YAMAGUCHI UNIVERSITY
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Studies on Glycoproteins of Equine Herpesviruses

馬ヘルペスウイルスの糖蛋白に関する研究

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General introduction

History of equine herpesvirus (EHV)-1 and EHV-4

In 1932, EHV-1 was discovered at Kentucky, as a result of necropsy examination of an aborted fetus (Dimock and Edwards, 1933). Retrospective examination of preserved specimens indicated that the disease had been present in the mare population as early as 1921 but the description of neurological disease was start in 1988 as infectious paralysis of horses, however the link between the disease and EHV-1 was made in 1966 by Saxegaard, who isolated the virus at necropsy from the nervous tissue of horse (Saxegaard, 1966).

Until 1981, two subtypes of the virus were described; EHV subtype 1 was mainly associated with abortion and nervous disorders and EHV subtype 2 was mainly responsible for respiratory disorders.

EHV-1 and EHV-4 were considered as two subtypes of the same virus, namely EHV-1, and the differentiation between EHV-1 and EHV-4 came after their genomic DNAs were compared by restriction endonuclease digestion pattern (Sabine et al., 1981; Studdert et al., 1981; Turtinen et al., 1981).

Classification

Order *Herpesviridae* are classified into four subfamilies based on host cell range, replication cycle, ability to destroy cells and site of latency. The subfamily *Alphaherpesvirinae* are characterized by a variable host cell range, a relatively short reproductive cycle, rapid spread in cell culture, efficient destruction of infected cells and the capacity to establish latent infections. The subfamily *Betaherpesvirinae* has a restricted host range, the reproductive cycle is long and the infection progresses slowly in cell culture, infected cells frequently become enlarged and latent virus can be maintained in secretary glands, lymphoreticular cells, kidneys and other tissues. The subfamily *Gammaherpesvirinae* has limited host range of family which the natural host belongs to, the replication causes lytic infection and latent virus is frequently demonstrated in lymphoid tissue. The subfamily unnamed, comprising the channel catfish herpesvirus-like viruses (Murphy et al., 1999)

The horse is the natural host of EHV-1, EHV-2, EHV-3, EHV-4 and EHV-5. EHV-2 has been linked to respiratory disease and conjunctivitis, but also has a role in general malaise (Palfi et al., 1978; Collinson et al., 1994; Schlocker et al., 1995), EHV-3 causes a self-limiting venereal infection of the external genitalia (Blanchard et al., 1992) and both EHV-2 and EHV-3 are widely prevalent compared to EHV-1 and EHV-4 (Palfi et al., 1978; Blanchard et al., 1992; Borchers et al., 1997). EHV-2 and EHV-3 are considered of less economic and veterinary importance (Borchers et al., 1997). EHV-5 has a role in the development of equine multinodular pulmonary fibrosis (Williams et al., 2007; Wong et al., 2008). A new equine herpesvirus, EHV-9, was isolated from diseased gazelle (Fukushi et al., 1997; Yanai et al., 1998) and seems to display only mild pathogenicity in horses (Taniguchi et al., 2000).

EHV-1 and EHV-4 are clinically, economically and epidemiologically the most relevant pathogens in horse. EHV-1 is a major pathogen in horses responsible for respiratory disorders, neonatal foal disease, abortion and neurological disorders.

Structure of virus

Complete genomic maps had been published for EHV-1 (Whalley et al., 1981) and EHV-4 (Cullinane et al., 1988; Nagesha et al., 1992) and the genomes are linear double-stranded DNA molecules with 57% G + C (Darlington and Randall, 1963; Soehner et al., 1965). The complete genome of EHV-1 strain Ab4 was composed of 150,223 bp containing at least 80 open reading frames (ORFs) (Telford et al., 1992) The genomes were composed of unique long (U_L) and short (U_S) regions (Fig. 1), and a set of inverted repeat sequences, internal and terminal repeat sequences flanking Us region (IR_S and TR_S). Although EHV-1 genome contains 80 ORFs encoding proteins, two ORFs are duplicated in the IR_S and TR_S, so EHV-1 is considered to contain 76 unique genes (Telford et al., 1992). The possibility remains that EHV-1 contains other less obvious ORFs. EHV-1 and EHV-4 are genetically collinear, although some variations in the arrangements of the genes with respect to the repeat structures have been recognized (Nagesha et al., 1993). EHV-1 genes 65, 66, and 67 are contained completely within the repeats, whereas gene 68 appears to straddle the junction (Telford et al., 1992). EHV-4 genes 65 and 66 are located completely within the repeats and gene 67 straddles the junction (Nagesha et al., 1993). Functions of the repeat structures are not completely understood, although they are isomerization of the S region relative to the L region. Also, a terminal element of the repeat sequences allows circularization of the genome for rolling circle replication (Roizman and Sears, 1990).

The core contains the linear double-stranded DNA in the form of a torus (Furlong et al., 1972; Nazerian, 1974) and is enclosed in an icosadeltahedral capsid, approximately 100 to 110 nm in diameter containing 162 capsomeres (12 pentameric and 150 hexameric). The pentameric capsomeres have not been well characterized and the hexameric capsomeres have a cylindrical shape with a channel running through the long axis (Wildy and Watson, 1962). Core and capsid form the nucleocapsid, which is surrounded by the tegument and the tegument is enclosed by a lipoprotein envelope derived from the host cell (Armstrong et al., 1961), (Fig. 2). Until now, 12 different glycoproteins have been described for EHV-1; gB, gC, gD, gE, gG, gH, gI, gK, gL, gM, gN and gp2 which are involved in virus attachment, penetration, egress and cell-to-cell spread (Table 1).

Epidemiology and diagnosis

Both EHV-1 and EHV-4 are endemic in horse population worldwide (Allen and Bryans, 1986; Allen et al., 1999). Due to antigenic similarity between EHV-1 and EHV-4, the interpretation of data from serological surveys was complicated by the lack of availability of a type specific antibody test until the early 1990. Especially, seroepidemiological studies on EHV-1 and EHV-4 infection were, pre complicated in horse populations in which EHV-1 and/or EHV-4 vaccination is practiced. Retrospective testing of sera from Thoroughbred horses collected from 1967–1974 and 1993 showed values of 9% and 28% for EHV-1 specific antibodies, respectively, and 100% for EHV-4 specific antibodies (Crabb and Studdert, 1995). An earlier study found an incidence of EHV-1 antibody in mares and foals of 26.2% and 11.4%, respectively, whereas >99% of mares and foals tested were EHV-4 antibody positive (Gilkerson et al., 1999).

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In the USA, 85% of foals tested 6–8 months after weaning were initially thought to have seroconverted to EHV-1 (Doll and Bryans, 1963), but the seroconversion was subsequently amended to EHV-4 (Allen and Bryans, 1986). There are only limited data available to explain the difference in relative incidence of EHV-4 and EHV-1. One study of respiratory disease in racehorses during the period 1979–1990 in Japan concluded that the EHV-1 infections occurred predominantly in winter season whereas those with EHV-4 occurred throughout the year (Matsumura et al., 1992).

EHV-1 infection to sucking foals can occur as early as 30 days of age (Gilkerson et al., 1997), because lactating mares may be the primary source of EHV-1 infection to foals and the latter further transmit the virus to other mares and foals (Gilkerson et al., 1999) and also stress that activate latent virus is likely to be the source of such transmissions. EHV-4 infection to the horse probably also starts early in life.

Classical tests such as virus isolation, various serological methods and more recently developed PCR assays are all used for diagnosis (Crabb and Studdert, 1995). However, virus isolation is still important as this is the way to secure the isolate for any further comparative analysis.

Diseases and pathogenesis

Acute respiratory diseases due to EHV-1 and EHV-4 are characterized by fever, anorexia, nasal discharge, ocular discharge and bacterial proliferation in nasal (Thompson et al., 1979). EHV-1 causes severe disease than EHV-4 (Burrows and Goodridge, 1973; Edington et al., 1986; Tewari et al., 1993; Patel et al., 2003b).

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Horses are repeatedly infected by both viruses in nature and the disease signs become less severe with progressive episodes later in life.

Primary EHV-1 replication occurs in the upper respiratory tract epithelial cells and local lymph nodes (Patel et al., 1982; Kydd et al., 1994a, b), resulting in leukocyte-associated viraemia (Patel et al., 1982; Dutta and Myrup, 1983; Scott et al., 1983; Edington et al., 1986). This leukocyte-associated viraemia in an acute infection has been shown to be a prerequisite for abortion and paresis by initiating replication of EHV-1 in endothelial cell lining of blood vessels in the central nervous system and pregnant uterus (Patel et al., 1982; Edington et al., 1986; Edington et al., 1991; Smith et al., 1993). It is likely that this mechanism also operates in abortions and paresis occurring long after an acute primary infection. Recrudescent virus from latently infected leukocytes spread to endometrial blood vessels, resulting in severe vasculitis and multifocal thrombosis (Edington et al., 1991; Smith et al., 1992) and abortion of a virus negative fetus (Smith et al., 1992; Patel et al., 2003a). Less extensive uterine vascular pathology may allow focal transfer of virus across the uteroplacental barrier and abortion of a virus- infected fetus (Smith et al., 1993; Smith, 1997). Transplacental EHV-1 infection could result in the birth of live infected foals, which usually die a few days afterwards a condition known as neonatal foal disease. In pony colts and pony stallions, EHV-1 was found in the epididymis and testis with virus replication in endothelial cells of these organs accompanied by necrotizing vasculitis and thrombosis, and also in semen collected after the cessation of viraemia (Tearle et al., 1996).

Acute EHV-1 infection can result in leukopenia (Bumgardner et al., 1982; Allen and Bryans, 1986), but this is not characteristic for all virus strains since EHV-1 strain C147 (Patel et al., 2003a) did not show any leukopenia indicating that not all

EHV-1 strains are immunosuppressive. Also the biological characteristics of EHV-1 strains vary in the other respects as is indicated by differences in abortigenic potential (Gleeson and Coggins, 1980; Mumford et al., 1994). Differences in abortigenic potential may relate to differences in the level of viraemia or endothelial cell infection induced (Mumford et al., 1994; Smith et al., 2000) and up to 95% of EHV-1 abortions occur in the last third of pregnancy and rarely at other stages (Doll, 1952; Allen and Bryans, 1986).

In the pathogenesis of EHV-4 leukocyte-associated viraemia, abortions and paresis are not a consistent feature of EHV-4 (Edington et al., 1986; Matsumura et al., 1992; Tewari et al., 1993; Patel et al., 2003b). EHV-4 replicated in vascular endothelial cells and it was suggested that the pathogenesis of EHV-4 abortion might have a vascular basis similar to that of EHV-1 (Edington et al., 1991; Smith et al., 1992). Whether difference in the incidence of EHV-4 induced abortions can be put down to strain differences of circulating viruses is unknown and is difficult to assess since DNA fingerprints of EHV-4 isolates from around the world have been found to be similar and EHV-4 is considered highly stable genetically (Studdert, 1983). As EHV-4 is not commonly associated with equine abortions, evidence for its role in paresis is limited (Meyer et al., 1987; Verheyan et al., 1998).

Glycoproteins

Envelope glycoproteins of EHV-1 and EHV-4 play important roles in viral replication by mediating virus attachment to cells and entry into cells, cell-to-cell spread, pathogenesis and induction of host humoral immune responses. These glycoproteins play a major role in the immunogenicity of EHV-1 and EHV-4 and serve as major targets for neutralization of virus infectivity by antibodies (Bridges et

al., 1987; Bridges et al., 1988; Ostlund et al., 1992; Papp et al., 1979; Shimizu et al., 1989). It was found that glycoproteins B, C, and D were considered as immunodominant antigens for generating antiviral serological responses to EHV-1 and EHV-4 in infected horses (Allen et al., 1992). EHV-1 glycoproteins, gB, gD, gH, gL, and gK, are essential for replication of the virus and the others, gC, gE, gG, gI, gM, and gp2, are not required for viral growth in cell culture and have been termed as non-essential glycoproteins. These non-essential envelope glycoprotein genes are maintained in virulent field isolates of EHV-1 and EHV-4 and their deletion results in a strong reduction of virulence (Matsumura et al., 1996, 1998).

The structural of virion proteins and glycoproteins of EHV-1 and EHV-4 are similar, but not identical for stable intertypic differences in their viral electrophoretic mobilities that can be used to differentiate the two viruses (Turtinen, 1983).

gp2

The gene encoding gp2 of EHV-1 and EHV-4 has been identified only in one other alphaherpesviruses, asinine herpesvirus 3 (AHV-3) (Colle et al., 1995) and there is no report of the related gene in other herpesviruses of any subfamily. The gp2 also known as gp300 (Rudolph et al., 2002a) appears to be abundant in EHV-1 and 4 virions and to be one of the most immunogenic viral antigens for the natural host (Allen et al., 1986; Colle et al., 1995). *In vitro* studies using an EHV-1 gene 71 deletion mutant demonstrated that gp2 was not essential for virus growth in cell culture (Marshall et al., 1997) and that it was involved in facilitating virus entry and egress (Matsumura et al., 1996). In mice, this deletion mutant lacked full expression of virulence (Fitzmaurice, 1997).

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The open reading frame of EHV-1 gp2 encodes a polypeptide of 797 amino acids (O'Callaghan et al., 1968). The gp2 is rich in serine and threonine residues and is a heavily O-glycosylated protein with a molecular mass in the range of 192 to >400 kDa (Sun et al., 1994; Telford, 1992; Whittaker, 1990). In contrast to EHV-4 gp2, the EHV-1 gp2 was shown to be partially cleaved into two polypeptides in infected cells (Learmonth et al., 2002). Endoproteolytic cleavage occurs after each of two adjacent arginine (R) residues at positions 506 and 507 in the sequence HRGRAGGR506R507G, and results in a 42-kDa carboxy (C)-terminal subunit, which contains the transmembrane anchor, and an *N*-terminal serine/threonine-rich component that is highly O-glycosylated (Learmonth et al., 2002; Wellington et al., 1996a). In the absence of gp2, EHV-1 was shown to be impaired in virus egress, while secondary envelopment appears to occur with unaltered kinetics. The EHV-1 with a deletion of gp2 gene conferred protection against pulmonary disease in mice after challenge with wild-type virus (Marshall et al., 1997; Rudolph et al., 2002a; Rudolph and Osterrieder, 2002b; Sun et al., 1996).

gB

EHV-1 gB is involved in penetration and in cell-to-cell spread of infection (Wellington et al., 1996b; Neubauer et al., 1997) and has been identified as a major target for virus specific antibody in the EHV-1 infected horse (Allen et al., 1992). Homologues of gB in other herpesviruses are known to be targets for cytotoxic T-lymphocytes (Blacklaws et al., 1987; Omar et al., 1998). EHV-1 gB as a component of a subunit vaccine has been supported by a number of experiments, mainly in small animal models. Monoclonal antibodies to EHV-1 gB conferred passive protection against EHV-1-induced disease in Syrian hamsters (Shimizu et al., 1989) and

immunization of mice with recombinant vaccinia virus expressing EHV-1 gB elicited EHV-1 specific antibodies (Bell et al., 1990) and protected hamsters against lethal EHV-1 challenge (Guo et al., 1990). A truncated EHV-1 gB co-expressed with HIV-1 gag virus-like particles in insect cells, protected immunized mice against intranasal challenge with EHV-1 (Osterrieder et al., 1995, 1996).

