Proteolytic cleavage of glycoprotein B is dispensable for in vitro replication, but required for syncytium formation of pseudorabies virus

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Glycoprotein B (gB) is the most conserved glycoprotein among herpesviruses and it plays important roles in virus infectivity. In most herpesviruses, including pseudorabies virus (PRV), gB is cleaved by a cellular protease into two disulfide-linked subunits. In the present study, I found that the PRV gB generated in human colon carcinoma LoVo cells, which lack the ubiquitous protease furin, remained in the uncleaved form and the virus replicated in these cells without cell fusion. The uncleaved gB was converted into its subunits after furin digestion. The virus also replicated in Madin–Darby bovine kidney cells without cell fusion in the presence of a furin inhibitor, whereas distinct syncytia were formed in the absence of the inhibitor. LoVo cells constitutively expressing furin showed cell fusion when they were infected with the virus. Penetration kinetics assays revealed that the virus carrying uncleaved gB penetrated the cells at the same rate as the virus carrying cleaved gB. These results indicate that PRV gB is cleaved by furin and that the cleavage is dispensable for virus replication in vitro. Furthermore, gB cleavage is involved in syncytium formation but not in penetration kinetics, suggesting that different mechanisms operate between cell–cell fusion and virus–cell fusion by PRV.

INTRODUCTION

Pseudorabies virus (PRV), a member of the subfamily Alphaherpesvirinae in the family Herpesviridae, is a causative agent of Aujeszky’s disease in pigs and also capable of causing lethal diseases in a variety of other animals (Ben-Porat & Kaplan, 1985). The virus produces at least 11 glycoproteins, of which glycoproteins B (gB), gD and gH/gL are essential for virus replication. PRV gB is one of the most abundant proteins in the viral membrane and plays essential roles in the penetration and cell-to-cell spread of the virus (Rauh & Mettenleiter, 1991). The primary translation product of PRV gB consists of 913 aa, while the mature form of the glycoprotein after removal of the signal sequence has 855 aa. The fully glycosylated gB (gBa) is a 120 kDa polypeptide that is cleaved between Arg444 and Ser445 by a cellular protease to yield two subunits, gBb (68 kDa) and gBc (55 kDa) (Hampl et al., 1984; Whealy et al., 1990; Wolfer et al., 1990).

gB homologues exhibit the highest amino acid sequence conservation among herpesvirus glycoproteins, and most of these homologues, including those of alpha-, beta- and gammaherpesviruses, are processed to yield two subunits (Baghian et al., 2000; Ben-Porat & Kaplan, 1985; Britt & Vugler, 1989; Johannsen et al., 2004; Meredith et al., 1989; Okazaki et al., 1986, 1990; Ross et al., 1989; van Drunen Littel-van den Hurk & Babaiuk, 1986). It was also reported that the processing of human cytomegalovirus (HCMV) gB is mediated by the ubiquitous protease furin (Vey et al., 1995). Although such proteolytic cleavage is necessary for the activation of other viral fusion proteins, such as influenza virus haemagglutinin and Newcastle disease virus (NDV) fusion (F) protein (Klenk et al., 1975; Nagai et al., 1976), cleavage of bovine herpesvirus 1 (BoHV-1) and HCMV gBs is not required for their functions in vitro (Kopp et al., 1994; Strive et al., 2002). Moreover, the gB of herpes simplex virus (HSV) is not cleaved at all (Claesson-Welsh & Spear, 1986).

In the present study, I found that furin is responsible for the cleavage of PRV gB and investigated the effects of cleavage of the glycoprotein on its functions. The results indicate that the cleavage is responsible for syncytium formation but not involved in cell entry of PRV.

