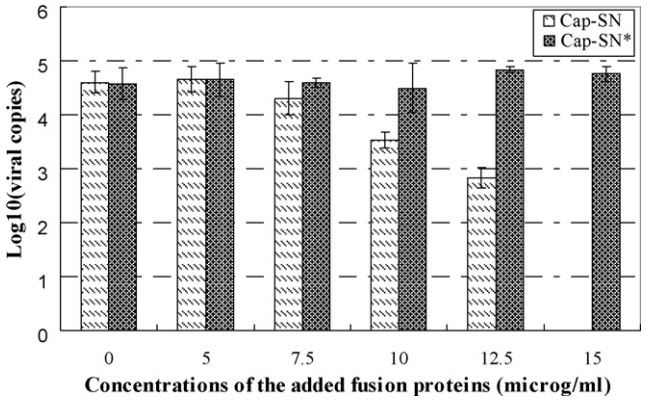

Fig. 3. Inhibition of CSFV replication in PK-15 cells by the fusion proteins quantitatively detected by real-time RT-PCR. The PK-15 cell cultures in a 96-well microplate were infected with 100 $CCID_{50}$ of CSFV Shimen strain for 1 h. The fusion proteins of indicated final concentrations were added to the wells. After 60 h incubation, the cells were harvested and subjected to detection of viral RNA by real-time RT-PCR as described previously (Zhao et al., 2008). Data represent the mean ± standard deviation of triplicate cultures.

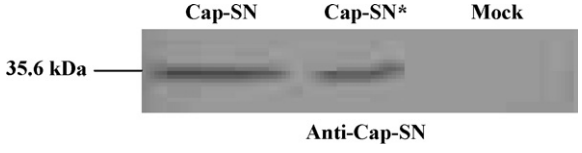

Fig. 4. Analysis of the protein within nascent virions by Western blot. Sixty hours post-infection, the nascent virions from the supernatants of PK-15 cells treated with Cap-SN, Cap-SN*, or mock-treated were collected by ultracentrifugation and probed with rabbit antisera raised against staphylococcal nuclease by Western blot.

Cap-SN-fusion protein to be non-toxic up to 1 mg/ml, which is in accordance with earlier studies using SN-fusion proteins (Natsoulis et al., 1995; Schumann et al., 1996; VanBrocklin and Federspiel, 2000).

The present study indicated that the fusion protein Cap-SN was transported into the cells efficiently with the aid of the HIV-1 Tat PTD to inhibit the replication of CSFV. We conclude that the nuclease activity of the incorporated SN was responsible for the major antiviral effects. It has been proven that the SN degrades both RNA and DNA, and more importantly, has a strict requirement for Ca^{2+} for activity (Tucker et al., 1979), which is very important for its practical antiviral applications. SN is non-toxic to PK-15 cells, as seen in many other cell types (Natsoulis and Boeke, 1991; Natsoulis et al., 1995; Wu et al., 1995; VanBrocklin and Federspiel, 2000; Qin et al., 2005). Therefore, SN-based CTVI seems to be a safe antiviral strategy and might be applied to the control of CSFV infections after further validation of *in vivo* safety and efficacy.

References

Beer, M., Reimann, I., Hoffmann, B., Depner, K., 2007. Novel marker vaccines against classical swine fever. Vaccine 25, 5665–5670.

Beterams, G., Nassal, M., 2001. Significant interference with hepatitis B virus replication by a core-nuclease fusion protein. J. Biol. Chem. 276, 8875–8883.

ICTV, 2006. The International Committee on Taxonomy of Viruses Online., http://www.virustaxonomyonline.com/virtax/lpext.dll?f=templates&fn=main-h.htm.

Liu, J., Li, Y.H., Xue, C.F., Ding, J., Gong, W.D., Zhao, Y., Huang, Y.X., 2003. Targeted ribonuclease can inhibit replication of hepatitis B virus. World J. Gastroenterol. 9, 295–299.

Moennig, V., 2000. Introduction to classical swine fever: virus, disease and control policy. Vet. Microbiol. 73, 93–102.

Natsoulis, G., Boeke, J.D., 1991. New antiviral strategy using capsid-nuclease fusion proteins. Nature 352, 632–635.

Natsoulis, G., Seshaiah, P., Federspiel, M.J., Rein, A., Hughes, S.H., Boeke, J.D., 1995. Targeting of a nuclease to murine leukemia virus capsids inhibits viral multiplication. Proc. Natl. Acad. Sci. U.S.A. 92, 364–368.