The open reading frame of EHV-1 gB encodes a polypeptide of 980 amino acids, including a conserved central region of over 600 residues that has 50 to 60% amino acid sequence identity with the gB homologues of herpes simplex virus (HSV) and other alphaherpesviruses (Whalley et al., 1989). The EHV-1 gB polypeptide contains characteristic hydrophobic sequences corresponding to signal peptide and membrane anchor regions, and it is processed in EHV-1 infected mammalian cells to a glycosylated precursor form reported to have apparent molecular mass of 112-138 kDa (Meredith et al., 1989; Sullivan et al., 1989). This precursor is cleaved into two subunits of approximately 75-77 kDa and 53-58 kDa, which associate to form a disulfide linked heterodimer of 145 kDa.

EHV-4 gB and HSV-1 gB proteins are 47% homologous and possess highly conserved hydrophilic surface, hydrophobic transmembrane and cytoplasmic anchor domains. EHV-4 gB contains five more potential N-linked glycosylation sites than HSV-1 gB, and it is interesting to note the presence of three such sites immediately adjacent to each other (residues 493 to 501). In comparison of predicted amino acid sequence of EHV-4 gB with the varicella zoster virus (VZV) gB, pseudorabies virus (PRV) gB, Epstein-Barr virus (EBV) gB and human cytomegalovirus gB, the identities were 51, 54, 30, and 29%, respectively. The gB glycoproteins of the alphaherpesviruses show a similar degree of homology with each other (47 to 54%) and significantly less homology with the gB protein of the betaherpesvirus human

cytomegalovirus (29%) and the gammaherpesvirus Epstein-Barr virus (30%). Epstein-Barr virus gB and human cytomegalovirus gB are only 30% homologous, suggesting that each family of herpesviruses has separate ancestral origins. EHV-4 gB glycoprotein shows 88% homology to the gB glycoprotein of EHV-1 (Riggio et al., 1989).

gC

Glycoprotein C (gC) was responsible for the first interaction between the virion and the cell surface. The primary attachment of both HSV-1 and PRV is mediated by cell surface glycosaminoglycans and is sensitive to treatment with heparin or heparinase (Mettenleiter, 1994). gC is dispensable for virus growth *in vitro* and gC-negative mutants only exhibit marginal defects in their growth characteristics in cultured cells when compared to wild-type viruses (Schreurs et al., 1988; Whealy et al., 1988; WuDunn and Spear, 1989; Mettenleiter and Rauh, 1990). Moreover, gC-negative PRV was shown to retain its virulence for laboratory animals and the natural host, indicating that gC function is not required or can be compensated by other viral glycoproteins *in vitro* and *in vivo* (Mettenleiter et al., 1988; Kritas et al., 1994a; Kritas et al., 1994b).

There are high sequence and structural similarity between EHV-1 gC and its EHV-4 homologue, but also some differences. These differences may help explain the difference in cell tropism between both viruses, and the difference in cell tropism between EHV-1 and EHV-4 may be associated with differences in the contributions of different glycoproteins, at least gC, gD and gG, which involved in virus entry or spread from an infected to a neighboring uninfected cell. EHV-4 gC plays a role in the interaction of the virus with cell surface heparan sulfate. It seems likely that gB,

another heparin-binding glycoprotein, could also mediate the adsorption of EHV-4 to the cells. In addition, direct interaction between EHV- 4 gC and equine C3 has been demonstrated and this interaction exhibited species-specificity (Huemer et al., 1993). EHV-4 gC protects EHV-4 from complement-mediated lysis (Azab et al., 2010).

gD

EHV-1 gD was involved in virus entry (Whittaker et al., 1992) and was identified as a potent immunogen. Intramuscular inoculation of plasmid DNA encoding the gene for EHV-1 gD induced a long-term neutralizing antibody response and led to accelerated clearance of infectious virus from lungs of mice in a model of EHV-1 respiratory disease (Audonnet et al., 1990; Flowers et al., 1991; Whalley et al., 1991; Ruitenberg et al., 1999). In these animals immunized EHV-1 gD, lymphocyte proliferation to EHV-1 and a T-helper type 1 isotype profile were demonstrated, indicating the induction of cell-mediated immune responses. It has also been reported that DNA encoding a truncated form of EHV-1 gB could provide some protection from EHV-1 challenge in the murine respiratory model (Osterrieder et al., 1995). The amino acid sequence of EHV-4 gD was compared with those of other alphaherpesviruses and EHV-4 gD shares 83 % sequence identity with EHV-I gD, 38 % with BHV-1 gD, 34% with PRV gD, 29% with MDV gD, 25 % with HSV-1 gD and 24 % with HSV-2 gD.

gE and gI

Glycoproteins gE and gI of herpesviruses have been shown to facilitate cell-to-cell spread since the deleted viruses yield small plaques compared to those of the parent viruses in the case of HSV-1 (Neidhardt et al., 1987; Dingwell et al., 1994),

PRV (Jacobs et al., 1993), BHV-1 (Otsuka and Xuan, 1996) and FHV-1 (Sussman et al., 1995). EHV-1 gE and/or gI also facilitate cell-to-cell spread, but are not involved in either the process of virus maturation and release or virus attachment and penetration (Matsumura et al., 1998).

EHV-4 with a deletion in the genes encoding gE and gI affected cell-to-cell spread of the virus *in vitro* and clinical signs in foals inoculated with the recombinant were milder than that for the revertant. This suggests that intact gE and/or gI genes are important factors in the expression of virulence in EHV-4 as in other herpesviruses (Damiani et al., 2000). HSV-1 mouse model demonstrated that HSV-1 gI and gE deletion mutants are less virulent and are impaired in their ability to establish latency (Meignier et al., 1988).

gG

EHV-1 and -4 gG were secreted after proteolytic processing and the homologues of gG were found in HSV-1 (Richman et al., 1986), EHV-3 (Hartley et al., 1999), bovine herpesvirus 1 (BHV-1) (Keil et al., 1996) and bovine herpesvirus 5 (BHV-5) (Engelhardt and Keil, 1996), but not in VZV (Gomi et al., 2002). EHV-1 gG was shown to bind to chemokines of human and mouse origin and to act as a viral chemokine binding protein by blocking interaction of chemokines with both chemokine specific receptors and glycosaminoglycans (Bryant et al., 2003). EHV-1 with deletion of gG based on the EHV-1 strain RacL11 showed a more pronounced inflammatory response in comparison with wild-type virus (Von Einem et al., 2007). Chemotaxis experiments demonstrated *in vitro* that EHV-1 gG was indeed able to inhibit migration of equine neutrophils in response to recombinant equine IL-8 (Van de Walle et al., 2007).

EHV-4 gG is also secreted into the medium of infected cells and shares an overall identity of 58% with its EHV-1 counterpart. In EHV-4 gG, no chemokine binding activity induced interference with neutrophil migration was found (Bryant et al., 2003; Crabb and Studdert, 1993; Van de Walle et al., 2007). The immunomodulatory property of EHV-1 gG might contribute to ability of EHV-1 to induce a more severe and systemic outcome of infection in contrast to EHV-4 (Bryant et al., 2003; Van de Walle et al., 2007). There is a hypervariable region, comprising amino acid 287–382 in EHV-4 gG and amino acid 288–350 in EHV-1 gG, which shared only 21% sequence identity (Crabb et al., 1995). The hypervariable region was responsible for the potential of EHV-1 gG, but not EHV-4 gG, to bind chemokines by using hybrid proteins in which the hypervariable region of EHV-1 and EHV-4 gG was exchanged. The epitope comprising amino acid 301–340 of EHV-1 gG were shown to be responsible for binding to chemokines *in vitro* (Van de Walle et al., 2009).

gH and gL

EHV-1 gH is essential for EHV-1 replication, plays a role in cell-to-cell spread and significantly affects plaque size and growth kinetics. EHV-1 entry into cells is presumed to require five viral envelope glycoproteins (gB, gC, gD and the heterodimer gH/gL) and is mediated through different cell surface receptors (Azab et al., 2012b; Sasaki et al., 2011). Depending on the type of cell, entry of EHV-1 can occur via endocytosis or fusion at the plasma membrane (Van de Walle et al., 2008; Frampton, et al., 2007; Hasebe et al., 2009). Among herpesviral glycoproteins, gB, gH, and gL are conserved across all herpesviruses and consequently essential for virus entry and cell fusion. Several studies suggested that gH/gL itself has fusogenic properties (Galdiero et al., 2008; Subramanian, 2007).

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The interact between gB and gH/gL during *alphaherpesviral* fusion is still not fully understood but recent studies suggest that fusion is a stepwise process starting with gD binding to its cognate receptors, followed by activation of gH/gL to prime gB for fusion (Atanasiu et al., 2007; Avitabile et al., 2007). However, the crystal structures of HSV-2 gH/gL, EBV gH/gL and PRV gH revealed that gH/gL does not resemble any known fusion protein. Instead, it may act as a fusion regulator (Atanasiu et al., 2010; Fan et al., 2009).

The structural studies showed that gH has three distinct domains with the N-terminal domain (domain H1) shown to bind to gL (Chowdary et al., 2010). It has long been known that in the case of EBV and HSV, gL is required for correct folding, trafficking and function of gH (Backovic et al., 2010; Fan et al., 2009).

gK

EHV-1 gK is either involved in an early step of virus assembly before the cell to-cell spread and virus egress pathways diverge, or gK is involved in membrane fusion processes that are important for both pathways (Neubauer et al., 2004). EHV-4 gK is essential for virus replication *in vitro* and that the gK-negative strain was not able to be reconstituted in equine cells (Azab et al., 2012a).

PRV gK might be necessary to avoid immediate re-fusion of mature virions with the virus-producing cells. gK facilitates virus penetration (Klupp et al., 1998), maturation of PRV gK was proven to depend on yet another multi-hydrophobic protein, as gK is only fully glycosylated in the presence of the UL20 product (Dietz et al., 2000). HSV-1 showed that presence of gK on the cell surface was dependent on the expression of UL20, indicating that this interaction might be conserved (Foster et al., 2003).

gM

Glycoprotein M is a nonessential glycoprotein that is conserved throughout all herpesviral subfamilies (JoĒns et al., 1998), EHV-1 gM like its counterparts in other herpesviruses represents a multiply hydrophobic class III membrane protein that contains eight putative transmembrane domains (Osterrieder et al., 1996; Telford et al., 1992). EHV-1 and EHV-4 gM carry more than one linear epitope between amino acid 376–450 and the major epitopes have to be distinct, although the amino acid sequences of EHV-1 and EHV-4 gM are 86% identical (Telford et al., 1998). EHV-4 gM is more important for a carefully balanced set of functional and structural interactions than the respective homologues in most other herpesviruses. Only the gM-homologue of the strictly cell associated MDV is essential for virus replication (Tischer et al., 2002). It could be hypothesized that EHV-4 might be more cell-associated than EHV-1.

In some alphaherpesviruses, including EHV-1, the simultaneous deletion of gM, gE and gI increases the defects in plaque formation and virus growth, demonstrating that these three proteins fulfil somewhat overlapping functions in secondary envelopment (Brack et al., 1999; Seyboldt et al., 2000), but none is essential by itself. One of the reasons to initiate electron microscopic analysis was the idea that gM alone might be so important for secondary envelopment that an accumulation of particles in the Golgi area might occur. EHV-1 gM or the gM/UL49.5 complex and the gE/gI complex play in the life cycle.

Latency

Latency was considered as an important epidemiological strategy ensuring survival and spread within the natural host population (Whitley and Gnann, 1993). For both EHV-1 and EHV-4, latency has been demonstrated in lymphoid as well as in neural tissues (Welsh et al., 1992; Gibson et al., 1992; Edington et al., 1994; Slater et al., 1994; Baxi et al., 1995; Borchers et al., 1999). Experimentally, EHV-1 has been reactivated from both naturally reared (Edington et al., 1994) and experimentally infected conventional (Edington et al., 1985) and specific pathogen free (Slater et al., 1994) horses upon immunosuppression. Infectious virus was recovered principally from leukocytes and only occasionally from nasal mucus after corticosteroid treatment of conventional horses (Edington et al., 1985), whereas considerable shedding in nasal mucus without recurrence of viraemia both after corticosteroid and cyclophosphamide treatment was recorded in specific pathogen free horses (Slater et al., 1994). The CD5+/CD8+ T lymphocytes were defined as the predominant site for EHV-1 latency, which was independently and indirectly activated by both IL-2 and equine chorionic gonadotrophin (Smith et al., 1998). Predominantly nasal virus shedding was recorded for reactivated EHV-4 from field-infected horses upon corticosteroid treatment (Browning et al., 1988). It is assumed that reactivated latent virus shed in nasal mucus plays a key role in the epidemiology of EHV-1 and EHV-4 infections.

Control and vaccination

In the last 50 years, many attempts have been made to immunize horses against both EHV-1 and EHV-4 with the objective of mimicing the resistance to disease induced by natural infection and thereby reducing the impact of disease caused by these two equine pathogens. Those efforts have been extensively described

(Allen et al., 1986; Bryan et al., 1982 and1986; Doll et al., 1963; Mumford et al., 1984) and have culminated in the availability of more than a dozen commercially manufactured products that are currently marketed for use in controlling the diseases caused by EHV-1 and EHV4 (Allen, 2002).

Adult horses respond to vaccination by these commercial immunogens by developing high titres of serum antibodies directed against the viruses (Bryans et al., 1980 and1982; Burrows et al., 1984; Crandell et al., 1980; Doll et al., 1963; Purdy et al., 1978). Weaker antibody responses are elicited in young, immunologically naïve animals. Antibody response may be undetectable in immunized foals possessing maternally-derived antibodies directed against the viruses at the time of vaccination. (Wilson et al., 1999; Breathnach et al., 1999). Vaccination of horses for EHV-1 and EHV-4 should be considered as only a supplemental tool. No currently registered vaccine for EHV-1 or EHV- 4 claims to be completely effective in preventing either infection or disease that may result from exposure. The primary beneficial effects derived from vaccination of horses against EHV-1 and EHV-4 are reduction in the severity and/or duration of respiratory disease in the young vaccinated animal and reduction of the overall incidence of fetal loss during outbreaks of abortion.