METHODS

Cells and viruses. Madin–Darby bovine kidney (MDBK), Madin–Darby canine kidney (MDCK) and rabbit kidney RK13 cells were maintained in minimal essential medium (MEM; Nissui) containing 10% calf serum. Human colon carcinoma LoVo, human embryonic kidney 293T, African green monkey kidney Vero E6 and cloned porcine kidney (CPK) cells were maintained in Dulbecco’s modified MEM (Gibco-BRL) containing 10% fetal calf serum. PRV strain YS-81 and NDV strain TCND were propagated in MDBK cells.
**RESULTS**

**Immunoprecipitation.** A mouse monoclonal antibody, 18/4, specific for PRV gB was kindly provided by Dr A. Takada (Department of Global Epidemiology, Hokkaido University, Japan) and immobilized on protein A–Sepharose (Pharmacia) by dimethylpimeliladate as described previously (Harlow & Lane, 1988). Virus-infected cells were treated with lysis buffer (1 % sodium deoxycholate, 1 % Triton X-100, 140 mM NaCl, 10 mM Tris/HCl pH 7.4, 1 mM EDTA) and clarified by centrifugation. The supernatant was incubated with the antibody-coupled Sepharose beads for 1 h at 37 °C, followed by five washes by centrifugation in the lysis buffer. After washing, the beads were boiled in Laemmli sample buffer (Laemmli, 1970) and subjected to SDS-PAGE. The immunoprecipitated proteins were visualized by silver staining (Wako).

**Digestion of immunoprecipitated gB by furin.** The gB-bound beads were washed with saline and incubated with 100 U furin (Sigma) ml⁻¹ in 150 mM NaCl, 4 mM CaCl₂ and 40 mM Bis-Tris (pH 5.8) for 1 h at 30 °C. After washing with the lysis buffer, the beads were subjected to SDS-PAGE under reducing conditions.

**Virus growth in the presence of a furin inhibitor.** A peptide furin inhibitor, decanoyl-Arg–Val–Lys–Arg-chloromethylketone (Vey et al., 1995), was purchased from Wako. MDBK cells were infected with PRV or NDV at an m.o.i. of 0.1 and incubated with different concentrations of the inhibitor. To 60 h post-infection, the supernatants were collected and clarified by centrifugation, followed by determination of the viral titres by plaque assays.

**Establishment of LoVo cells stably expressing furin.** The complete coding sequence of human furin was excised from the plasmid pSG5-hfurin (Takahashi et al., 1993), which was kindly provided by Dr Y. Misumi (Department of Cell Biology, School of Medicine, Fukuoka University, Japan). Following complete digestion of the plasmid with EcoRV and partial digestion with BamHI, a 2.7 kb fragment was obtained and cloned into the PvuII/BamHI sites of pCEP4 (Invitrogen). The resultant plasmid was designated pCEP4-hfurin. Transfection of LoVo cells with pCEP4-hfurin was carried out using LipofectAMINE 2000 (Gibco-BRL) as described previously (Okazaki et al., 1993). At 48 h post-transfection, the cells were transferred into fresh medium containing 40 µg hygromycin B ml⁻¹. The hygromycin-resistant cells were screened for furin production by immunofluorescence staining using a rabbit polyclonal IgG against furin (Santa Cruz Biotechnology) as described previously (Okazaki et al., 1987). The positive cells were isolated and subjected to single-cell cloning.

**Penetration assay.** The penetration kinetics were measured by inactivation of the extracellular virus with acid solution as described previously (Okazaki et al., 1991). Approximately, 1000 p.f.u. of the virus grown in LoVo or MDBK cells was allowed to adsorb onto monolayers of MDBK cells in 60 mm dishes on ice. After washing with cold MEM, the monolayers were covered with pre-warmed MEM and shifted to 37 °C. At different times after the temperature shift, the cells were treated with acid solution (0.1 M HCl, 0.1 M sodium citrate, pH 2.5) for 5 min, extensively washed and overlaid with agar.