Okui, N., Kitamura, Y., Kobayashi, N., Sakuma, R., Ishikawa, T., Kitamura, T., 2001. Virion-targeted viral inactivation: new therapy against viral infection. Mol. Urol. 5, 59–66.

OIE, 2004. Classical Swine Fever (Hog Cholera), Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees), 5th ed. Office International des Epizooties, Paris, France, pp. 244–252 [Part 2, Chapter 2.1.13].

Qin, C.F., Qin, E., Yu, M., Chen, S.P., Jiang, T., Deng, Y.Q., Duan, H.Y., Zhao, H., 2005. Therapeutic effects of dengue 2 virus capsid protein and staphylococcal nuclease fusion protein on dengue-infected cell cultures. Arch. Virol. 150, 659–669.

Schumann, G., Cannon, K., Ma, W.P., Crouch, R.J., Boeke, J.D., 1997. Antiretroviral effect of a gag-RNase HI fusion gene. Gene Ther. 4, 593–599.

Schumann, G., Hermankova, M., Cannon, K., Mankowski, J.L., Boeke, J.D., 2001. Therapeutic effect of a Gag-nuclease fusion protein against retroviral infection in vivo. J. Virol. 75, 7030–7041.

Schumann, G., Qin, L., Rein, A., Natsoulis, G., Boeke, J.D., 1996. Therapeutic effect of gag-nuclease fusion protein on retrovirus-infected cell cultures. J. Virol. 70, 4329–4337.

Schwarze, S.R., Ho, A., Vocero-Akbani, A., Dowdy, S.F., 1999. In vivo protein transduction: delivery of a biologically active protein into the mouse. Science 285, 1569–1572.

Tucker, P.W., Hazen Jr., E.E., Cotton, F.A., 1979. Staphylococcal nuclease reviewed: a prototypic study in contemporary enzymology. III. Correlation of the three-dimensional structure with the mechanisms of enzymatic action. Mol. Cell Biochem. 23, 67–86.

VanBrocklin, M., Ferris, A.L., Hughes, S.H., Federspiel, M.J., 1997. Expression of a murine leukemia virus Gag-*Escherichia coli* RNase HI fusion polyprotein significantly inhibits virus spread. J. Virol. 71, 3312–3318.

VanBrocklin, M., Federspiel, M.J., 2000. Capsid-targeted viral inactivation can eliminate the production of infectious murine leukemia virus in vitro. Virology 267, 111–123.

Vrancken, R., Paeshuyse, J., Haegeman, A., Puerstinger, G., Froeyen, M., Herdewijn, P., Kerkhofs, P., Neyts, J., Koenen, F., 2008. Imidazo[4,5-c]pyridines inhibit the in vitro replication of the classical swine fever virus and target the viral polymerase. Antiviral Res. 77, 114–119.

Vrancken, R., Haegeman, A., Paeshuyse, J., Puerstinger, G., Rozenski, J., Wright, M., Tignon, M., Le Potier, M.F., Neyts, J., Koenen, F., 2009. A proof of concept for the reduction of classical swine fever infection in pigs by a novel viral polymerase inhibitor. J. Gen. Virol. 90, 1335–1342.

Weber, D.J., Meeker, A.K., Mildvan, A.S., 1991. Interactions of the acid and base catalysts on staphylococcal nuclease as studied in a double mutant. Biochemistry 30, 6103–6114.

Wheeler, D.S., Dunsmore, K.D., Wong, H.R., 2003. Intracellular delivery of HSP70 using HIV-1 tat protein transduction domain. Biochem. Biophys. Res. Commun. 301, 54–59.

Wu, X., Liu, H., Xiao, H., Kim, J., Seshaiah, P., Natsoulis, G., Boeke, J.D., Hahn, B.H., Kappes, J.C., 1995. Targeting foreign proteins to human immunodeficiency virus particles via fusion with Vpr and Vpx. J. Virol. 69, 3389–3398.

Zhao, J.J., Cheng, D., Li, N., Sun, Y., Shi, Z., Zhu, Q.H., Tu, C., Tong, G.Z., Qiu, H.J., 2008. Evaluation of a multiplex real-time RT-PCR for quantitative and differential detection of wild-type viruses and c-strain vaccine of classical swine fever virus. Vet. Microbiol. 126, 1–10.