The aim of our study

In spite of many trials to develop the effective vaccine, there is no complete vaccine and EHV-1 and EHV-4 infection still threaten horses all over the world and lead to highly economic losses in horse industries. In this study, we try to understand the glycoproteins of these viruses because these glycoproteins play important role in the virus structure and infection to cells.

At first, we produced MAbs to EHV-1 and EHV-4and these MAbs were used as a tool to characterize the glycoproteins of EHV-1 and EHV-4. Total fourteen MAbs were produce for EHV-1; Four MAbs had virus-neutralizing activity without complement. Seven MAbs were EHV-1-specific, while the other two recognized EHV-4. Four MAbs recognized gB, four did gC and one did gp2 by immunoblot analysis and indirect immunofluroscent assay. Eight MAbs to EHV-4 were produced. Both MAbs were EHV-4-specific One MAb was specific to gB and one was to gC by indirect immunofluroscent assay. Furthermore, total of 23 glycoproteins of EHV-1 and EHV-4 were expressed in mammalian cells and characterized their immunogenicity.

Table 1. Function of glycoproteins for EHV-1 and EHV-4

Glycoprotein	Essential	Function
gp2	No	Virulence factor role in inflammation
gB	Yes	Cell penetration and cell-to-cell spreading
gC	No	Attachment egress
gD	Yes	Cell-to-cell spread role in endocytic entry
gE	No	Cell-to-cell spread
gG	No	Viral chemokine binding protein
gH	Yes	Unclear
gI	No	Cell-to-cell spread
gK	Yes	Cell-to-cell spread egress
gL	Yes	Unclear
gM	No	Penetration cell-to-cell spread
gN	No	gM processing

Fig. 1. Genomic organization of EHV-1

Abbreviations: TR, terminal repeat; IR, internal repeat; U, unique; L, long; S, short.

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Envelope

Tegument

Capsid

DNA
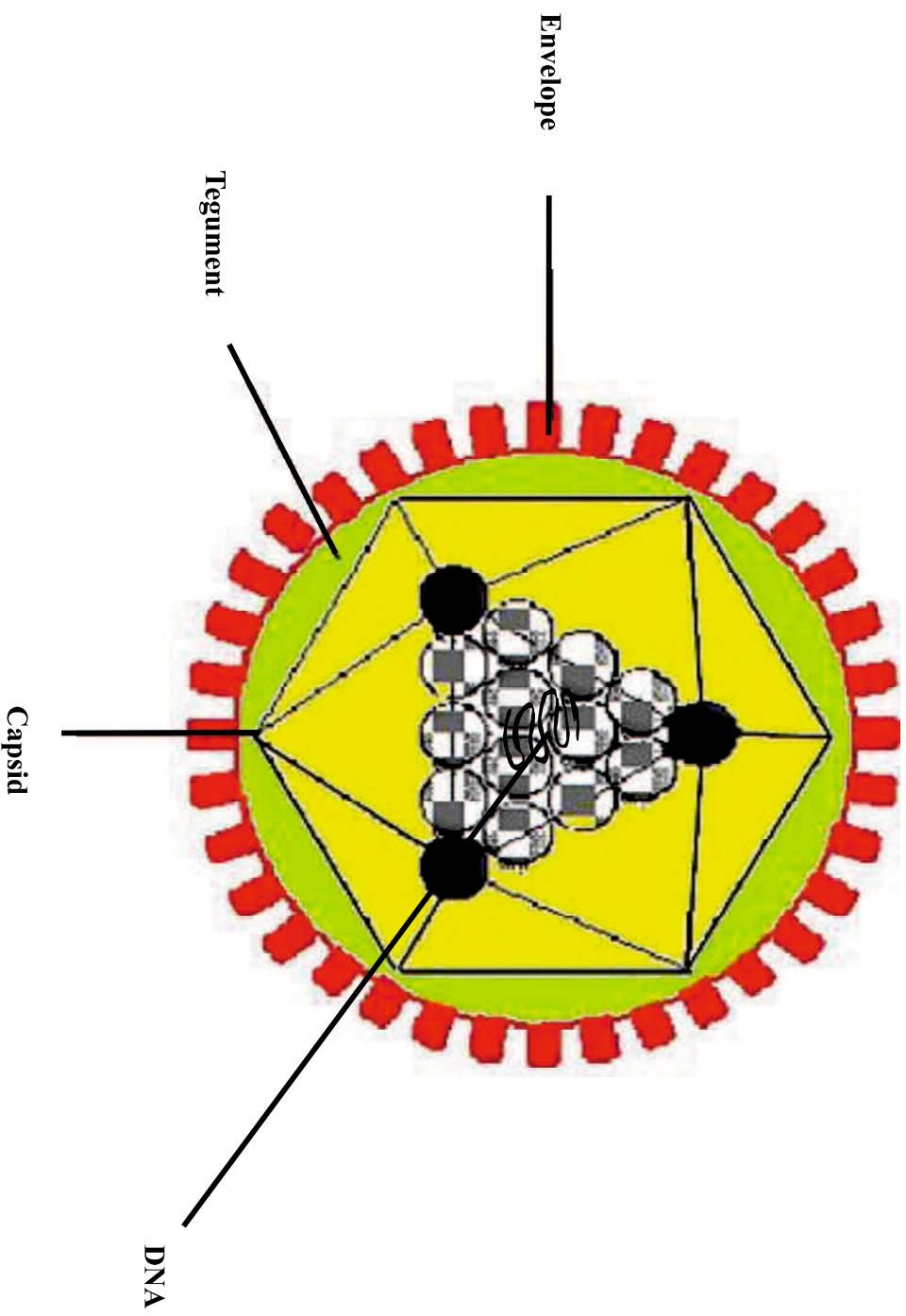
Fig.2. Schematic of EHV-1structure

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Chapter 1

Characterization of glycoproteins in EHV-1

1.1. Summary

In this study, we attempted to express twelve glycoproteins of equine herpesvirus-1 (EHV-1) in 293T cells, and to characterize these using monoclonal antibodies (MAbs) and horse sera against EHV-1. Expression of glycoprotein B (gB), gC, gD, gG, gI and gp2 were recognized by immunoblot analysis using horse sera, but gE, gH, gK, gL, gM and gN was not. Four MAbs recognized gB, four recognized gC and one recognized gp2. Two MAbs against gB cross-reacted with EHV-4. Interestingly, coexpression of gE and gI and gM and gN enhanced their antigenicity. Furthermore, immunoblot analysis of gp2 showed that different molecular masses of gp2 were recognized by the MAb against gp2 and horse sera against EHV-1. In this study, it was demonstrated that at least six glycoproteins were immunogenic to horses and coexpression of gE and gI and gM and gN was important for enhancement of antigenicity.

1.2. Introduction

Equine herpesvirus-1 (EHV-1) is a member of genus *Varicellovirus*, subfamily *Alphaherpesvirinae*, family *Herpesviridae* and order *Herpesvirales*. EHV-1 distributed worldwide (Allen et al., 1999) and is a major cause of respiratory disease in horses, resulting in serious economic losses in the horse industry. In addition, EHV-1 causes epidemic or sporadic abortion in mares and myeloencephalopathy (Allen et al., 1986; Crabb et al., 1995; Studdert et al., 2003). Acquired immunity induced by natural infection or vaccination against EHV-1 is generally short-lived, and consequently, horses may suffer from repeated infections (Turtinen et al., 1982). Herpesviral glycoproteins are essential in infection processes including virus adsorption, penetration and cell-to-cell spread, and plays significant roles in eliciting both humoral and cellular immune responses in horses (Norrild et al., 1985; Paillot et al., 2008). In EHV-1, eleven viral glycoproteins, gB (gp14), gC (gp13), gD (gp18), gE, gG, gH, gI, gK, gL, gM and gN are conserved in comparison with those of other alphaherpesviruses. However, glycoprotein2 (gp2) is encoded only in equine alphaherpesviruses, EHV-1, equine herpesvirus-4 (EHV-4) and asinine herpesvirus 3 (AHV-3) (Paillot et al., 2008). These twelve glycoproteins of EHV-1 are possible candidates for vaccine antigens. In some glycoproteins of herpesviruses, co-expression with different glycoproteins was often required for efficient expression and protein folding (Crump et al., 2004; Farnsworth et al., 2003; Kukreja et al., 1998; Rudolph et al., 2002; Norrild et al., 1985), but the detail in EHV-1was still unclear. To characterize their immunogenicity and functions, twelve glycoproteins of EHV-1 were expressed *in vitro* and characterized using established monoclonal antibodies (MAbs) against EHV-1 and sera collected from EHV-1-infected horses.

1.3. Materials and methods

1.3.1. Cells

FHK-Tcl3.1 (Andoh et al., 2009; Maeda et al., 2007) and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated fetal calf serum (FCS; JR Scientific, Woodland, CA, U.S.A.), 100 units/ml penicillin and 100 μg/ml streptomycin (GIBCO) at 37°C under 5% CO_2. The myeloma cell line P3U1 was maintained in RPMI1640 medium (GIBCO) supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 55 μM of 2-mercaptoethanol (2-ME) (GIBCO) at 37°C under 5% CO_2.

1.3.2. Viruses

EHV-1 strain 89c25 was isolated from a racehorse with respiratory disease caused by EHV-1 respiratory infection (Matsumura et al., 1992). 89c25 was plaque-purified three times in primary fetal horse kidney (FHK) cells and designated as 89c25p. EHV-1 was propagated in FHK-Tcl3.1 cells. EHV-4 TH20p strain (Kawakami et al., 1962; Damiani et al., 1998) was propagated in FHK-Tcl3.1 cells and was used for indirect immunofluorescence assay (IFA).

1.3.3. Construction of expression plasmids

For expression of glycoproteins of EHV-1, each gene was amplified from the genome of EHV-1 89c25p by polymerase chain reaction (PCR) using primers (Table 1. 1). PCR was performed by using Takara LA PCR Kit Ver. 2.1 (Takara, Otsu, Japan) and the amplified fragments were digested with restriction enzymes, and were then cloned into the expression plasmid pCAGGS, which was kindly provided by Dr.

Miyazaki (Osaka University). The nucleotide sequences of at least two constructed plasmids were determined using a Big Dye Terminator v3.1 kit (Applied Biosystems, Foster City, CA, U.S.A.). All plasmids were purified by QIAprep Spin Miniprep Kit (QIAGEN, Maryland, U.S.A.) or QIAfilter plasmid Midi Kit (QIAGEN).

1.3.4. Expression in 293T cells

293T cells were transfected with purified plasmids using polyethylenimine (PEI). Briefly, 3.2 µg of plasmid were mixed with 8 µl of PEI (2 mg/ml) and were then transfected to 293T cells in 6 wells plate (Sumitomo Bakelite, Tokyo, Japan), as reported previously (Boussif et al., 1995).

1.3.5. Production of MAbs

BALB/c mice (female, aged six weeks) were intraperitoneally inoculated with 1×10^5 PFU of EHV-1 three times at an interval of three or four weeks. At 3 to 7 days after final immunization, splenocytes were collected and fused with P3U1 myeloma cells using 50% polyethylene glycol solution (Hybri-Max^{TM}; Sigma, St. Louis, MO, U.S.A.). Hybridoma cells were selected in GIT media (Wako, Osaka, Japan) containing hypoxanthine aminopterin thymidine supplement (HAT) (GIBCO), 10% BM-condimed H1 hybridoma cloning supplement (Roche Diagnostics, Mannheim, Germany) and 10% FCS for 7 days at 37°C under 5% CO_2. Supernatants of hybridomas were screened by virus-neutralization (VN) test and/or IFA. Hybridoma cells secreting EHV-1-specific MAbs were cloned twice by limiting dilution method and were then inoculated into BALB/c mice pretreated with pristane (Sigma) for preparation of ascites.

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1.3.6. VN test

In order to detect VN activity, supernatants of hybridoma or diluted ascites were mixed with EHV-1 for 60 min at 37°C, and then the mixtures were directly inoculated into FHK-Tcl3.1 cells that were washed with DMEM without FCS. Control was carried out without hybridoma or ascites. After incubation for 60 min at 37°C under 5% CO_2, cells were washed twice with DMEM without FCS and overlaid with 0.8% agarose (SeaPlaque GTG agarose; Lonza, Rockland, ME, U.S.A.) in DMEM containing 10% FCS. Plates were placed at 37°C in 5% CO_2 for 3 days and cells were fixed with 5% buffered formaldehyde. Agarose layers were removed, and cells were stained with crystal violet. The number of plaques was counted, and hybridoma or diluted ascites that reduced the number of plaques by more than 50% in comparison with the mean number of plaques in control wells were considered to be positive.

1.3.7. IFA

Virus-infected FHK-Tcl3.1 cells or plasmid-transfected 293T cells was collected, washed with PBS three times and placed on 24-well microscope slides (Matsunami Glass, Osaka, Japan). After fixation with cold acetone for 30 min, cells were incubated with MAbs for 60 min at 37°C. Cells were then washed three times in PBS and incubated with goat anti-mouse Ig (H+L)-FITC human-adsorbed (Southern Biotech, Birmingham, AL, U.S.A.) for 30 min at 37°C. After washing three times, fluorescence was observed under a Nikon Optiphot 2 EFD3 fluorescence phase contrast microscope (Nikon, Tokyo, Japan).

1.3.8. Immunoblot analysis

Virus-infected FHK-Tcl3.1 cells or plasmid-transfected 293T cells were extracted with RIPA (25mM Tris-HCl (pH 7.6), 150 mM sodium chloride (NaCl), 1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate and 1% Triton X-100) and then dissolved in 2×SDS sample buffer (125 mM Tris-HCl, 4% SDS, 40% glycerol and 0.002% bromophenol blue) with or without 2-ME or directly dissolved in 1×SDS sample buffer with or without 5% 2-ME. After boiling for 3 min, samples were loaded on SDS-polyacrylamide gel (PAGE), and electrophoresis was carried out in SDS buffer (25 mM Tris, 192 mM glycine and 0.1% SDS). Then, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Immobillon; Millipore, Billerica, MA, U.S.A.) by semi-dry blotting apparatus (Biocraft, BE-310, Tokyo, Japan). After membranes were reacted with 3% gelatin (Bio-rad, Hercules, CA, U.S.A.) in TBS (20 mM Tris-HCl and 150 mM NaCl, pH 7.5) for 30 min at 37°C, they were then washed three times with TBS containing 0.05% Tween 20. After washing, the membrane was incubated with diluted horse sera or MAbs for 1 hr at 37°C as primary antibody, followed by incubation with the peroxidase-conjugated F (ab)$_2$ fragment of anti-horse IgG (H&L) goat (Rockland, Gilbertsville, PA, U.S.A.) or peroxidase-conjugated goat affinity purified antibody against mouse imunoglobulins IgG, IgA and IgM (Cappel, Solon, OH, U.S.A.) for 30 min at 37°C as secondary antibody. All antibodies were diluted with TBS containing 0.05% Tween 20 and 1% gelatin. The reaction was visualized using 0.03% diaminobenzidine (Wako) and 0.009% H$_2$O$_2$ (Wako) in TBS.