**Furin cleaves gBa into gBb and gBc**

Since only LoVo cells showed a lack of gB cleavage, I examined whether furin was able to process their gB on the antibody-coupled beads. Uncleaved gB precipitated from virus-infected LoVo cells by the antibody was incubated with furin and analysed by SDS-PAGE under reducing conditions. As shown in Fig. 2, the gB on the beads was converted into two subunits by furin digestion. The molecular masses of the subunits obtained from gB coincided with those obtained from MDBK cells infected with PRV. These findings suggest that the PRV gB from LoVo cells is cleaved by furin.
A furin inhibitor has little effect on replication but does affect syncytium formation by PRV

In order to ascertain the function of furin, MDBK cells infected with NDV or PRV were incubated in the presence of a furin inhibitor. As shown in Fig. 3(a), the inhibitor decreased the infectious titre of NDV in the culture fluid in a concentration-dependent manner. At a concentration of 80 μM the titre was decreased to less than 1/10 000 of the control. The virus generated in the presence of the inhibitor at concentrations of more than 20 μM was activated up to 10^3 p.f.u. ml\(^{-1}\) by trypsin, whereas the enzyme had no effect on the virus generated at lower concentrations of the inhibitor. These findings indicate that the F protein of the virus particles released from the cells was mostly cleaved at the inhibitor concentrations below 20 μM, while the inhibitor interfered with the cleavage and decreased the infectious titres in a concentration-dependent manner between 20 and 80 μM. In contrast, the titres of PRV in the culture fluid were scarcely affected by the inhibitor (Fig. 3b). Even at the inhibitor concentration of 80 μM, the titre was only decreased to 1/10 of the control. Furthermore, no increase in the titre was observed in the presence of trypsin. These results confirm that furin does not play a role in generating infectious particles of PRV.

Since NDV only grew in a single-step manner in the presence of the inhibitor, a small number of cells exhibited CPE (data not shown). On the other hand, the cells infected with PRV exhibited extensive CPE, even in the presence of the inhibitor, where no cleaved product of gB was detected (Fig. 4). However, it is noteworthy that the cells incubated with the inhibitor only showed rounding CPE, whereas syncytia were formed in the absence of the inhibitor, where gBa was cleaved into gBb and gBc. These findings may indicate that cleavage of gB is responsible for the cell fusion caused by PRV.

Furin-expressing LoVo cells show syncytium formation by PRV

In order to confirm the correlation between gB cleavage and syncytium formation, LoVo cells constitutively expressing furin were established and infected with PRV. Fig. 5(a) shows that, in the cells expressing furin, gBa was definitely converted into gBb and gBc, while in naïve LoVo cells the
glycoprotein was scarcely cleaved. PRV-infected LoVo cells expressing furin also exhibited cell fusion clearly, while the cells not expressing the enzyme showed no such fusion (Fig. 5b). These results demonstrate that cleavage of gB is responsible for syncytium formation by PRV.

Cleavage of gB has no influence on the penetration kinetics of PRV

Since gB plays a role in the cell entry of PRV, the penetration kinetics were compared between ordinary virions and uncleaved gB-carrying particles. Following incubation of the two types of virus attached to the cell surface at 37°C for different periods of time and subsequent treatment with acid solution, the numbers of plaques were counted (Fig. 6).

The virus grown in LoVo cells developed full tolerance to the acid treatment at 20 min after the incubation, and the virus grown in MDBK cells required 30 min to achieve such tolerance. These results may indicate that the penetration efficiency of PRV is separate from the cleavage of gB.

DISCUSSION

HSV1 gB homologues exhibit the highest amino acid sequence conservation among herpesvirus glycoproteins, and most of these homologues are modified by proteolytic cleavage due to the highly basic consensus sequence, RXR/
KR. In fact, a recent study demonstrated that Epstein–Barr virus abundantly expresses cleaved gB on its extracellular particles (Johannsen et al., 2004), although virus-infected cells contain the uncleaved form (Gong et al., 1987). On the other hand, the gBs of BoHV-1 and HCMV do not require cleavage for their functions, as evaluated using genetically engineered viruses (Kopp et al., 1994; Strive et al., 2002). The present study using the naïve virus has demonstrated for the first time that cleavage of gB is not essential for replication of PRV, but is required for the syncytium formation. We also found that furin serves as the cleavage enzyme for the glycoprotein.

