Characterization and localization of the unique Marek’s disease virus type 2 ORF873 gene product

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Studies on Marek’s disease virus (MDV)-unique genes are important for understanding the biological nature of the virus. Based on complete DNA sequence analyses of the MDV genomes, the MDV genomes contain presumably at least five MDV-unique genes, which are commonly conserved among the three MDV serotypes. A recombinant baculovirus that contains the MDV serotype 2 (MDV2)-unique gene, ORF873, under the polyhedrin promoter was constructed and designated rAcORF873. Polyclonal and monoclonal antibodies, which recognize the recombinant MDV2 ORF873 protein in Spodoptera frugiperda clone 9 (Sf9) cells infected with rAcORF873, were prepared by immunizing mice with a recombinant fusion protein expressed in Escherichia coli. Immunoblot analyses with the antibodies revealed a major protein band with a molecular mass of 108-kDa in both MDV2-infected chick embryo fibroblasts (CEF) and rAcORF873-infected Sf9 cells. By indirect immunofluorescence analyses using monoclonal antibody, the authentic ORF873 protein was localized in the cytoplasm of MDV2-infected CEF cells. The monoclonal and polyclonal sera, which were generated in the present study and reacted effectively to MDV2 ORF873 protein, are considered to be useful reagents for further studying the role(s) of the ORF873 protein in MDV2 infection.

Key words: MDV, MDV2, MDV unique protein, MDV2 ORF873 protein

Introduction

Marek’s disease virus (MDV) is an oncogenic herpesvirus, which causes a highly contagious neoplastic disease in chickens. Marek’s disease is characterized by the development of T cell lymphomas, neurological disorders, immune-deficiency, and for some strains, atherosclerosis [4]. This disease can be successfully prevented by vaccination with antigenically related nonpathogenic or attenuated virus strains [19]. Three serotypes of MDV can be recognized by respective monoclonal antibodies (MAbs) raised against these viruses [2]. The oncogenic MDV, the prototype of this group, is designated as serotype 1 (MDV1). Serotypes 2 and 3 designate nonpathogenic but antigenically related herpesviruses from chickens (MDV2) and turkeys (MDV3 or HVT), respectively. The exceptionally short latency of the MDV-induced lymphomas makes it a valuable model to study herpesviral oncogenesis and to define viral genes involved in T cell transformation. A comparative analysis of the genome structure and sequences between the oncogenic and vaccine strains would be most valuable in identifying genes that are responsible for the pathogenic phenotypes of the virus. The genomic structure of MDV is similar to that of herpes simplex virus (HSV) and consists of a long and short region, each flanked by inverted repeat sequences and terminal repeats [6]. Early works, based on partial DNA sequences of MDV, confirmed that its genome is collinear with and closely related to that of α-herpesviruses [3,15]. This is surprising, as MDV exhibits biological properties more closely resembling γ-herpesviruses. This provides impetus to identify genes unique to MDV, which may be responsible for the lymphotropic and oncogenic phenotypes.

Recently, the entire genome sequences of the three MDV serotypes were determined [1,8,11] and analysis of these sequences revealed that most of the identified MDV genes were found in collinear arrangement compared to the completely sequenced α-herpesvirus genomes of HSV type 1 [12], varicella-zoster virus [5], equine herpesvirus type 1 [17], and bovine herpesvirus type 1 [16]. Based on sequence data comparisons between MDV’s and other α-herpesviruses, at least five genes are found to be uniquely conserved in the genomes of the three MDV serotypes, which may be responsible for the unique biological nature of the virus [1,8,11]. Nonetheless, no protein products of the MDV-unique genes have been identified to date.

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The ORF873 gene of MDV2 strain HPRS24 was mapped to the right part end of the unique long region of the genome. In addition, the ORF873 gene transcript is post-transcriptionally modified by splicing events. Based on primary amino acid sequence analysis, the MDV2 ORF873 protein was previously predicted to be a membrane-associated glycoprotein with a stretch of a hydrophobic transmembrane domain in the C-terminus and three putative N-linked glycosylation sites [18]. However, the properties of this protein and its biological roles are unclear. Therefore, to determine the properties of the MDV2 ORF873 protein and its biological roles, this protein was firstly identified in the MDV2-infected cells and characterized in the present report.