1.3.9. Sera

Sera were collected from three foals that were experimentally infected with EHV-1 89c25p (Tsujimura et al., 2009). Serum specific to EHV-1 gE was collected

from BALB/c mice immunized with fusion protein of glutathione S-transferase and gE (169-201) (Andoh et al., 2013).

1.3.10. Immunoglobulin class and subclass

Immunoglobulin class and subclass of MAbs were determined using mouse monoclonal antibody isotyping kit (Roche).

1.4. Results

1.4.1. Sequence analysis of each glycoprotein of EHV-1 strain 89c25p

Nucleotide sequences of at least two cloned genes encoding each glycoprotein of 89c25p were determined and compared with those of EHV-1 strain Ab4 (Telford et al., 1992). The results showed that the amino acid sequences of gC, gD, gE, gG, gH, gI, gK, gL and gN of 89c25p were identical to those of Ab4, but those of gp2, gB and gM were different at positions 95 (Serine to Phenylalanine), 938 (Lysine to Glutamic acid) and 84 (Methionine to Lysine), respectively (Table 1. 2). These plasmids were designated as pCAG-gp2-1 pCAG-gB-1, pCAG-gC-1, pCAG-gD-1, pCAG-gE-1 pCAG-gG-1, pCAG-gH-1, pCAG-gI-1, pCAG-gK-1, pCAG-gL-1, pCAG-gM-1 and pCAG-gN-1.

1.4.2. Expression of EHV-1 glycoproteins in 293T cells

Twelve expression plasmids encoding each glycoprotein were transfected into 293T cells, and expression was examined by immunoblot analysis using horse sera. Expressed gp2, gB, gC, gD, gG and gI were detected, but gE, gH, gK, gL, gM and gN were not. Three bands with molecular masses of approximately 230, 65 and 42 kilo Daltons (kDa) were detected in pCAG-gp2-1-transfected cells, two bands of 148 and

135 kDa were detected in pCAG-gB-1-transfected cells, two bands of 150 and 68-75 kDa were detected in pCAG-gC-1-transfected cells, one band of 50 kDa was detected in pCAG-gD-1-transfected cells, two bands of 100 and 50 kDa were detected in pCAG-gG-1-transfected cells, and two bands of 140 and 65 kDa were detected in pCAG-gI-1-transfected cells (Fig. 1. 1).

1.4.3. Establishment and characterization of MAbs

In order to characterize EHV-1 glycoproteins, fourteen MAbs against EHV-1 strain 89C25p were produced (Table 1. 3). Four MAbs, 5F8, 1G10, 8H4 and 8B2, had VN activity against EHV-1 and all were specific to gC (Table 1. 3 and Fig. 1. 2). Four MAbs, 5F12, 6F4, 6G12 and 7E11, were specific to gB (Table 1. 3 and Fig. 1. 3A) and two, 6F4 and 6G12, cross-reacted with EHV-4 (Table 1. 3). One MAb 8H11 was specific to gp2 (Table 1. 3 and Fig. 1. 3B). All MAbs did not recognize glycoproteins on immunoblot analysis under reducing conditions. In addition, the molecular masses of gp2, gB and gC expressed in 293T cells were similar to those in EHV-1-infected cells (Figs. 1. 4A, B and C). Interestingly, the molecular mass of gp2 detected by MAb 8H11 was over 250 kDa and was different from those detected in horse sera, 230, 65 and 42 kDa (Fig. 1. 5B).

1.4.4. Co-expression of glycoproteins

EHV-1 gE and gI, gM and gN and gH and gL were co-expressed in 293T cells, meaning that co-expressed gE and gI, and gM and gN reacted strongly with horse sera, but co-expressed gH and gL did not (Fig. 1. 6C). With co-expression of gE and gI, two additional bands other than gI-specific bands with molecular masses of 80-90 and 250 kDa were seen (Figs. 1. 5A and 1. 6A). Furthermore, mouse antibody specific to

gE also recognized two bands with molecular masses of 80-90 and 250 kDa with co-expressed gE and gI, but not with individually expressed gE and gI (Fig. 1. 5A). With co-expression of gM and gN, one band with a molecular mass of over 250 kDa was observed (Fig. 1. 6B).

1.5. Discussion

In this study, twelve glycoproteins in EHV-1 were analyzed using horse sera against EHV-1 and established MAbs. In expression systems for single glycoproteins, gp2, gB, gC, gD, gG and gI reacted strongly with horse sera (Fig.1. 1). Although high immunogenicity has been reported for gp2, gB, gC, gD and gG (Crabb et al., 1991, 1993 and 1995), it is interesting that the immunogenicity of gI was also high. Furthermore, gp2, gB and gC expressed in 293T cells were similar to those expressed in EHV-1-infected cells (Figs. 1. 4A, B and C), indicating that these proteins were mature when expressed *in vitro*.

We reported that EHV-1 gE is able to induce antibodies in horses and that the 20 amino acids of gE at positions 169 to188 were a useful antigen for ELISA to differentiate EHV-1 and EHV-4 infections (Andoh et al., 2013). However, gE expressed in 293T cells was not recognized by horse sera (Figs. 1. 1, 1. 5A and 1. 6A). After co-expression with gI, gE strongly reacted with horse sera against EHV-1 and mouse antibody specific to gE (Fig. 1. 6A), indicating that gI might be required for efficient expression of gE. In EHV-4, gE (95kDa) was co-precipitated with gI (75kDa) by immunoprecipiration using anti-gI serum, but not by anti-gE serum (Damiani et al., 2000a). It is unknown whether two bands, 80-90 and 250 kDa, in co-expressed gE and gI contain only gE or complex of gE and gI. Further experiment will be required to clarify the role of gI in expression of gE.

As shown in (Fig. 1. 6B), over 250 kDa proteins in co-expressed gM and gN strongly reacted with horse sera against EHV-1, indicating that gM/gN complex is important for antigenicity in horses. In a previous study, it was reported that gN is necessary and sufficient for functional processing of gM (Rudolph et al., 2002b). Therefore, we speculated that immature gM and gN are expressed in a single expression system and that coexpression of gM and gN induces maturation of gM and enhances the antigenicity. However, it is still unknown whether over 250kDa protein contains only gM or both gM and gN.

In EHV-1, it is reported that co-expression of gH and gL is important for efficient expression and for induction of protective immunity (Kukreja et al., 1998; Stokes et al., 1996). Therefore, co-expression of gH and gL was examined, but reactions with horse sera were not observed (Fig. 1. 6C). It is unknown whether antigenicity of EHV-1 gH and gL against horse is low, or whether another protein is required for maturation of EHV-1 gH and gL.

Horse sera against EHV-1 and MAb against gp2 recognized different molecular masses of gp2, 230, 65 and 42 kDa, and over 250 kDa, respectively (Fig. 1. 5B). EHV-1 gp2 is rich in serine and threonine residues and is a heavily *O*-glycosylated protein with a molecular mass in the range of 192 to 400 kDa (Sun et al., 1994; Telford et al., 1992; Whittaker et al., 1990). Furthermore, it has been reported that gp2 is cleaved into a highly glycosylated *N*-terminal subunit (over 200 kDa) and *C*-terminal subunit (42 kDa) (Learmonth et al., 2002). Therefore, horse sera against EHV-1 and MAb 8H11 might recognize different positions of gp2, the *C*-terminal subunit and glycosylated *N*-terminal subunit, respectively.

1.6. Conclusion

In conclusion, our results showed that efficient expression and/or maturation of two glycoproteins, gE and gM, requires co-expression of gI and gN, respectively. In addition, these materials, expression plasmids and MAbs, may be useful for further analysis of EHV-1 glycoproteins.

1.7. Figure legends

Fig. 1. 1. Immunoblot analysis of EHV-1 glycoproteins expressed in 293T cells. 293T cells were transfected with each plasmid and FHK-Tcl3.1 cells were infected with EHV-1. Immunoblot analysis was carried out under non-reducing conditions. Sera collected from horses experimentally infected with EHV-1 were used as primary antibody.

Fig. 1. 2. EHV-1 glycoprotein C (gC) in 293T cells by IFA, MAbs 5F8, 8H4, IG10 and 8B2 were used as a primary antibodies.

Fig. 1. 3. EHV-1 glycoprotein B (gB) in 293Tcells by IFA. MAbs 6F4, 5F12, 6G12 and 7E11 were used as primary antibodies. (B) EHV-1 glycoprotein 2 (gp2) in 293Tcells by IFA. MAb 8H11 was used as a primary antibody.

Fig. 1. 4. Comparison of gp2, gB and gC expressed in plasmid-transfected 293T cells and EHV-1-4-infected FHK-Tcl3.1 cells. Immunoblot analysis was carried out under non-reducing conditions. MAbs, 8H11(A), 5F12 (B) and 1G10 (C) were used as primary antibodies.

Fig. 1. 5. (A) Comparison of co-expressed gE and gI by immunoblot analysis using mouse sera specific to gE (169-201) and horse sera specific to EHV-1. Black arrowheads show gE-specific bands and open arrowheads do gI-specific bands. (B) Comparison of molecular masses of gp2 detected by MAb 8H11 and horse sera specific to EHV-1. Immunoblot analysis was carried out under non-reducing conditions. Black arrowheads show gp2-specific bands.

Fig. 1. 6. Co-expression of EHV-1 glycoproteins. Plasmids pCAG-gE-1 and pCAG-gI-1 (A), pCAG-gM-1 and pCAG-gN-1 (B), and pCAG-gH-1 and pCAG-gL-1 (C) were co-transfected into 293T cells. Immunoblot analysis was carried out under non-reducing conditions. Sera collected from horses experimentally infected with EHV-1 were used as primary antibody. The amount of plasmid DNA transfected into 293T cells in 35mm dishes is noted under each figure.

Table 1. 1. Primers based on the complete genome of Ab4 strain

Genes	Primers		Position[a]	
	Name	Sequences[b]	Start	stop
gB	gB1-F	5´-ACGAATTCatgtcctgttgccgttc -3´	61432	61452
	gB1-R	5´-ACCTCGAGttaaaccatttttcatg -3´	64374	64349
gC	gC1-F	5´-ACAGATCTatgtggttgcctaatctcgtg -3´	22851	22830
	gC1-R	5´-ACGAATTCctaaaagtcagacttcttgtac -3´	21445	21467
gD	gD1-F	5´-ACCTCGAGttacggaagctggtatatt -3´	132791	132771
	gD1-R	5´-ACAGATCTatgcctgctgctgcttgt -3´	131583	131603
gE	gE1-F	5´-ACAGATCTatggagcgttggccgcaag -3´	134406	134426
	gE1-R	5´-ACGAATTCtcatttagtacgacttttagct -3´	136058	136035
gG	gG1-F	5´-ACAGATCTatgttgactgtcttagcagcc -3´	127681	127702
	gG1-R	5´-ACGAATTCttaagcaacgtactcaagtcg -3´	128916	128896
gH	gH1-F	5´-ACAGATCTatgttcaaccgtatcgaaaat -3´	71192	71215
	gH1-R	5´-ACCTCGAGttactactcaataactaacaatttat -3´	73738	73712
gI	gI1-F	5´-ACAGATCTatggccaaactcactggatg -3´	132899	132920
	gI1-R	5´-ACGAATTCctatgattcttctttgatggcct -3´	134173	134150
gK	gK1-F	5´-ACAGATCTatgttactcggtggtagaacgg -3´	7042	7020
	gK1-R	5´-ACGAATTCttaggacgagaggactcgc -3´	6011	6031
gL	gL1-F	5´-ACAGATCTatgtatcaaatcctaattgggtg -3´	108803	108780
	gL1-R	5´-ACCTCGAGttaggtctttgaagattggcg -3´	108147	108169
gM	gM1-F	5´-ACAGATCTatggcacgacgggagagc -3´	94472	94492
	gM1-R	5´-ACGAATTCctatcggtactccgggcat -3´	93120	93100
gN	gN1-F	5´-ACAGATCTatgctgtccacgagattcgt -3´	12084	12104
	gN1-R	5´-ACCTCGAGtcaatgcaaggtgttgcaacat -3´	12386	12365
gp2	gp2-F	5´-ACAGATCTatgggggttcaatctatgtcg -3´	129097	129117
	gp2-R	5´-ACGAATTCttaatatacagacgctcggggg -3´	131490	131468

a: Position in Ab4 strain (Accession No. AY665713.1)

b: Capital letters indicate the restriction endonuclease digestion site and additional two nucleotides.

A final polymerization step for 7 min at 72°C served for the completion of fragmental DNA strands. Finally, the reaction was finished by a 1 min cooling step to 4°C.

