Influence of Cell Surface Glycoprotein gC Produced by Pseudorabies Virus on Cytopathic Effect

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ABSTRACT. The wild-type pseudorabies virus (WT-PRV) produced a round-type cytopathic effect (CPE) in PK-15 cell line of porcine kidney origin, while PRVgCs lacking in gC-transmembrane-anchor region and PRVgC- defecting in gC gene produced a syncytium-type CPE. The mouse embryo cell line (BALB/3T3 clone A31) were transfected with recombinant plasmid of pcDNA3 which incorporated with gC gene. The transfected A31/gC cells were stably expressing gC. Only a round-type CPE was observed in these cells infected with WT-PRV, while a syncytium-type CPE was observed in the cells infected with each of the PRVgCs and PRVgC-. Any viruses described above induced a syncytium-type CPE in A31/pcDNA cells transfected with a plasmid without gC gene. By WT-PRV infection, PK-15 cells generated about 2- or 8-fold more gC than the A31/gC and A31/pcDNA cells when gC was measured by hemagglutination test. Flowcytometric analysis revealed that amount of gC on the cell surface of A31/gC and PK-15 cells increased after infection with WT-PRV. Round-type CPE was observed with the increase of gC. These results suggest that the type of CPE formation induced by PRV is dominated by the amount of gC on the infected cell surface. — **KEY WORDS:** cytopathic effect, glycoprotein gC, pseudorabies virus.

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Pseudorabies virus (PRV) is the causative agent of pseudorabies in several animal species. The disease is primarily a disease of pigs, characterized by symptoms of the nervous system and/or death in newborn pigs, respiratory disorders in older pigs, and reproductive failures such as abortions and stillbirths in sows [1, 4, 5]. Pigs serves as a reservoir and principal source of natural infection. Other animals are susceptible to PRV and almost all die.

PRV is able to produce a round-type or syncytium-type cytopathic effect (CPE) in various kinds of cell lines. In a wild-type (WT) PRV infection, pig cell lines show a round-type CPE, while other animal cell lines show a syncytium-type CPE [13]. Wathen and Wathen [12] and Yamada *et al.* [13] reported that a round-type CPE was observed in WT-PRV-infected fetal pig kidney cells (MVPK-1) and porcine kidney cells (PK-15), and that gC-mar (monoclonal-antibody-resistant) mutant induced a syncytium-type CPE in both these cell lines. From these findings we suggest that not only the virus but the host cells have a role in determination of the type of CPE produced.

To solve the mechanism of the CPE formation, we constructed two PRV mutants truncated coding region or transmembrane-anchor region of gC gene and a mouse cell line stably expressing gC. The aim of this study was to investigate whether the formation of CPE was influenced by the location and the amount of gC associated with the infected cell.

MATERIALS AND METHODS

Cells and viruses: Cell lines used in this investigation are shown in Table 1. BALB/3T3 clone A31 cells of mouse embryo cell line were transfected with plasmid pcDNA3 (Invitrogen) with or without the gC gene. Both A31/gC cell

line [6] stably expressing gC and A31/pcDNA cell without gC gene were used. These cell lines were cultured in Eagle's minimum essential medium (Eagle's MEM) supplemented with 5% FCS or Doulbecco's modified Eagle medium (D-MEM) supplemented with 10% FCS.

PRV Iwate strain (wild-type: WT) was isolated from the tonsil of a pig with symptoms of pseudorabies in our laboratory in 1981. WT-PRV and two mutants, PRVgC-defecting in gC gene and PRVgCs lacking in transmembrane-anchor region were propagated in the porcine kidney cell line, PK-15, and titrated in the same cells by the plaque method.

Construction of PRV mutants: For isolation of PRVgC-, a plasmid DNA pgCdel-LUC was constructed by replacing codons 2 to 458 of gC with the luciferase gene from the plasmid vector pMAM-neo-Luc (CLONTECH). For isolation of PRVgCs, a plasmid DNA pcGs was constructed by truncating the membrane-anchor region and 16 amino-

Table 1. CPE type and HA antigen produced by wild-type PRV infection in various cell lines

Cell line	Tissue derived from:	CPE type	HA titer ^{a)}
BALB/3T3 clone A31	Mouse embryo	Syncytium	320
MDBK	Bovine kidney	Syncytium	160
HmLu-1	Hamster lung	Syncytium	640
RK-13	Rabbit kidney	Syncytium	160
MDCK	Dog kidney	Syncytium	80
CRFK	Cat kidney	Syncytium	80
Vero	Monkey kidney	Syncytium	80
PK-15	Pig kidney	Round	2,560
CPK	Pig kidney	Round	2,560
ESK	Pig kidney	Round	1,280

a) HA titer (units/25 μ l) was measured for cell lysate antigens.