Materials and Methods

Cells and viruses
Autographa californica nuclear polyhedrosis virus (AcNPV) and recombinant AcNPV (rAcNPV) were grown in Spodoptera frugiperda clone 9 (Sf9) cells in TC-100 medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 0.3% tryptose phosphate broth (TPB), penicillin (100 μg/ml), and streptomycin (100 μg/ml). Three serotypes of MDV1 (strain GA), MDV2 (strain HPRS24), and HVT (strain FC126) were grown in primary chicken embryo fibroblast (CEF) cells in an equal amount of Medium 199 and Ham’s F-10 nutrient mixture (Gibco BRL) containing 5% calf serum, 10% TPB, penicillin (100 μg/ml), and streptomycin (100 μg/ml). MDV2 (strain HPRS24) was passaged in CEF cells at least 30 times prior to use in this study.

Preparation of immune sera against MDV
Antiserum against MDV2 (HPRS24) and HVT (FC126) were prepared by repeated inoculations of each of the virus-infected CEF cells (1000 PFU per chicken) in to separate specific-pathogen-free chickens. Chicken polyclonal antiserum against MDV1 (GA) was kindly provided by Dr. K. Imai (National Institute of Animal Health, Japan).

Preparation of MDV2 ORF873-monospecific polyclonal antiserum
To detect and identify the MDV2 ORF873 protein, codons 255 to 448 of the ORF873 gene were expressed as a glutathione S-transferase (GST) fusion protein in E. coli (Fig. 1) and used as an immunogen. A 585 bp EcoRI-HindIII fragment that contains an immunogenic sequence was blunted by the Klenow fragment (Takara) and then inserted into the SmaI site of the expression vector pGEX 4T-3 (Pharmacia). The pGEX expression vector encoding a part of the ORF873 gene was transformed into the E. coli BL21(DE3) pLysE strain. The transformed bacteria were grown overnight at 37°C in 2× YT (yeast extract and tryptone) medium supplemented with 2% glucose and the appropriate antibiotics (100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol). The cultures were diluted 1 : 100 in 250 ml of fresh, pre-warmed 2× YT medium containing the appropriate antibiotics and grown for 1.5 to 2 h at 37°C. Fusion protein synthesis was then induced by incubating the cells with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) for 2 to 3 h at 37°C. The cells were lysed, and the proteins were purified with the BugBuster GST-Bind Purification Kit (Novagen) according to the manufacturer’s instructions with slight modifications. Cells were lysed in 12 ml of protein extraction reagent containing 25 U/ml of Benzonase nuclease for 20 min by shaking at room temperature (RT). Insoluble debris were removed by centrifugation at 12,000 rpm for 20 min in the HANIL A50S-8 rotor, and GST proteins were purified from the soluble extract by batch-binding the supernatant with GST-bind resin (1 ml of resin.
per 5-8 mg of protein) at RT for 40 min. The beads were then washed twice with 5 ml of GST bind/wash buffer. The bound proteins were eluted from the resin in 1.5 to 2 ml of GST elute buffer. Eluates were then loaded into Centricron columns (Amicon) as directed by the manufacturer to both desalt and concentrate the purified proteins by ultrafiltration. Protein purity was determined by SDS-PAGE, and concentrations were estimated by densitometric analysis using the Darkroom CN-FTX gel documentation system (Vilber Lourmat) by comparing protein intensity to known amounts of bovine serum albumin (Pierce). Aliquots of proteins were stored at -70°C. For immunization with the purified protein, 6-week-old female BALB/c mice received four doses of 100 µg protein at 2-week intervals via intraperitoneal injection. Polyclonal antibodies from five mice were pooled and used in immunoblot analysis.

Insertion of MDV2 ORF873 DNA into transfer vector pVL1392

The entire MDV2 ORF873 coding region was amplified by the polymerase chain reaction (PCR) in the conditions as described in our previous study [18] using the primers 5'-CGCTCTAGA and 5'-CATATGATCTTCGTTTTCTCGTAT-3' (ORF873F) and 5'-CGCTCTAGACTACTGTTTCCTCGTAT-3' (ORF873R), which created PstI and XbaI sites (underlined) on the 5' and 3' termini of the fragment, respectively (Fig. 1). The PCR product was cleaved with restriction enzymes PstI and XbaI, and the resulting fragment was cloned in frame into the same sites of the pVL1392 vector (Invitrogen). The recombinant vector was designated pVLORF873. The expected size and orientation of the inserted fragment were confirmed by restriction enzyme digestion and sequencing of the 5'-junction region by the chain-terminating method.