The amplified DNA fragments were separated in a 0.8 % agarose gel, stained with ethidium bromide solution

# Table 1. 2. Differences of amino acid sequences of glycoproteins

Glycoproteins	Change of amino acid
EHV-1 gp2	S95F[a]
EHV-1 gB	K938E[a]
EHV-1 gM	M84K[a]

a: Comparison with Ab4 strain (Accession No. AY665713.1)

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Table 1.3. Characterization of monoclonal antibodies to EHV-1

MAbs	Recognized proteins	Isotype	VN test EHV-1	VN test EHV-4	IFA EHV-1	IFA EHV-4	Expression on cell surface
8H11	gp2	IgG2a	<1:10	<1:10	1:1280	<1:10	+
5F8	gC	IgM	1:80	<1:10	1:1600	<1:10	+
1G10	gC	IgM	1:640	<1:10	1:800	<1:10	+
8H4	gC	IgM	1:1280	<1:10	1:800	<1:10	+
8B2	gC	IgM	1:3200	<1:10	1:1600	<1:10	+
5F12	gB	IgM	<1:10	<1:10	1:1280	<1:10	+
6F4	gB	IgG2a	<1:10	<1:10	1:20480	1:800	+
6G12	gB	IgM	<1:10	<1:10	1:1280	1:800	+
7E11	gB	IgM	<1:10	<1:10	1:1280	<1:10	+
8C11	unknown	IgG1	<1:10	<1:10	1:640	1:80	+
5D1	unknown	IgM	<1:10	<1:10	1:5120	1:800	+
4G9	unknown	IgG2b	<1:10	<1:10	1:10240	1:800	+
1G9	unknown	IgG1	<1:10	<1:10	1:10240	1:320	-
1A1	unknown	IgG2b	<1:10	<1:10	1:1280	<1:10	-

Fig. 1.1

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Fig. 1.2

pCAG-gC-1-transfected 293 T cells

5F8

8H4

1G10

8B2
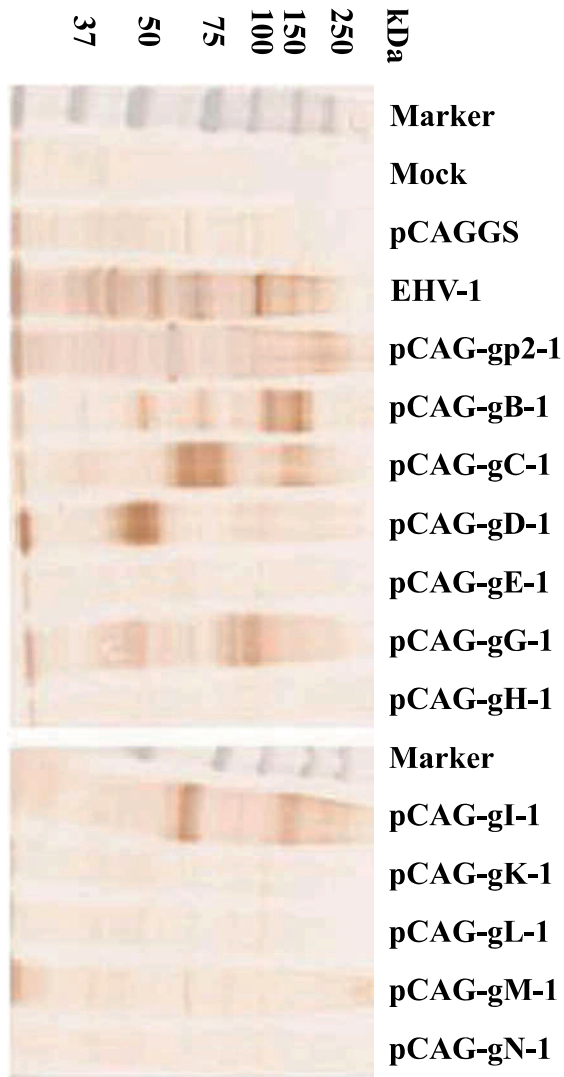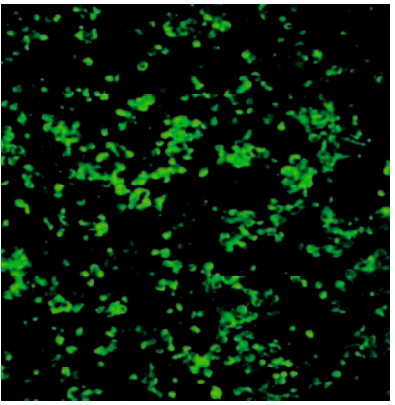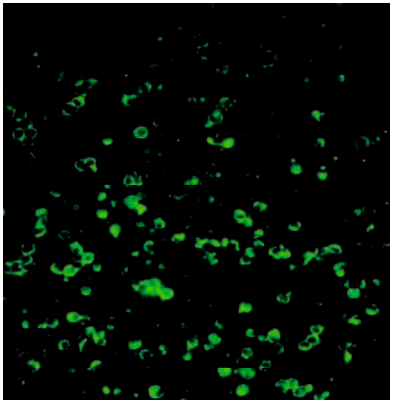
pCAGGS-transfected 293 T cells
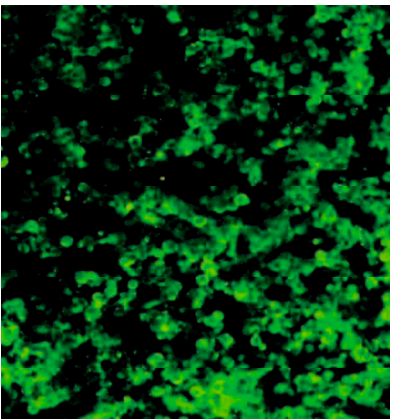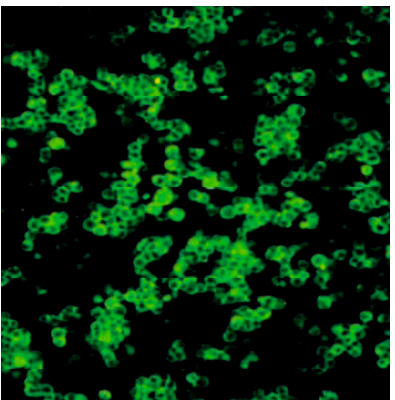
5F8 M
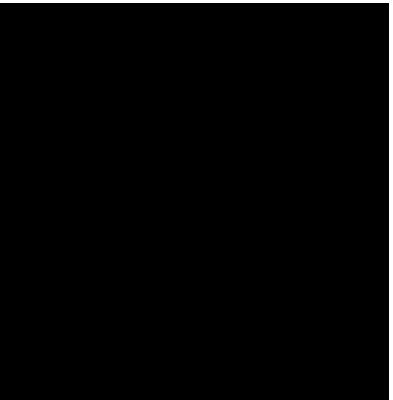
8H4 M
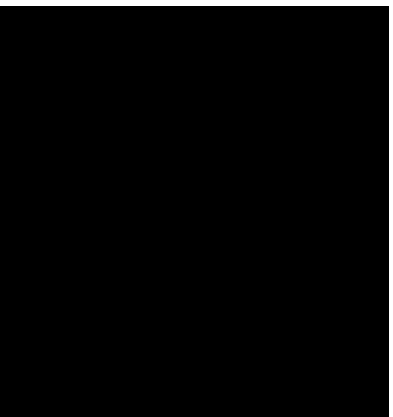
1G10 M
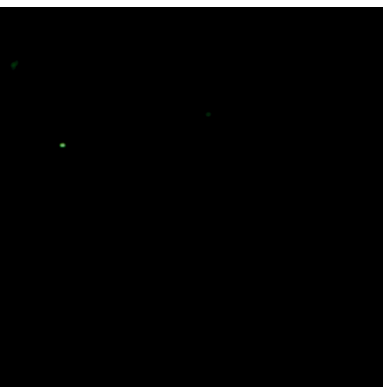
8B2 M

pCAGGS-transfected 293T cells

pCAG-gB-1-transfected 293T cells

Fig. 1. 3

A

6F4 Mock

6F4

5F12 Mock

5F12

6G12 Mock

6G12

7E11 Mock

7E11

8H11 Mock

B
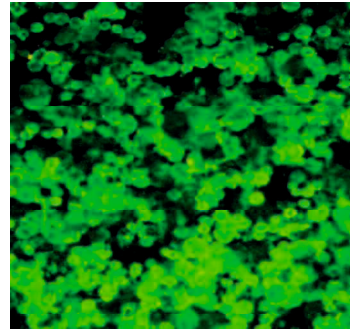
8H11
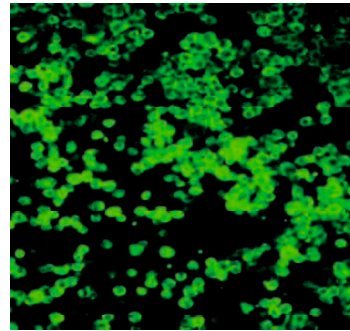
pCAGGS-transfected 293T cells
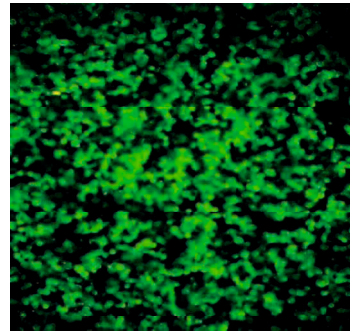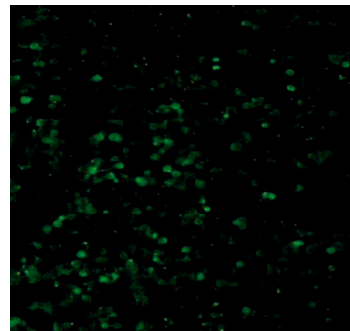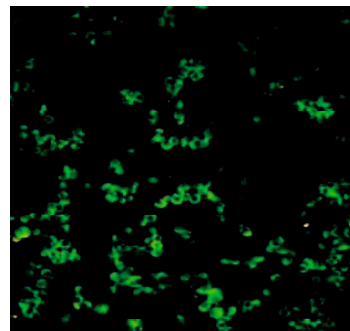
pCAG-gp2-1- transfected   293T cells

43

Fig. 1.4
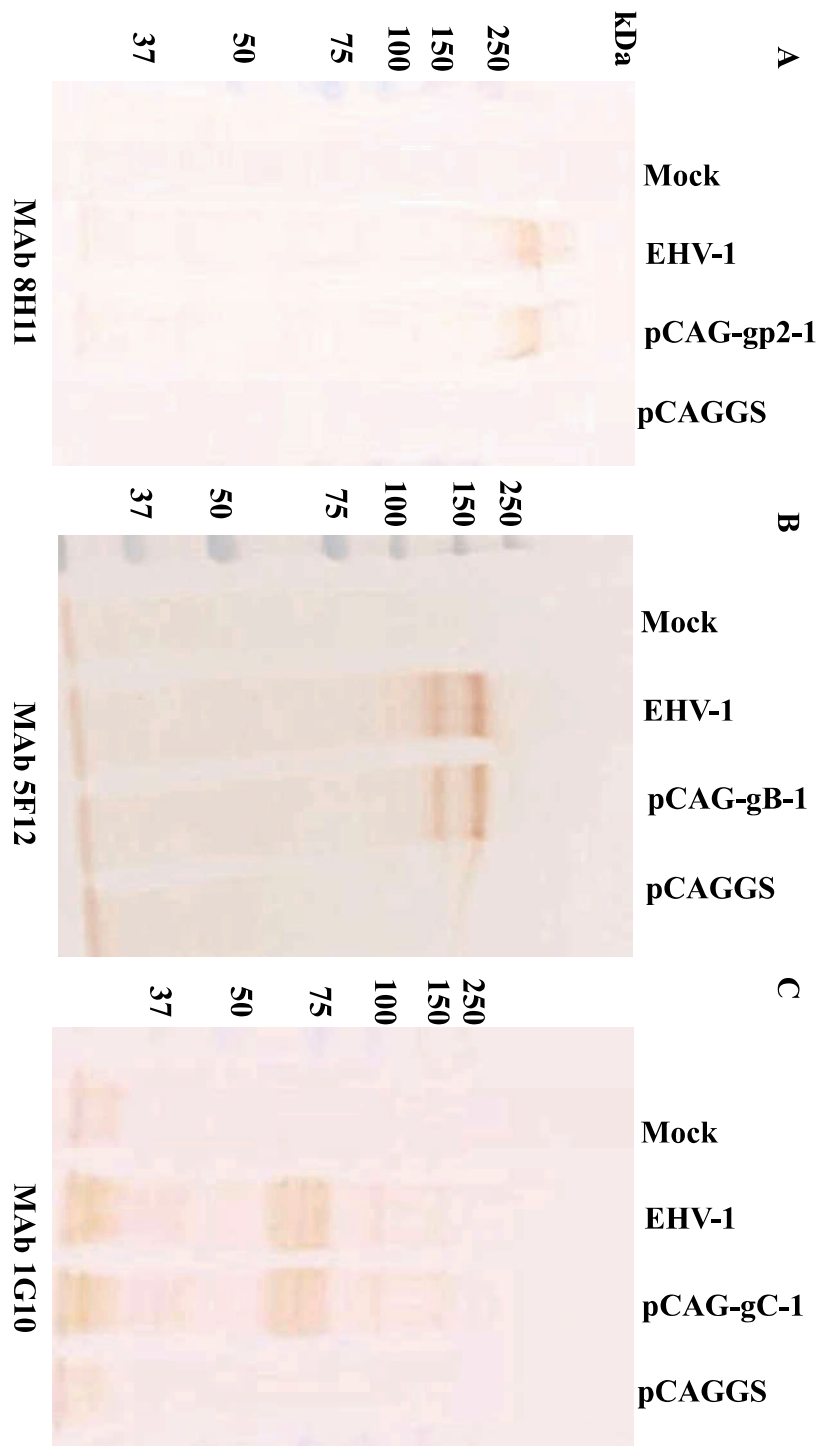
Fig. 1.5
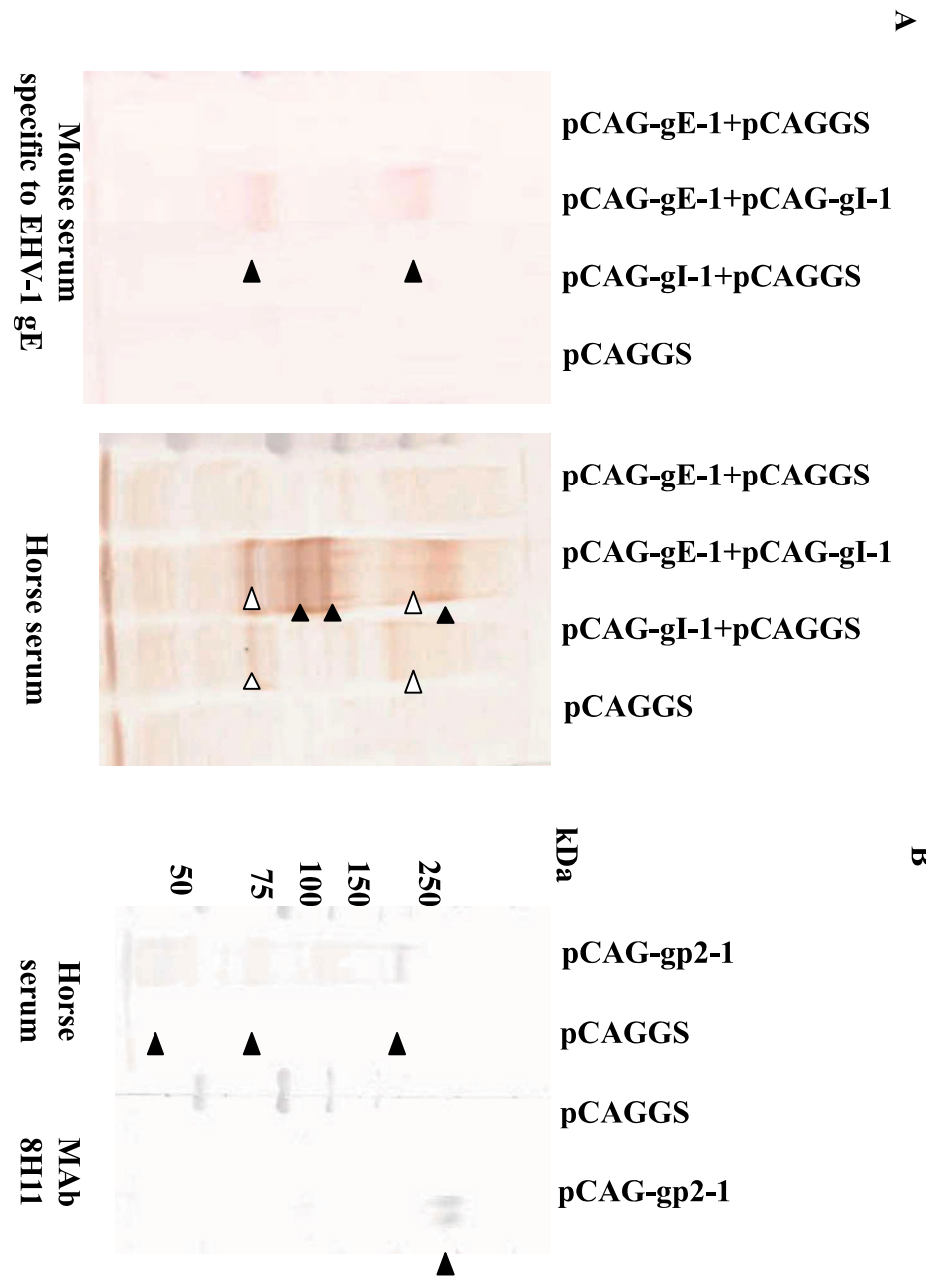
Fig. 1.6
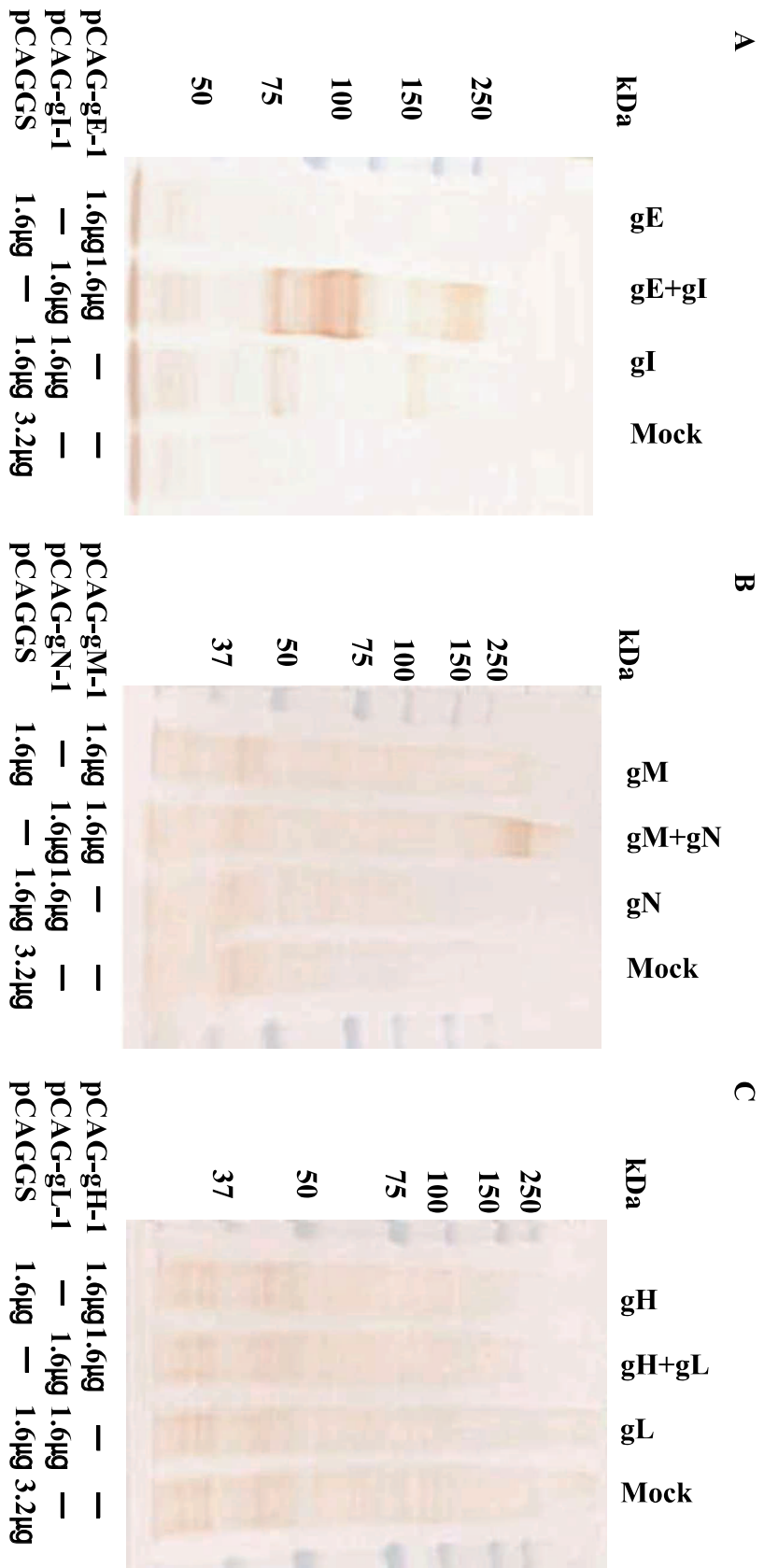
Chapter 2