acids of the C-terminal extracellular domain of gC protein. PK-15 cells were seeded into a 6 well plate at 3×10^5 cells/well in growth medium and cultured at 37°C for 24 hr. The monolayers were washed three times with serum-free D-MEM and 2 ml of serum-free D-MEM added. Viral DNA from WT-PRV (100 ng) and 1 µg of each plasmid DNA were suspended in 100 µl serum-free D-MEM and mixed with an equal volume of 10% lipofectin reagent (GIBCO BRL). The DNA-lipofectin mixture was incubated at room temperature for 15 min and then added to the washed PK-15 cells. The culture was incubated at 37°C until the CPE was detected. PRVgC- and PRVgCs were characterized for the production of gC protein by western blot analysis.

Inoculation of WT-PRV into various cell lines: Each cell line indicated in Table 1 was cultured in a 25-cm² culture bottle. The confluent monolayers were inoculated at a multiplicity of infection (moi) of 0.1 pfu/cell. After adsorption for 1 hr at 37°C, 5 ml Eagle's MEM supplemented with 1% FCS was added into each culture bottle, and the cultures were incubated at 37°C for the appropriate times. Induced CPE was observed.

Western blot analysis: Each virus-infected PK-15 cell was lysed with phosphate buffered saline (PBS) containing 1% Triton X-100. The lysate was centrifuged and the supernatant was used as the sample. Culture supernatant from PRVgCs-infected cells was precipitated with 50% saturation of ammonium sulfate, and then dialyzed against PBS. Each sample was mixed with equal volume of 2 × Lammili sample buffer (100 mM Tris-HCl, pH6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 10% β-mercaptoethanol) and then heated for 3 min in boiling water. Samples were electrophoresed in a 10% SDS-polyacrylamide gel, and the separated protein bands transferred onto polyvinylidene (PVDF) membrane (Millipore, U.S.A.). The membrane was blocked with 5% skimmed milk and incubated with the mouse anti-gC MAb. The membrane was washed three times for 10 min with PBS containing 0.05% Tween-20 and then incubated with a peroxidase conjugated anti-mouse Ig antibody (Amersham). After washing in the same way as above, the protein bands were visualized by using a western blotting detection kit (Amersham).

Inoculation of WT-PRV and two viral mutants to PK-15, A31/gC and A31/pcDNA cell line: Each cell line of PK-15, A31/gC and A31/pcDNA was infected with WT-PRV, PRVgC- and PRVgCs respectively, as described in the above protocol. CPE produced by each virus was photographed.

Preparation of cell lysate antigen: Virus-infected cells were harvested when extensive CPE was observed. The cells were washed once with PBS and then lysed with PBS containing 1% Triton X-100 for 10 min at room temperature. They were centrifuged for 10 min at 7,400 × g and the supernatants were used as the cell lysate antigens.

Hemagglutination (HA) test: Hemagglutination titer was determined by using glutaraldehyde-fixed mouse erythrocytes [6, 11]. Two-fold serial dilutions of the cell lysate antigens were prepared with gelatin-veronal buffer

containing 0.2% bovine serum albumin in a V-bottom microtiter plate. Twenty five µl of the same buffer was added into all wells, and subsequently 50 µl of 0.5% fixed erythrocytes was added. The HA titer was determined as the highest reciprocal dilution which indicated complete agglutination.

Flow cytofluorographic analysis: Monolayers of PK-15, A31/gC and A31/pcDNA cells were inoculated with WT-PRV, PRVgCs or PRVgC- at a moi of 5 pfu/cell. After cultivation for 24 hr at 37°C, the infected and mock-infected cells were harvested, and each was divided into two parts. The one part was incubated with anti-gC mouse MAb for 30 min at room temperature, and the other was incubated with PBS. Each sample was washed twice with PBS and then was incubated with FITC conjugated anti-mouse IgG antibody (Amersham) for 30 min at room temperature. The cells were washed twice with PBS, and suspended in 1 ml of PBS. Each sample was scanned on as Epics XL System II flow cytometer (Coulter). Sizing gates were set to include all nucleated cells, and 5×10^3 cells were analyzed for each sample.

RESULTS

Morphology of the CPE and HA antigen produced by WT-PRV in the various cell lines: The CPE-types and HA antigen equivalent to gC produced by WT-PRV in the various cell lines are shown in Table 1. A round-type CPE was observed in PK-15, CPK and ESK cell lines of pig origin infected with WT-PRV. HA titers of these cell lysates were 1,280 HA units/25 µl for ESK to 2,560 for PK-15 and CPK. On the other hand, a syncytium-type CPE was observed in the cell lines of BALB/3T3 clone A31 (320 HA units/25 µl), MDBK (160), HmLu-1 (640), RK-13 (160), MDCK (80), CRFK (80), and Vero (80) derived from animals except the pig. HA titers of these cell lysate were 80 to 640, which revealed considerably lower titer than that of pig cells.