Transfection and selection of the recombinant baculovirus expressing the MDV2 ORF873 protein

To introduce the MDV2 ORF873 gene into the AcNPV genomic DNA, Sf9 cells (1.5 × 10^6) were cotransfected with 50 ng of BaculoGold-linearized baculovirus DNA (Pharmingen) and 5 g of pVLORF873 transfer DNA with Lipofectin (Gibco BRL). The rAcNPV expressing the ORF873 protein released into the supernatant fluid from the transfected cell cultures was subjected to plaque purification. After plaque purification, recombinant baculovirus stocks were obtained from Sf9 cells and designated as rAcORF873. As a negative control, the baculovirus recombinant cAcNPV was prepared by cotransfection with the parent vector pVL1392 and BaculoGold-linearized baculovirus genomic DNA.

Preparation of MDV2 ORF873-specific MAbs

Primary immunization was carried out by an intraperitoneal injection of 1 × 10^7 rAcORF873-infected cells per a BALB/c mouse. Successive immunization was done intraperitoneally with the same antigens. The last immunization was given intravenously 3-5 days before collecting hyperimmune antisera and removing the mouse spleen cells for fusion with P3-X63-Ag8-U1 myeloma cells. The antibody-secreting hybridomas which reacted by indirect immunofluorescence analysis with rAcORF873-infected Sf9 cells but not with cAcNPV-infected Sf9 cells were screened, and cloned by limiting dilution. One MAb from three mice was pooled and used in the present IFA experiments.

Immunoblot analysis

For immunoblot analyses, mock- or three serotypes of each MDV-infected CEF cells, and cAcNPV- or rAcORF873-infected Sf9 cells were collected at 72 h post-infection (PI), washed three times with phosphate-buffered saline (PBS), and resuspended in PBS. These cells were lysed by three times of freezing and thawing cycles, and the proteins were eluted in 1× Laemmli sample buffer (62.5 mM Tris-HCl at pH 6.8, 2% SDS, 20% glycerol, 5% 2-ME, 0.01% bromophenol blue). After boiling for 5 min, the proteins were separated by 10% SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane (Millipore) for 1 h. After transfer, the membrane was blocked for 1 h at RT in TBST buffer (10 mM Tris-HCl at pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat powdered milk. The membrane was then incubated either with anti-ORF873 mouse polyclonal antibody at a dilution of 1 : 400 or MDV2 (strain HPRS24)-infected antiserum at a dilution of 1 : 10 in TBST for 1 h at RT. After three times of washing each for 10-min with TBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse or chicken antibody (Cappel), diluted in TBST at a dilution of 1 : 1,000 for 1 h at RT, and followed with three TBST washes to remove unbound antibody. Immunocomplexes were visualized by incubation in TBST containing a diaminobenzidine-hydrogen peroxide substrate.

Immunofluorescence analysis

The CEF and Sf9 cells were maintained under the conditions as described previously [13], with the following modifications. Monolayers of primary CEF cells were grown on coverslips, infected with MDV2 (strain HPRS24) or mock-infected for 48 h, fixed with methanol-acetone (1 : 1) for 15 min at RT, and washed three times with PBS. The Sf9 cells were infected with rAcORF873 or cAcNPV at a multiplicity of infection (MOI) 0.1 PFU per cell. After 48 h PI, the cells were collected, washed three times with PBS, smeared onto coverslips, and then fixed. The cells were then incubated for 45 min at 37°C either with the anti-ORF873 MAb or preimmune mouse serum as a negative control. After three washes with PBS, the cells were further incubated for 45 min at 37°C with fluorescein-conjugated goat anti-mouse antibody (Cappel), and the cells were washed three times with PBS to remove unbound fluorophore.
antibodies. The cells were then counterstained with propidium iodide (2 µg per ml in 2× SSC) for 3 min at RT. Finally, the cells were washed three times with PBS containing 0.05% Tween-20. Fluorescence microscopy was then performed to ascertain the intracellular distribution of the fluorescein-labeled ORF873 protein.

**Immunoprecipitation analysis**

Antisera against MDV2 (HERS24) and HVT (FC126) were prepared by repeated inoculations of each of the virus-infected CEF (1000 PFU per chicken) into specific-pathogen-free chickens. Chicken polyclonal antisera against MDV1 (GA) and MDV2 (SB-1) were kindly provided by Dr. K. Imai (National Institute of Animal Health, Japan).