PRV was found to replicate in a variety of cell lines accompanied by extensive CPE. With the exception of LoVo cells, all the cells examined generated cleaved gB and formed syncyta, whereas LoVo cells, which lacks furin, neither cleaved the glycoprotein nor formed syncytia. MDBK cells treated with a furin inhibitor also generated the uncleaved form of gB and exhibited only rounding CPE when the cells were infected with PRV. Although gD and gH of PRV contain the proteolytic cleavage consensus sequence, RXR/KR, no cleaved product was observed in either virus-infected cells or virions (Klupp et al., 1992; Whealy et al., 1991). No other glycoproteins in addition to gB, gD and gH possess the consensus sequence. In spite of the possibility that an unknown number of accessory molecules play a role in syncytium formation, the present findings may indicate that cleavage of gB is involved in the function of the glycoprotein. Kopp et al. (1994) described that BoHV-1 carrying uncleavable gB is inferior to the wild-type virus in its ability to spread from infected cells to adjacent uninfected cells. The present results appear to be consistent with this observation, although BoHV-1 scarcely forms syncytia in MDBK cells. In contrast, the cleavability of BoHV-1 gB has no influence on the capacity for direct cell-to-cell spreading of the virus in a PRV background (Kopp et al., 1994).

In the presence of a furin inhibitor, PRV gB remained in the uncleaved form even in MDBK cells. Uncleaved gB isolated from LoVo cells was successfully converted into two subunits after furin digestion. Moreover, constitutional expression of furin allowed LoVo cells to process the

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Table 1. Distribution of the consensus sequence motif for furin in the gBs of herpesviruses

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<th>GenBank accession no.</th>
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glycoprotein. Since pCEP4 is maintained episomally in the nucleus, the endogenous gene expression of the host cells is barely affected. Therefore, expression of furin must solely cause the cleavage of the glycoprotein to form syncytia in LoVo cells after virus infection. The ubiquitous protease furin is responsible for activation of the glycoproteins of many enveloped viruses (de Haan et al., 2004; Ortmann et al., 1994; Volchkov et al., 1998; Zhang et al., 2003), as well as the capsid protein of non-enveloped papillomavirus, for entry into cells (Richards et al., 2006). It was also demonstrated that HCMV gB was cleaved by furin and that the same inhibitor as used in this study had no influence on release of infectious particles carrying uncleaved gB (Vey et al., 1995). On the other hand, Jean et al. (2000) reported that a different furin inhibitor caused mislocalization of gB and blocked generation of infectious particles of HCMV.

Since the virus achieved multistep replication in LoVo cells or in the presence of the inhibitor, cleavage of gB is dispensable for the adsorption, penetration and release of PRV. In particular, the penetration kinetics were unaffected by proteolytic modification of the glycoprotein. The observed reduction in the virus titres in the presence of the inhibitor is probably due to the deficiency in syncytium formation, since no difference in the titre was found between the drug-treated and untreated cells at an m.o.i. of 5 (data not shown). Vey et al. (1995) reported no cytotoxicity of decanoyl-Arg–Val–Lys–Arg-chloromethylketone for human fibroblast cells. Cleavage of PRV gB does not seem to be involved in virus–cell fusion, but does seem to be required for cell–cell fusion. Although gB takes part in membrane fusion events by PRV, different mechanisms must participate in virus–cell and cell–cell fusion. Different regions of gB were reported to determine the penetration kinetics and syncytium formation phenotype of HSV1 (Bzik et al., 1984). Moreover, a synthetic peptide corresponding to a heptad repeat region of gB does not affect the penetration of BoHV-1, but does interfere with its cell-to-cell infection (Okazaki & Kida, 2004).

As shown in Table 1, only viruses belonging to the genus Simplexvirus lack the consensus sequence motif for furin, RXR/KR, in their gB molecules. It is interesting to consider the relationship between the functions of gB and virus evolution. Co-expression of gB, gD, gH and gL is necessary for HSV1 to induce sufficient cell fusion (Turner et al., 1998), whereas gB, gH and gL are sufficient for PRV (Klupp et al., 2000). Furthermore, HSV1 gB does not complement the lethal defect in gB PRV although reciprocal complementation is affected (Mettenleiter & Spear, 1994). Further investigations into this essential glycoprotein may provide insights into the mechanisms of membrane fusion by herpesviruses.

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Syncytium formation by PRV requires gB cleavage