Characterization of glycoproteins in EHV-4

2.1. Summary

Eleven glycoproteins in EHV-4 were expressed in 293T cells and eight MAbs to EHV-4 were developed. One MAb recognized gB4, one did gC4 and the other six did unknown proteins. Only three glycoproteins, gB4, gD4 and gG4, expressed in 293T cells reacted with sera from EHV-4-infected foals, indicating that these three glycoproteins were immunogenic to horses. However, the other eight glycoproteins were not recognized by horse sera, suggesting that *in vitro* expression of EHV-4 glycoproteins might not be efficient for recognition by horse sera.

2.2. Introduction

EHV-4 is also a member of the *Alphaherpesvirinae* subfamily, genus *Varicellovirus* (Davison et al., 2009; Roizman, 1996). The complete genome sequences of EHV-4 was reported and the virus has a linear double stranded DNA genome of approximately 145 kbp and 57% of G+C content (Cullinane et al., 1988, Darlington and Randall, 1963; Roizmann et al., 1992; Telford et al., 1998). Sequence analysis showed that there is 55–84% DNA homology depending on the gene and that the homology at the amino acid level ranged from 55% to 96% (Telford et al., 1998) between EHV-4 and EHV-1.

EHV-4 mainly causes respiratory disease in horse and rare cases causing abortion, while EHV-1 is responsible for abortion and myeoloencephalopthy in addition to respiratory disease. Both EHV-4 and EHV-1 cause economic losses and negative impact on equine welfare as a result of morbidity or mortality from virus-induced abortion and respiratory disease. However, EHV-4-specific vaccine has not yet been available in Japan.

EHV-4 encodes twelve envelope glycoproteins which are involved in virus attachment, penetration, egress and cell-to-cell spread. Although these glycoproteins were candidates of subunit vaccine, EHV-4 glycoproteins have not been characterized well. In this study, EHV-4-specific MAbs were produced and EHV-4 glycoproteins were characterized *in vitro*.

2.3. Materials and methods

2.3.1. Cells

FHK-Tcl3.1 (Andoh et al., 2009; Maeda et al., 2007) and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island,

NY, U.S.A.) supplemented with 10% heat-inactivated fetal calf serum (FCS; JR Scientific, Woodland, CA, U.S.A.), 100 units/ml penicillin and 100 μg/ml streptomycin (GIBCO) at 37°C under 5% CO_2. The myeloma cell line P3U1 was maintained in RPMI1640 medium (GIBCO) supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 55 μM of 2-mercaptoethanol (2-ME) (GIBCO) at 37°C under 5% CO_2.

2.3.2. Viruses

EHV-4 TH20 strain was isolated from a colt suffering from respiratory disease in1952 (Kawakami et al., 1962) was plaque-purified three times in primary fetal horse kidney (FHK) cells and termed as TH20p.

2.3.3. Construction of expression plasmids

For expression of glycoproteins of EHV-4 each gene was amplified from the genome of EHV-4 TH20p by polymerase chain reaction (PCR) using primers (Table 2.1). PCR was performed by using Takara LA PCR Kit Ver. 2.1 (Takara, Otsu, Japan), and the amplified fragments were digested with restriction enzymes, and were then cloned into the expression plasmid pCAGGS, which was kindly provided by Dr. Miyazaki (Osaka University). The nucleotide sequences of at least two constructed plasmids were determined using a Big Dye Terminator v3.1 kit (Applied Biosystems, Foster City, CA, U.S.A.). All plasmids were purified by QIAprep Spin Miniprep Kit (QIAGEN, Maryland, U.S.A.). or QIAfilter plasmid Midi Kit (QIAGEN).

2.3.4. Expression in 293T cells

293T cells were transfected with purified plasmids using polyethylenimine (PEI). Briefly, 3.2 μg of plasmid were mixed with 8 μl of PEI (2 mg/ml), and were then transfected to 293T cells in 6 wells plate (Sumitomo Bakelite, Tokyo, Japan), as reported previously (Boussif et al., 1995).

2.3.5. Production of MAbs

BALB/c mice (female, aged six weeks) were intraperitoneally inoculated with 1×10^5 PFU of EHV-4 three times at an interval of three or four weeks. At 3 to 7 days after final immunization, splenocytes were collected and fused with P3U1 myeloma cells using 50% polyethylene glycol solution (Hybri-Max^{TM}; Sigma, St. Louis, MO). Hybridoma cells were selected in GIT media (Wako, Osaka, Japan) containing hypoxanthine aminopterin thymidine supplement (HAT) (GIBCO), 10% BM-condimed H1 hybridoma cloning supplement (Roche Diagnostics, Mannheim, Germany) and 10% FCS for 7 days at 37°C under 5% CO_2. Supernatants of hybridomas were screened by virus-neutralization (VN) test and/or IFA. Hybridoma cells secreting EHV-4-specific MAbs were cloned twice by limiting dilution method and were then inoculated into BALB/c mice pretreated with pristane (Sigma) for preparation of ascites.

2.3.6. VN test

In order to detect VN activity, supernatants of hybridoma or diluted ascites were mixed with EHV-4 for 60 min at 37°C, and then the mixtures were directly inoculated into FHK-Tcl3.1 cells that were washed with DMEM without FCS. Control was carried out without hybridoma or ascites. After incubation for 60 min at 37°C under 5% CO_2, cells were washed twice with DMEM without FCS and overlaid

with 0.8% agarose (SeaPlaque GTG agarose; Lonza, Rockland, ME, U.S.A) in DMEM containing 10% FCS. Plates were placed at 37°C in 5% CO_2 for 3 days and cells were fixed with 5% buffered formaldehyde. Agarose layers were removed and cells were stained with crystal violet. The number of plaques was counted and hybridoma or diluted ascites that reduced the number of plaques by more than 50% in comparison with the mean number of plaques in control wells were considered to be positive.

2.3.7. In direct fluorescent assay (IFA)

Virus-infected FHK-Tcl3.1 cells or plasmid-transfected 293T cells were collected, washed with PBS three times and placed on 24-well microscope slides (Matsunami Glass, Osaka, Japan). After fixation with cold acetone for 30 min, cells were incubated with MAbs for 60 min at 37°C. Cells were then washed three times in PBS and incubated with goat anti-mouse Ig (H+L)-FITC human-adsorbed (Southern Biotech, Birmingham, AL, U.S.A.) for 30 min at 37°C. After washing three times, fluorescence was observed under a Nikon Optiphot 2 EFD3 fluorescence phase contrast microscope (Nikon, Tokyo, Japan).

2.3.7. Immunoblot analysis

Virus-infected FHK-Tcl3.1 cells or plasmid-transfected 293T cells were extracted with RIPA (25mM Tris-HCl (pH 7.6), 150 mM sodium chloride (NaCl), 1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate and 1% Triton X-100) and then dissolved in 2×SDS sample buffer (125 mM Tris-HCl, 4% SDS, 40% glycerol and 0.002% bromophenol blue) with or without 2-ME or directly dissolved in 1×SDS sample buffer with or without 5% 2-ME. After boiling for 3 min, samples were loaded

on SDS-polyacrylamide gel (PAGE) and electrophoresis was carried out in SDS buffer (25 mM Tris, 192 mM glycine and 0.1% SDS). Then, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Immobillon; Millipore, Billerica, MA, U.S.A.) by semi-dry blotting apparatus (BIOCRAFT, BE-310, Tokyo, Japan). After membranes were reacted with 3% gelatin (Bio-rad, Hercules, CA, U.S.A.) in TBS (20 mM Tris-HCl and 150 mM NaCl, pH 7.5) for 30 min at 37°C, and were then washed three times with TBS containing 0.05% Tween 20. After washing, the membrane was incubated with diluted horse sera or MAbs for one hour at 37°C as primary antibody, followed by incubation with the peroxidase-conjugated F(ab)$_2$ fragment of anti-horse IgG (H&L) goat (Rockland, Gilbertsville, PA, U.S.A) or peroxidase-conjugated goat affinity purified antibody against mouse imunoglobulins IgG, IgA and IgM (Cappel, Solon, OH, U.S.A) for 30 min at 37°C as secondary antibody. All antibodies were diluted with TBS containing 0.05% Tween 20 and 1% gelatin. The reaction was visualized using 0.03% diaminobenzidine (Wako) and 0.009% H$_2$O$_2$ (Wako) in TBS.

2.3.10. Sera

Sera were collected from six foals, Three foals were challenged with the EHV-4 TH20 revertant and three were challenged with the EHV-4 TH20p (Damiani et al., 2000).

2.3.11. Immunoglobulin class and subclass

Immunoglobulin class and subclass of MAbs were determined using mouse monoclonal antibody isotyping kit (Roche).

2.4. Results

2.4.1. Establishment and characterization of MAbs

Eight MAbs to EHV-4 (3-1 F3A9, 3-3C12E5, 3-3E4H10, 3-2G2A7, 3-2C1H5, 3-1E5A83-1D4H8 and 3-2C10C8) were produced and all of them were only EHV-4-specific by IFA (Table 2. 2). By IFA using each glycoprotein-transfected cells, one MAb 3-1D4H8 was specific to EHV-4 gB and one MAb 3-2C10C8 was specific to gC by IFA (Table 2. 2 and Fig. 2. 1). All MAbs could not detect EHV-4 proteins by immunoblot analysis (Table 2. 2).