Sf9 cells (1 × 10⁶ per 35-mm-diameter dish) were infected with rAcORF873 or cAcNPV at a MOI of 5 PFU per cell. At 24 h PI, the cells were radiolabeled for 12 h with 50 Ci of [³⁵S]methionine (1175.0 Ci/mmol; New England Nuclear) per dish in Grace’s methionine-free insect cell medium (Gibco BRL) containing 1/10th the normal concentration of methionine and 5% FBS. At 36 h PI, the cells were lysed in ice-cold lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 1 mM PMSF, 0.15 M NaCl, and 0.02% sodium azide in 50 mM Tris-HCl, pH 8.0) with aprotinin (0.2 U/ml), and immunoprecipitated with chicken polyclonal antibody and rabbit anti-chicken IgG (Cappel) as previously described [7]. The immune complexes were precipitated with 2 to 4 mg of Protein A-Sepharose CL-4B beads (Pharmacia), and analysed by SDS-PAGE as previously described [13]. For tunicamycin (Sigma) treatment, rAcORF873- and cAcNPV-infected cells were cultured in the presence of 10 µg of tunicamycin per ml as previously described [13].

**Results**

**Recombinant baculovirus expression and characterization of MDV2 ORF873 proteins**

The MDV2 ORF873 proteins expressed by the rAcORF873 and MDV2 strain HPRS24 were identified and characterized by immunoblot analysis. The results of immunoblot analysis using the anti-ORF873-monspecific polyclonal serum are shown in Fig. 2. The molecular mass of a 108-kDa protein was specifically detected in both the rAcORF873-infected Sf9 and the MDV2-infected CEF cells (Fig. 2, lanes 2 and 5), whereas the 108-kDa protein was not observed in cAcNPV-infected Sf9 and mock-infected CEF cells (Fig. 2, lanes 1 and 3). This protein was not detected with preimmune mouse serum (data not shown). The domain (residues 255 to 448) of the MDV2 ORF873, which was expressed as a GST fusion protein and used as an immunogen to create ORF873-monspecific mouse polyclonal antibody, showed significantly higher identities with both the corresponding region of MDV1 ORF21 (46.5%) and HVT ORF1 (31.5%). However, the anti-ORF873 mouse polyclonal antibody used in this study did not display recognition of the homologous MDV1 and HVT proteins (Fig. 2, lanes 4 and 6).

**Antigenic property of recombinant MDV2 ORF873 protein**

To confirm whether the expressed MDV2 ORF873 protein was glycosylated, Sf9 cells were additionally infected with the rAcORF873 in the presence of 10 µg per ml of tunicamycin (Sigma) to prevent glycosylation as described in the Materials and Methods. However, no reduction of the molecular mass of the protein was observed (data not shown), suggesting that the 108-kDa polypeptide apparently does not contain N-linked sugars. Furthermore, the antigenic properties of the rAcORF873 protein were also examined by immunoblotting and immunoprecipitation analyses using chicken antisera raised against MDV1 (strain GA), MDV2 (strains HPRS24 and SB-1), or HVT (strain FC126) as previously described [9,10,14]. However, the baculovirus-expressed recombinant MDV2 ORF873 protein was not detected with the three serotype-specific antisera (data not shown).

**Localization of the unique MDV2 ORF873 protein**

To examine the localization of the ORF873 protein in MDV2-infected cells, indirect immunofluorescence microscopy experiments were performed using anti-ORF873 MAb. The results in Fig. 3 show that the anti-ORF873 MAb recognized the recombinant MDV2 ORF873 protein in the cytoplasm of rAcORF873-infected Sf9 cells. Using the MAb, it has additionally demonstrated that the authentic MDV2...
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ORF873 protein was expressed in the cytoplasm of MDV2-infected CEF cells (Fig. 4C). These results were controlled by monitoring the reactivity of the MAb with cAcNPV-infected (A) or rAcORF873-infected (B) Sf9 cells (Fig. 3A) and mock-infected CEF cells (Fig. 4A), and preimmune mouse serum with rAcORF873-infected Sf9 cells (data not shown) and MDV2-infected CEF cells (Fig. 4B).

Discussion

Studies on MDV2 are important for understanding the natural nononcogenic phenotypes of MDV. Especially, studies on MDV-unique genes will be made possible, helping to understand its oncogenic nature and characterize the biological nature of the virus. The goals in the present and previous studies [8-10,14,18] have been to dissect the genomic structure and to understand the functions of the different viral proteins among the three MDV serotypes. In this communication, it is described that the unique MDV2 ORF873 protein is being expressed and localized in the cytoplasm of the virus-infected cells as the molecular mass of a 108-kDa protein.