Characterization of PRV glycoproteins: Glycoprotein gC prepared from PK-15 cell infected with WT-PRV or the two mutants was analyzed by western blotting (Fig. 1). WT-PRV led to gC protein synthesis of a 92-kDa mature form and 74-kDa precursor form in the infected cells (Fig. 1, lane 1). PRVgCs synthesized a 80-kDa mature form of the gC protein and 65-kDa precursor form (Fig. 1, lane 2), and the 80-kDa mature form was secreted in the culture supernatant (Fig. 1, lane 4). No gC was detected in the PRVgC- infected cell lysate (Fig. 1, lane 3).

CPE-types produced by PRV mutants in PK-15 cell line: WT-PRV produced a round-type CPE in PK-15 cells. On the other hand, PRVgC- or PRVgCs produced a syncytium-type CPE in these cells (Fig. 2).

CPE-types produced by PRV mutants in the cell lines of A31/gC and A31/pcDNA: WT-PRV induced a round-type CPE in A31/gC cells, while the PRVgC- or PRVgCs induced syncytium-type CPE in these cells (Fig. 2).

When A31/pcDNA cells were infected with WT-PRV,

PRVgC- or PRVgCs respectively, a syncytium-type CPE was observed in all cases (Fig. 2).

Amount of gC in WT-PRV- infected cell lysates: HA titers of WT-PRV infected cell lysates were 2,560 HA units/25 μ l

Fig. 1. Western blot analysis of the gC in virus-infected PK-15 cell lysates and in medium. PK-15 cells were infected with WT-PRV (lane 1), PRVgC- (lane 3) or PRVgCs (lane 2 and 4). Each virus-infected cell lysate (lane 1, 2 and 3) and concentrated medium (lane 4) were electrophoresed in a 10% SDS-PAGE under reducing condition, and then transferred onto PVDF membrane. The gC protein bands were visualized by an immunoenzymatic reaction using a mouse anti-gC monoclonal antibody and peroxidase-conjugated anti-mouse Ig antibody. Numbers on the left indicate molecular masses.

for PK-15, 1,280 for A31/gC, and 320 for A31/pcDNA.

Analysis of the presence of gC on the cell surface: When the round-type CPE produced by WT-PRV was observed in A31/gC cells, gC expression in the cells reached at the same level as that of PK-15 cells. On the other hand, PRVgC- produced the syncytium-type CPE in the A31/gC cells, and the level of gC expression of the infected cells was maintained at the same as that of uninfected A31/gC cells (Fig. 3). Only a slight increase of gC was observed by PRVgCs.

PK-15 cells showed a high level of gC expression by WT-PRV but only a slight increase was noticed in the PRVgCs infection. A slight increase of gC of A31/pcDNA cells was observed after WT-PRV or PRVgCs infection, however no increase of gC was observed in these cells infected with PRVgC- (Fig. 3).

DISCUSSION

PRV is able to grow in various kinds of cell lines with a CPE. In pig cell lines WT-PRV produces a round-type CPE, but not in other cell lines, though it shows a syncytium-type CPE [13]. In PK-15 or MVPK, gC might be essential for formation of a round-type CPE because gC-mar mutant did not form this type of CPE and formed typical syncytium-type [12, 13]. Glycoprotein gC has been implicated in the adsorption of virion to host cells [15], and in binding of suspended virus-infected cells to uninfected monolayer cells [3]. The functional domain of gC concerned with the adsorption is located in the amino-terminal one-third [2]. Additionally, gC has hemagglutination activity

Fig. 2. Morphology of CPE caused by PRV infection. The PK-15, A31/gC and A31/pcDNA cell lines were infected with WT-PRV, PRVgC- or PRVgCs. Virus-infected cells were photographed.

Fig. 3. Flow cytofluorographic analysis. PK-15, A31/gC and A31/pcDNA cells were infected with WT-PRV, PRV gC- or PRVgCs. After cultivation for 24 hr at 37°C, the cells were harvested. The suspended cells were incubated with anti-gC mouse MAb and subsequently incubated with FITC-conjugated anti-mouse Ig antibody. Each sample (5×10^3 cells) was analyzed on a flow cytometer.

against mouse erythrocytes [7], and it plays a role in release of virion into the culture medium [10]. On the other hand, gB and gH are required for the cell fusion [8, 9], and gE promotes the cell fusion [14].