2.4.3. Sequence analysis of each glycoprotein of EHV-4 strain TH20p

Nucleotide sequences of genes encoding each glycoprotein showed that the amino acid sequences of gB, gE, gG, gH, gK, gL, gM and gN of TH20p were identical to those of NS80567, but those of gC, gD, gI and gp2 were different at positions 309 (Aspartic acid to Glutamic acid), 27 and 382 (leucine to valine and glycine to glutamic acid, respectively), 134 and 298 (arginine to tryptophan and aspartic acid to alanine, respectively) and 376, 463 and 509 (leucine to serine, lysine to arginine and Glutamine to Arginine, respectively), respectively (Table 2. 3).These genes were cloned into the expression plasmid, pCAGGS, and the resultant plasmids were designated as pCAG-gB-4, pCAG-gC-4, pCAG-gD-4, pCAG-gE-4 pCAG-gG-4, pCAG-gH-4, pCAG-gI-4, pCAG-gK-4, pCAG-gL-4, pCAG-gM-4 and pCAG-gN-4. We could not clone the gene encoding gp2 into pCAGGS.

2.4.5. Expression of EHV-4 glycoproteins in 293T cells

Eleven expression plasmids encoding each glycoprotein were transfected into 293T cells and expression was examined by immunoblot analysis using horse sera.

Expressed gB, gD, gG were detected, but gC, gE, gH, gI, gK, gL, gM and gN were not. Two bands of 148 and 135 kDa were detected in pCAG-gB-4-transfected cells, a broad band of 40-44 kDa was detected in pCAG-gD-4-transfected cells, and two bands of 100 and 47 kDa were detected in pCAG-gG-4-transfected cells (Fig. 2. 2 and 2. 3). Furthermore, proteins with molecular masses of 100 and 47 kDa were detected in supernatant from pCAG-gG-4-transfected cells (Fig. 2. 3).

2.5. Discussion

In this study, eleven glycoproteins were expressed *in vitro* and eight MAbs were produced. The results indicated only three glycoproteins, gB, gD and gG, reacted with horse sera to EHV-4. These three glycoproteins are well known to be highly immunogenic in EHV-1 and other herpesviruses. However, one of the major immunogenic proteins, gC, could not be detected by horse sera. Since one MAb 3-2C10C8 could detect the gC in pCAG-gC-4-transfected cells by IFA, the amount of gC in the transfected cells should be enough. These results indicated that EHV-4 gC might be not immunogenic to horses and might not induce antibody to horse. Further analysis will be required to clarify the immunogenicity of EHV-4 gC.

EHV-4 gB shared 47% homology with HSV-1 gB and possesses highly conserved hydrophilic surface, hydrophobic transmembrane and cytoplasmic anchor domains. EHV-4 gB contains five more potential *N*-linked glycosylation sites than HSV-1 gB, and it is interesting to note the presence of three such sites immediately adjacent to each other (residues 493 to 501). EHV-4 gB also shows 88% homology with EHV-1 gB (Riggio et al., 1989). gB expressed in 293T cells resulted in two bands of 148 and 135 kDa under non-reducing condition. This result was similar to the previous report (Sullivan et al., 1989). Comparison of nucleotide sequence of gB

with the reference strain NS80567 indicated that there is no change in amino acid of gB of EHV-4.

Two MAbs specific to EHV-1 gB recognized the proteins with similar molecular masses in EHV-4 and EHV-1-infected cells and EHV-1 gB- and EHV-4 gB-transfected cells, indicating that both EHV-1 and EHV-4 gBs shared similar immunogenic site. In amino acid sequences, EHV-4 gB have 85 to 90 % identity with EHV-1 gB.

EHV-4 gG was secreted from infected cells (Drummer et al., 1998) and highly immunogenic to horses (Allen et al., 1986; Maeda et al., 2004). In this study, two bands with molecular masses of 100 and 50 kDa were detected in supernatant from EHV-4 gG-transfected cells under non-reducing conduction and the two bands were also detected in supernatant by using horse sera for EHV-4. The result is similar to the previous report.

EHV-4 gE and gI are important factors in cell-to-cell spread *in vitro* and for the expression of EHV-4 virulence (Damiani et la., 2000b) and the gE and gI of other herpes viruses are associated with neurovirulence (Neidhardt et al., 1987; Card et al., 1992; Kritas et al., 1994b). In this study, gE or gI expressed *in vitro* was not recognized by horse sera to EHV-4, indicating that the immunogenicity of both gE and gI was low or their antigenicity was not complete. Further experiment will be required to clarify the complex of gE and gI. However, EHV-1 gI was recognized by horse sera to EHV-1, suggesting that at least EHV-4 gI was less immunogenic to horses than EHV-1 gI.

EHV-4 gH, gK, gL, gM and gN were also recognized by horse sera to EHV-4. In EHV-1, co-expression of gM and gN were recognized by horse sera to EHV-1, indicating that antigenicity of gM changed by co-expression with gN in Chapter 2.

Further analysis should be performed to clarify the antigenicity of these glycoproteins not recognized by sera from natural hosts.

In this study, some glycoproteins of EHV-4 were not recognized by horse sera to EHV-4 in spite that those of EHV-1 were recognized by horse sera to EHV-1. These results suggested that EHV-4 might possess immune evasion system than EHV-1.

2.6. Conclusion

Eight MAbs to EHV-4 were produced, and all of them were EHV-4-specific, one MAb was specific to gB and one was to gC by indirect immunofluroscent assay. In mammalian cells, total of 11 glycoproteins of EHV-4 were expressed. Although gB, gD and gG were recognized by immunoblot analysis using horse sera, gC, gE, gH, gI, gK, gL, gM and gN were not. In this study, EHV-4-specific MAbs were produced and these materials must be useful for research and diagnosis of EHV. In addition, it was suggested that EHV-4 might possess immune evasion system more than EHV-1.

2.7. Figure legends

Fig. 2. 1. (A) EHV-4 gB in 293Tcells by IFA using MAb 3-1D4H8. (B) EHV-4 gC in 293Tcells by IFA using MAb 3-2C10C8.

Fig. 2. 2. Immunoblot analysis of EHV-4 glycoproteins expressed in 293T cells. 293T cells were transfected with each plasmid and FHK-Tcl3.1 cells were infected with EHV-4. Immunoblot analysis was carried out under non-reducing conditions. Sera collected from horses experimentally infected with EHV-4 were used as primary antibody.

Fig. 2. 3. Immunoblot analysis of EHV-4 glycoprotein g (gG) and EHV-1 glycoprotein g (gG) in supernatant from all 293T cells. 293T cells were transfected with each plasmid and FHK-Tcl3.1 cells were infected with EHV-4 or and EHV-1. Immunoblot analysis was carried out under non-reducing conditions. Serum collected from horses experimentally infected with EHV-4 was used as primary antibody.

Table 2. 1. Primers based on the complete genome of EHV-4 NS80567

Genes	Name	Primers Sequences[b]	Position[a] Start	stop
gB	gB4 F	5´-ACGAATTCagtccacttgttgccgtgc-3´	61147	61168
gB	gB4 R	5´-ACCTCGAGttaaaccattttcgctttcca-3´	64074	64051
gC	gC4 F	5´-ACGAATTCatgggtttggtaaatataatgcg-3´	22396	22373
gC	gC4 R	5´-ACCTCGAGttagaagtctgctttcttgtac-3´	20939	20961
gD	g D4 F	5´-ACAGATCTatgtctaccttcaagcctatg-3´	129798	129777
gD	g D4 R	5´-ACCTCGAGttacggaagctgagtatattg-3´	131006	130984
gE	gE4-F	5´-ACAGATCTatgtgagctgttgactcccgc-3´	136058	136037
gE	gE4-R	5´-ACCTCGAGtcattttagagtagaccgagc-3´	134239	134262
gG	g G4 F	5´-ACAGATCTatgttggctgtgggagcaact-3´	125970	125991
gG	g G4 R	5´-ACCTCGAGttatgcaacgtactcaagtcg-3´	127277	127256
gH	gH4-F	5´-ACGAATTCatgtcaacctatctaaaaat-3´	70858	70881
gH	gH4-R	5´-ACCTCGAGttactcagagtttaataacaaact-3´	73425	73421
gI	gI 4-F	5´-ACGAATTCatggccaaacatactgtattgt-3´	131111	131133
gI	gI 4-R	5´-ACCTCGAGttagtcttcctcttttatggcc-3´	132373	132351
gK	gK4-F	5´-ACAGATCTatgttattcggggggagaacg-3´	6649	6628
gK	gK4-R	5´-ACGCATGCttattgtgacaaggactcgcc-3´	5618	5640
gL	gL4-F	5´-ACAGATCTatgtatcactcccaattgggtg-3´	108296	108274
gL	gL4-R	5´-ACCTCGAGtcattcgaagttcttgaatt-3´	107637	107615
gM	gM4-F	5´-ACAGATCTatggcacgacgtgggcgtgct-3´	94033	94012
gM	gM4-R	5´-ACCTCGAGTtaacggtaatctcgcgcgtag-3´	92681	92702
gN	g N4 F	5´-ACAGATCTatgttgtcaggagattagtg-3´	11671	11692
gN	g N4 R	5´-ACCTCGAGtcagtgtaggtgtcgcaacat-3´	11973	11952
gp2	gp2-F	5´-ACAGATCTatgggggttcatctatgttggc-3´	127455	129476
gp2	gp2-R	5´-ACCTCGAGttaatatacgggacgccggggg-3´	129707	129686

a: position of genome of Equine herpesvirus 4 strain NS80567, complete genome (Accession No. AF030027.1)

b: Capital letters indicate the restriction endonuclease digestion site and additional two nucleotides.

A final polymerization step for 7 min at 72 ˚ C served for the completion of fragmental DNA strands. Finally, the reaction was finished by a 1 min cooling step to 4 ˚ C. The amplified DNA fragments were separated in a 0.8 % agarose gel, stained with ethidium bromide solution.

Table 2. 2. Characterization of monoclonal antibodies to EHV-4

MAbs	Recognized proteins	Isotype	VN test		IFA		Expression on cell surface
			EHV-4	EHV-1	EHV-4	EHV-1	
3-1 F3A9	Unknown	IgM	<1:10	<1:10	1:640	<1:10	-
3-3C12E5	Unknown	IgM	<1:10	<1:10	1:320	<1:10	-
3-3E4H10	Unknown	IgM	<1:10	<1:10	1:640	<1:10	+
3-1D4H8	gB4	IgM	<1:10	<1:10	1:640	<1:10	+
3-2C10C8	gC4	IgM	<1:10	<1:10	1:320	1:10	+
3-2G2A7	Unknown	IgG3	<1:10	<1:10	1:640	<1:10	+
3-2C1H5	Unknown	IgM	<1:10	<1:10	1:640	<1:10	+
3-1E5A8	Unknown	IgM	<1:10	<1:10	1:640	<1:10	+

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Table 2. 3. Differences of amino acid sequences of glycoproteins

Glycoprotein	Change of amino acid
EHV-4 gC	D309E[a]
EHV-4 gD	L27V, G382E[a]
EHV-4 gI	R134W, D298A[a]
EHV-4 gp2	L376S, K463R, Q509R[a]

a: Comparison with NS80567 strain (Accession No. AF030027.1)

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Fig. 2. 1.

pCAGGS-transfected 293T cells   pCAG-gB-4-transfected 293T cells

A

3-1D4H8 Mock   3-1D4H8

B

3-2C10C8 Mock   3-2C10C8

pCAGGS-transfected 293T cells   pCAG-gC-4-transfected 293T cells
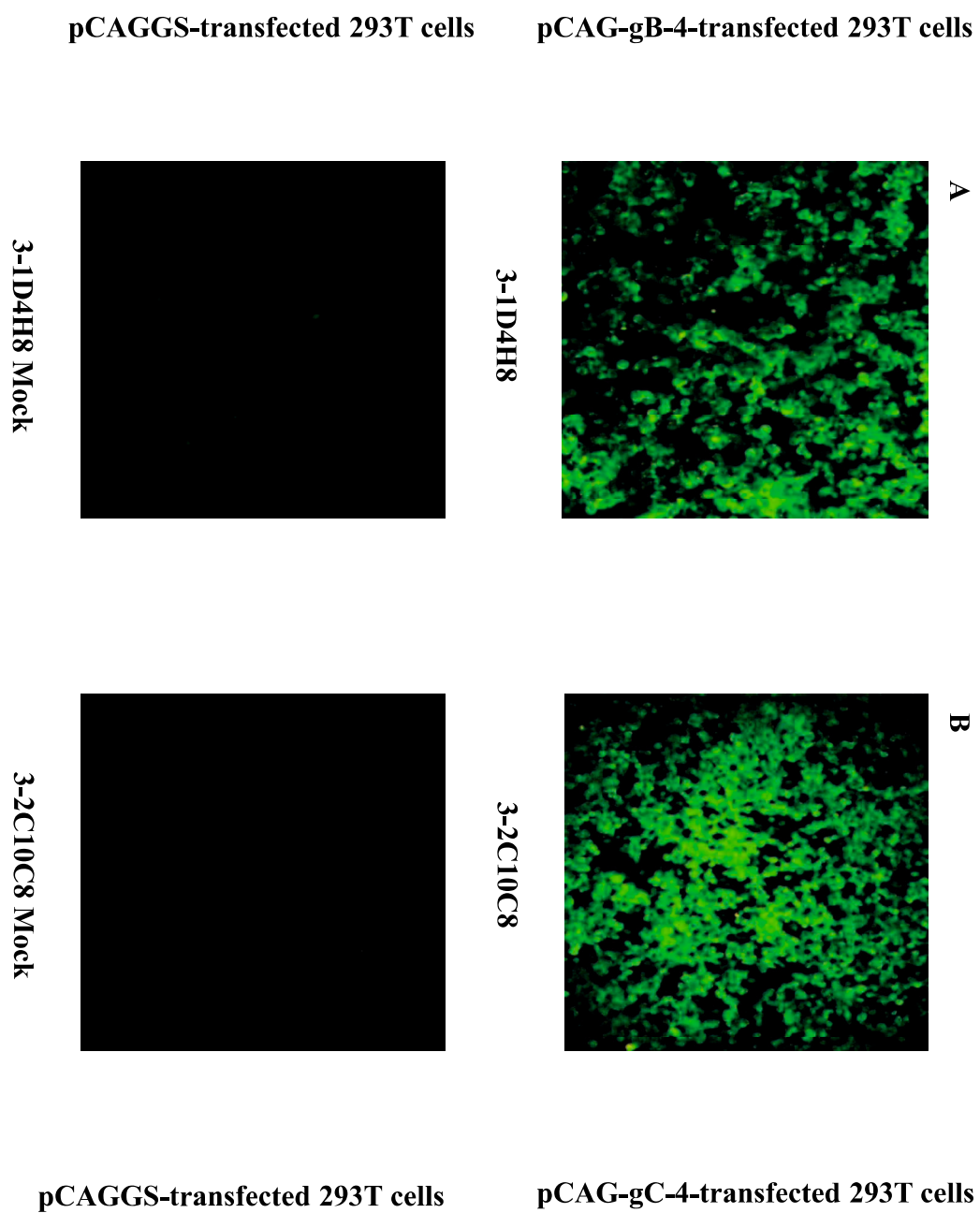
Fig. 2. 2.
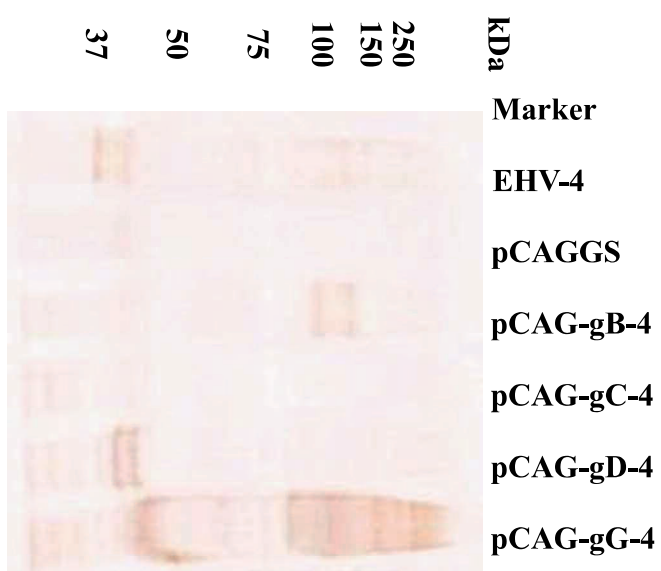
Fig. 2. 3.
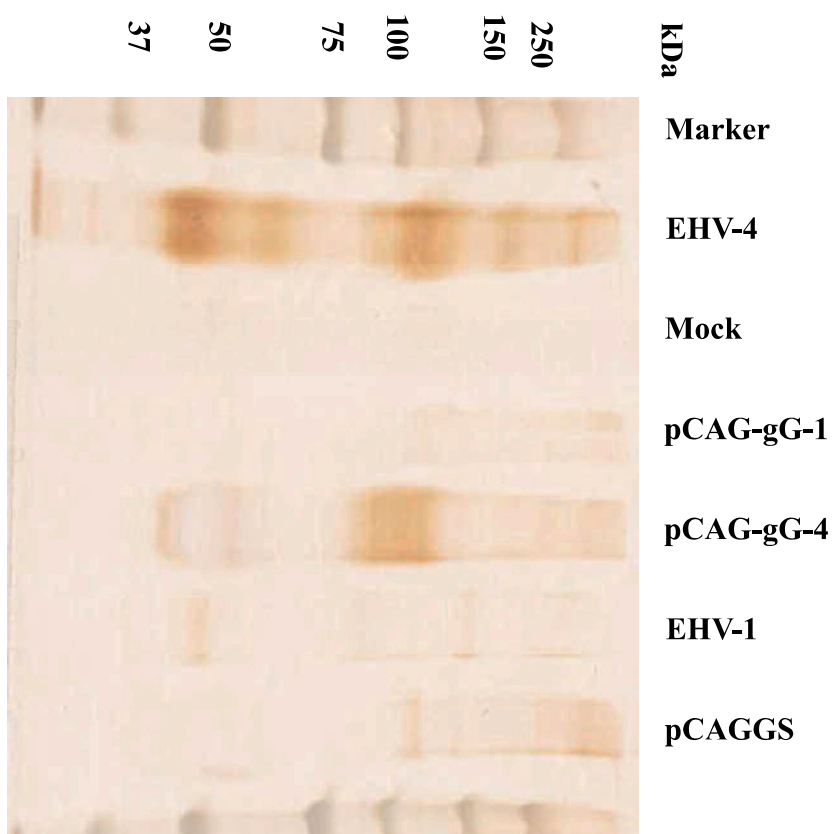
Chapter 3