A recombinant baculovirus expressing MDV2 ORF873 protein was constructed and used for the characterization of the protein. Interestingly, the apparent molecular mass of the protein detected was equal to the mass calculated from the deduced ORF873 amino acid sequence. This observation raises an important question as to whether the protein is modified by glycosylation as predicted in the computer analysis, and if so, whether the glycosylations can be blocked by treatment with glycosylation inhibitory reagents. However, the molecular mass of the recombinant MDV2 ORF873 protein detected by immunoblotting using anti-ORF873-monospecific polyclonal mouse serum identified as a 108-kDa polypeptide likely as that was detected in the MDV2-infected cells. These observations suggest that the 108-kDa polypeptide of the MDV2 ORF873 gene product apparently does not contain N-linked glycans.

Several MDV-specific genes were presumably identified within the unique long region, although most of the MDV-
specific genes were identified within the inverted and terminal repeat sequences, including pp38, pp24, and several oncogenes [1,8,11]. The MDV2 ORF873 gene was identified in the unique long region and was located upstream from the 3′-terminal part of the pp38 gene that showed no significant homology to any known herpesviral proteins [8,18]. Since the full-length genome of MDV1 and HVT sequences were recently reported [1,11], comparison study was performed in the present report that the predicted MDV2 ORF873 protein showed 53.1% and 39.1% identities to the homologous MDV1 LORF11 and HVT ORF1 proteins, respectively. Therefore, the antigenicity of the recombinant MDV2 ORF873 protein was also examined by immunoblotting and immunoprecipitation analyses by the antisera from chickens inoculated with each of the three serotypes of MDV. However, the baculovirus-expressed recombinant MDV2 ORF873 protein was not detected with the three serotype-specific antisera (data not shown). These results may indicate that the recombinant ORF873 protein is devoid of major epitopes as compared to the antigenicity of the homologous MDV1 LORF11 and HVT ORF1 proteins, respectively. Therefore, the antigenicity of the recombinant MDV2 ORF873 protein was also examined by immunoblotting and immunoprecipitation analyses by the antisera from chickens inoculated with each of the three serotypes of MDV. However, the baculovirus-expressed recombinant MDV2 ORF873 protein was not detected with the three serotype-specific antisera (data not shown). These results may indicate that the recombinant ORF873 protein is devoid of major epitopes as compared to the antigenicity of the glycoproteins gE and gI, and phosphorylated protein pp38 [9,10,14]. Alternatively, the recombinant ORF873 protein synthesized in the artificial expression systems may assume structural conformation that differs from that of the MDV-infected cell protein.

Next, the localization of the MDV2 ORF873 protein in the virus-infected cells was performed by indirect immunofluorescence microscopy experiments with an anti-MDV2 ORF873 MAb. The results showed that both of the recombinant MDV2 ORF873 and the authentic ORF873 protein localized in the cytoplasms of the recombinant baculovirus- or MDV2-infected cells. Although further studies need for addressing the observations, the MDV2 ORF873 proteins in both of the Sf9 and CEF cells additionally seem to interact with cytoskeletal components such as actin or microtubules. However, the anti-ORF873 MAb used in the present study failed to recognize the counterparts of the ORF873 protein in the MDV1- or HVT-infected CEF cells. To further characterize the ORF873 homologous proteins among the three serotypes of MDV, it may necessary for generating other antibodies reacting commonly to the ORF873 homologous proteins.

In summary, the ORF873 gene and its homologues are commonly conserved among the three MDV genomes. The MDV2 ORF873 protein was identified in the virus-infected CEF cells with an its specific MAb, raised against the recombinant GST-fusion ORF873 protein. Although the predicted ORF873 protein has 53.1% and 39.1% identities to the homologous MDV1 LORF11 and HVT ORF1 proteins, respectively, the counterpart was not identified in MDV1 strain GA- and HVT strain FC126-infected CEF cells. The MAb and anti-ORF873 mouse polyclonal serum generated in the present study are considered to be useful reagents for further studying the role(s) of the ORF873 protein in MDV2 infections. Although it is very difficult to generate mutant viruses of MDVs due to its highly cell-associated nature, characterization of the viruses lacking these genes will be a major topic of future studies to ascertain functions of the ORF873 homologous proteins following infection each of the virus in cultured cells and/or natural hosts.

Acknowledgments

This study was financially supported in part by research grant from Chonbuk National University.

References