In this study, we investigated whether the location and the amount of cell-associated gC influenced on the formation of a round-type CPE. The difference between two types of CPE produced by WT-PRV or PRV gCs might be dependent on the amount of gC on infected cell surface. Glycoprotein gC synthesized by PRVgCs differed from gC synthesized by WT-PRV in the absence of the transmembrane-anchor region. The extracellular domain of gC played a very important role in the formation of the round-type CPE in MVPK-1 cells [12]. It was suggested that the transmembrane-anchor region was essential to the function of extracellular domain for the CPE formation. These findings indicated that anchoring of gC on infected cell surface influenced on the CPE formation. The type of CPE produced by WT-PRV was related to the amount of gC expressed as HA titer of cell lysate such that; a round-type CPE was formed in PK-15 cells with HA titer of 2,560, a round-type CPE was formed in A31/gC cells with higher amount gC on the cell surface with HA titer of 1,280, and a syncytium-type CPE was formed in A31/pcDNA cells with HA titer of 320. These results suggest that the amount of gC produced on the cell surface plays an important role in determination of the CPE formation.

From these observations described above, we concluded that the anchoring of a large amount of gC on the surface of infected cells may be essential for the formation of the round-type CPE, and that the anchored gC may interact

with or interfere viral fusogens, relating to syncytium formation.

REFERENCES

1. Baskerville, A., McFerran, J. B. and Dow, C. 1973. Aujeszky's disease in pigs. *Vet. Bull.* 43: 465–480.
2. Flynn, S. J., Burgett, B. L., Stein, D. S., Wilkinson, K. S. and Ryan, P. 1993. The amino-terminal one-third of pseudorabies virus glycoprotein gIII contains a functional attachment domain, but this domain is not required for the efficient penetration of Vero cells. *J. Virol.* 67: 2646–2654.
3. Hanssens, F. P., Nauwynck, H. J. and Pensaert, M. B. 1993. Involvement of membrane-bound viral glycoproteins in adhesion of pseudorabies virus-infected cells. *J. Virol.* 67: 4492–4496.
4. Iglesias, G. J. and Harkness, J. W. 1988. Studies of transplacental and perinatal infection with two clones of a single Aujeszky's disease (pseudorabies) virus isolate. *Vet. Microbiol.* 16: 243–254.
5. Iglesias, G. J., Trujano, M., Lokensgard, J. and Molitor, T. 1992. Study of the potential involvement of pseudorabies virus in swine respiratory disease. *Can. J. Vet. Res.* 56: 74–77.
6. Katayama, S., Yoshiki, K., Okada, N. and Shimizu, Y. 1997. Protective effect of glycoprotein gC-rich antigen against pseudorabies virus. *J. Vet. Med. Sci.* 59: 657–663.
7. Matsuda-Tsuchida, A., Okada, N., Katayama, S., Okabe, T. and Sasaki, N. 1991. Characterization of protective viral glycoproteins for pseudorabies virus infection. *J. Vet. Med. Sci.* 53: 737–741.
8. Peeters, B., De Wind, N., Hooisma, M., Wagenaar, F., Gielkens, A. and Moormann, R. 1992. Pseudorabies virus

- envelope glycoproteins gp50 and gII are essential for virus penetration, but only gII is involved in membrane fusion. *J. Virol.* 66: 894–905.
9. Peeters, B., De Wind, N., Broer, R., Gielkens, A. and Moormann, R. 1992. Glycoprotein H of pseudorabies virus is essential for entry and cell-to-cell spread of the virus. *J. Virol.* 66: 3888–3892.
 10. Schreurs, C., Mettenleiter, T. C., Zuckermann, F., Sugg, N. and Ben-Porat, T. 1988. Glycoprotein gIII of pseudorabies virus is multifunctional. *J. Virol.* 62: 2251–2257.
 11. Tetsu, N., Inaba, Y., Yukawa, M., Yoshiki, K., Hirahara, T., Furuya, T., Ito, S., Yonemochi, A. and Ishikawa, H. 1989. Hemagglutination with pseudorabies virus. *Arch. Virol.* 106: 321–326.
 12. Wathen, M. W. and Wathen, L. M. K. 1986. Characterization and mapping of a nonessential pseudorabies virus glycoprotein. *J. Virol.* 58: 173–178.
 13. Yamada, S., Sekikawa, K. and Shimizu, Y. 1989. Molecular biology of Aujeszky's disease virus. pp. 63–84. *In: Progress in Veterinary Science 1989* (Izawa, H. and Shimizu, Y. eds.), Tokyo.
 14. Zsak, L., Zuckermann, F., Sugg, N. and Ben-Porat, T. 1992. Glycoprotein gI of pseudorabies virus promotes cell fusion and virus spread via direct cell-to-cell transmission. *J. Virol.* 66: 2316–2325.
 15. Zuckermann, F., Zsak, L., Reilly, L., Sugg, N. and Ben-Porat, T. 1989. Early interactions of pseudorabies virus with host cells: Functions of glycoprotein gIII. *J. Virol.* 63: 3323–3329.