Pseudorabies virus infection in wild boars in Japan

3.1. Summary

In Japan, most pig populations are now free from pseudorabies virus (PRV) due to the recent success of an extensive eradication program. However, PRV infection persists in Japanese wild boars (*Sus scrofa leucomystax*), representing another potential reservoir for the virus in Japan. In this study, the seroprevalence of PRV in wild boars captured in three different regions was ascertained. The virus-neutralization (VN) test showed that 6 of 173 (3%) were positive for VN antibody; glycoprotein E-ELISA revealed infection with the wild-type, but not the available vaccine strain, PRV. These results indicate that PRV has continued to spread among wild boars in Japan.

3.2. Introduction

Pseudorabies virus (PRV), a member of the genus *Varicellovirus* in the subfamily *Alphaherpesvirinae* of the family *Herpesviridae* (Klupp et al., 2004), is the causative agent of a contagious and epidemic disease, Aujeszky's disease, which affects swine at various production phases, causing high mortality in naïve and newborn piglets and abortion in pregnant sows. This disease results in significant economic losses for the swine industry (Cunha et al., 2006).

In Japan, a PRV eradication program in domestic pigs has been successfully implemented; consequently, most domestic pig populations are now free from wild-type PRV and therefore are not vaccinated with a live attenuated vaccine. As this vaccine virus has a deletion in the glycoprotein E (gE) gene, vaccinated pigs can be serologically differentiated from those infected with wild-type PRV by the gE-ELISA. In 1997, a PRV epidemic in Nara Prefecture, Japan resulted in the deaths of 24 hunting dogs due to PRV infection acquired after eating raw wild boar (*Sus scrofa leucomystax*) meat (Kouda et al., In the 129th Annual Meeting of the Japanese Society of Veterinary Science, Tsukuba, 2000). This event suggested that wild boars may represent a potential source of PRV infection for other animals. However, there is currently little information about the present situation of PRV prevalence among wild boars in Japan. Here we present the results of a seroepidemiological study of PRV in wild boars in Japan in order to clarify the seroprevalence of PRV in this population.

3.3. Materials and Methods

3.3.1. Sera

A total of 173 serum samples were collected from wild boars in three different prefectures of Japan. These three prefectures were located in the western part of Japan, were not neighbor each other and were free from PRV in their pig populations. Most animals were hunted with government permission during the winter season. Sera were inactivated by incubation at 56°C for 30 min and then kept at −20°C until use. Results of sample analysis are presented in (Table 3. 1).

3.3.2. Cell

Cloned porcine kidney (CPK) cells were kindly provided by the National Institute of Animal Health in Japan; these were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, NY, U.S.A.) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin.

3.3.3. Virus

PRV Indiana strain was propagated in the cultured CPK cells.

3.3.4. Virus-neutralization test

To assess the presence of virus-neutralization (VN) antibody to PRV, sera were serially two-fold diluted in DMEM containing 2% FCS. Diluted sera and medium (control) samples were mixed with equal volumes of a solution containing 50 plaque-forming units (PFU) /100 μl of PRV, and then incubated at 37°C for 1 hr. CPK cells were then inoculated with these samples. After adsorption for 60 min at 37°C in 5% CO_2, treated cells were washed twice with DMEM and then overlaid with 0.8% agarose (SeaPlaque GTG agarose, Lonza, USA) in DMEM containing 10% FCS. The plates were then incubated at 37°C in 5% CO_2 for 3 days. The cells were fixed with

5% buffered formaldehyde for 1 hr, and the agarose layers were removed. After staining with crystal violet, plaques were counted. Sera that reduced the number of plaques by more than 80%, as compared to the mean number of plaques in control wells, were considered positive.

3.3.5. ELISA

PRV gE-ELISA was carried out using HerdChek PRV g1 (gE) Antibody ELISA (IDEXX Lab., Westbrook, ME, U.S.A.) according to manufacturer's protocol.

3.4. Results

The results of VN assays revealed that at least 6 sera (3%) had VN antibodies against PRV (Table 3. 1). PRV seropositivity was found in 2 of 50 wild boars (4%) in area A, 4 of 71 (6%) in area B, and none of the 52 in region C. VN titers in the 6 positive sera ranged from 1:40 to 1:160 (Table 3. 2). PRV-positive boars were over three years old and relatively heavy (49-81 kg), indicating that infection may have occurred a few years before sampling took place and may not occur recently.

To confirm PRV seropositivity, PRV gE-ELISA was carried out using HerdChek PRV g1 (gE) Antibody ELISA. This assay detects antibody to wild-type PRV gE by competition with a monoclonal antibody, thereby distinguishing antibody induced by wild-type PRV from that induced by the vaccine strain (Mikulska et al., 2005). Results for all 6 VN-positive sera contained antibodies to wild-type, virulent PRV gE, and not the vaccine strain.

3.5. Discussion

The present results were consistent with the PRV epidemic in hunting dogs in 1997, because prefecture B is neighbor to Nara Prefecture, suggesting that virulent PRV continues to spread among wild boars in Japan. However, the reported seroprevalences of PRV in wild boars in western (10%) and eastern (9%) Germany (Lutz et al., 2003; Müller et al., 1998), France (6%) (Albina et al., 2000), south-central Spain (56%) (Gortázar et al., 2002), Croatia (55%) (Župančič et al., 2002) and the southern USA (34-61%) (Corn et al., 2004; Gresham et al., 2002; Hahn et al., 1997;Van Der Leek et al., 1993) seem to be higher than that in Japan (3%). In addition, only one previous report indicated that wild boars in Shikoku, in southern Japan, were free from PRV (Ishiguro et al., 2005). These results suggest that the low seroprevalence of PRV in wild boars in Japan might be indicative of the success of the PRV eradication program in domestic pigs.

PRV-seropositive wild boars can act as a source of infection for other wildlife species, including wild canids and hunting dogs (Tozzini et al., 1982). Mortality associated with PRV infection has also been documented in endangered species such as the Florida panther (*Felis concolor)* in the USA (Glass et al., 1994), and PRV has been suggested as a possible factor contributing to the declining numbers of the Eurasian lynx (*Lynx lynx*) in Slovenia (Vengust et al., 2005). Taken together with our results, these trends indicate that PRV infection in wild boars should become a target for eradication programs like the one implemented in the domestic pig population in Japan.

3.6. Conclusion

In conclusion, although PRV continues to infect wild boars in Japan, the prevalence is very low. Therefore, the possibility of transmission to domestic pigs may also be low, since PRV seems to be rare even within the wild boar population. Nevertheless, the continued threat of economic loss in the domestic pig industry due to this persistent virus reservoir should not be ignored.

Table 3. 1. Seroprevalence of PRV in wild boars.

Area	Period	Number of examined sera	Number of PRV-positive sera	Percentage of PRV-positive sera
A	September, 2009 to November, 2010	50	2	4
B	November, 2007 to March, 2010	71	4	6
C	January, 2010 to December, 2010	52	0	0
Total		173	6	3

Table 3. 2. VN titres and antibodies to PRV gE in PRV-positive sera

Prefecture	Date	Sex	Age[a] (Body weight)	gE ELISA S/N ratio[b]	VN titer
A	Nov. 26, 2010	F	Over 3 (65kg)	0.04	1:160
A	Oct. 4, 2010	F	Over 3 (73kg)	0.19	1:80
B	Feb. 15, 2008	M	N.A. (52kg)	0.05	1:160
B	Feb. 19, 2008	M	N.A. (53kg)	0.19	1:80
B	Mar. 3, 2010	M	N.A. (81kg)	0.10	1:40
B	Feb. 16, 2010	M	N.A. (49kg)	0.15	1:80

a: Age was presumed from their teeth.
b: S/N ratio was calculated as a ratio of absorbance of well with serum/absorbance of control well without serum.
Serum with S/N ratio of $\leqq 0.60$ is judged as positive.
N.A.: Data is not available

General Conclusion

In my PhD course, glycoproteins of EHV-1 and EHV-4 were expressed *in vitro* and characterized by our established monoclonal antibodies (MAbs). In addition, the availability of ELISA for detection of antibody to gE was examined using other herpesvirus.

Chapter 1. Characterization of glycoproteins in EHV-1

In this chapter, I attempted to express twelve glycoproteins of EHV-1 in 293T cells, and to characterize these using MAbs and horse sera against EHV-1. Expression of glycoprotein B (gB), gC, gD, gG, gI and gp2 was recognized by immunoblot analysis using horse sera, but that of gE, gH, gK, gL, gM and gN was not. Four MAbs recognized gB, four recognized gC and one recognized gp2. Two MAbs against gB cross-reacted with EHV-4. Interestingly, coexpression of gE and gI and gM and gN enhanced their antigenicity. Furthermore, immunoblot analysis of gp2 showed that different molecular masses of gp2 were recognized by the MAb against gp2 and horse sera against EHV-1. In this study, it was demonstrated that at least six glycoproteins were immunogenic to horses and coexpression of gE and gI and gM and gN was important for enhancement of antigenicity.

Chapter 2. Characterization of glycoproteins in EHV-4

In this chapter, eleven glycoproteins in EHV-4 were expressed in 293T cells and eight MAbs to EHV-4 were developed. One MAb recognized gB4, one did gC4 and the other six did unknown proteins. Only three glycoproteins, gB4, gD4 and gG4, expressed in 293T cells reacted with sera from EHV-4-infected foals, indicating that these three glycoproteins were immunogenic to horses. However, the other eight glycoproteins were not recognized by horse sera, suggesting that EHV-4

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glycoproteins might require other mechanisms for efficient expression and proper folding or that EHV-4 glycoproteins might be less immunogenic to horses than EHV-1 glycoproteins.

Chapter 3. Pseudorabies virus infection in wild boars in Japan

Pseudorabies virus (PRV) is the next candidate for eradication from Japanese swine industry. To eradicate PRV, the attenuated live vaccine, PRV with a deletion in gE gene, is available and the ELISA to detect antibody to PRV gE is used to serologically differentiate between wild type PRV infection and vaccination. In horses, EHV-1 with deletion in gE gene is developed as a new live attenuated vaccine and our laboratory developed ELISA for detection of antibody to EHV-1 gE and the ELISA made it possible to distinguish between wild EHV-1 infection and vaccine inoculation. In this study, the availability of ELISA to detect antibody to PRV gE was examined by using sera from wild bores (*Sus scrofa leucomystax*).

In Japan, most pig populations are now free from PRV due to the recent success of an extensive eradication program. However, PRV infection persists in Japanese wild boars, representing another potential reservoir for the virus in Japan. In this study, the seroprevalence of PRV in wild boars captured in three different regions was ascertained. The virus-neutralization (VN) test showed that 6 of 173 (3%) were positive for VN antibody; glycoprotein E-ELISA revealed infection with the wild-type, but not the available vaccine strain, PRV. These results indicate that PRV has continued to spread among wild boars in Japan and confirm that ELISA to detect antibody to gE is available in the field.

Conclusion

In these PhD studies, there are some novel findings.

1. EHV-1 gI plays an important role in expression of EHV-1 gE.

2. Immunogenicity of EHV-4 glycoproteins seems to be different from that of EHV-1 glycoproteins.

These novel findings, expression plasmids and MAbs must be available for understanding the pathogenicity of EHVs and for development of effective vaccine and diagnostic method.

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