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Heat-shock protein 70 is associated with the entry of Marek′s disease virus into fibroblast

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Summary. – Literature pertaining to the interactions between Marek′s disease virus (MDV) entry-related glycoproteins and corresponding receptors is still limited. Results from a Western blot analysis of cellular proteins for virus receptors and co-immunoprecipitation suggest that heat shock protein 70 (HSP70) is a potential cellular receptor for MDV glycoprotein gH. Plaque inhibition assays confirm the involvement of HSP70 in the early stages of MDV entry into chicken embryo fibroblasts (CEF). The present work supports that HSP70 is implicated in the MDV entry process by binding to gH, and enhances the understanding of multifunctional HSP70 and the MDV infection process.

Keywords: Marek′s disease virus (MDV); heat shock protein 70 (HSP70); glycoprotein H; virus entry

Introduction

Herpesviruses express many glycoproteins, various types of cellular receptors and a multipartite entry-fusion system (Campadelli-Fiume *et al*., 2007; Heldwein and Krummenacher, 2008). Recently, several studies have suggested that glycoprotein gB, expressed by herpes simplex virus 1, a member of alphaherpesviruses, interacts with the heparan sulfate proteoglycan and pair immunoglobulin-like type 2 receptor alpha to facilitate the initial attachment of the virus to the cell surface. The glycoprotein gD then binds to a specific surface receptor, such as nectin-1 or the herpesvirus entry mediator. This interaction alters the conformation of gD and enables the activation of gB and the dimer gH-gL. Finally, the gD-gB-gH-gL complex facilitates the fusion of the viral envelope with the plasma membrane (Maurer *et al*., 2008; Satoh *et al*., 2008). In the human cytomegalovirus of betaherpesviruses, the gH-gL dimer associates with other glycoproteins to form either a gH-gL-gO or a gH-gL-UL128-131 complex, and virus-cell fusion is triggered by interactions between gH-gL and integrins (Parry *et al*., 2005). In the case of the Epstein-Barr virus of gammaherpesviruses, the fusion of the virus with B lymphocytes requires gB and a 3-component complex of glycoproteins, gH-gL-gp42. Its formation is triggered by interactions between gp42 and MHC class II. However, the fusion of the virus with epithelial cells is impeded by gp42. This process is instead triggered by interactions between gH-gL and integrins alphavbeta6 and/or alphavbeta8 (Chesnokova *et al*., 2009).

MDV, a cell-associated virus has long been of interest as a model virus, particularly with respect to the pathogenesis and immune control of virus-induced lymphoma in an easily accessible small-animal system. Owing to its biological properties, particularly its ability to induce T-cell lymphoma and its slow growth in cell culture, MDV was long thought to be closely related to Epstein-Barr virus, a member of gammaherpesviruses. Electron-microscopy studies of the MDV genome provided the first evidence that this double-stranded DNA virus possesses repeat structures that are characteristic of alphaherpesviruses, which was confirmed by detailed restriction-enzyme mapping and sequencing, first of individual genes, and, later, of the en-

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Abbreviations: CEF = chicken embryo fibroblasts; gB = glycoprotein B; gD = glycoprotein D; gH = glycoprotein H; HSP70 = heat shock protein 70; HSP90 = heat shock protein 90; MDV = Marek′s disease virus; p.i. = post infection

tire genome (Osterrieder *et al*., 2006). In addition, unlike the majority of herpesviruses, MDV up-regulates MHC class II cell surface expression in infected cells both *in vitro* and *in vivo* (Nikura *et al*., 2007). Recent advances in our knowledge of MDV genetics and functional genomics have dramatically increased our understanding of the mechanisms leading to latency and tumor formation. However, a limited amount of work has been conducted investigating the molecular mechanisms involved in MDV entry into host cell.

Glycoprotein gD of MDV lacks functions typically associated with alphaherpesvirus gD homologues (Zelník *et al*., 1999). It has been hypothesized that the MDV gD homolog is not essential for cell-to-cell spread of MDV but is important for the generation of cell-free infectious MDV from feather follicle epithelium (Anderson *et al*., 1998). The secreted glycoprotein gC of MDV, one of the primary antigens that triggers a substantial serological response of the immune system, is required for full virulence of MDV and was speculatively considered to be a cellular receptor binding glycoprotein (Tischer *et al*., 2005). Some envelope and tegument proteins including tegument protein VP22 (Dorange *et al*., 2002), envelope glycoprotein gE-gI (Schumacher *et al*., 2001), glycoprotein gB (Schumacher *et al*., 2000), and glycoprotein gH-gL (Wu *et al*., 2001) are essentially required for cell-to-cell spread in cultured cells. Literature pertaining to the interactions between MDV glycoproteins and receptors is still limited. Our previous study demonstrated the presence of unidentified protein related to glycoprotein gB, with approximate molecular mass of 90 kDa. In addition, no potential receptor molecules related to gC and gE were identified. The current study focuses on recovery of potential receptor molecules corresponding to the glycoprotein gH in the early stages of MDV entry into fibroblast. The relationship between potential receptor and virus entry is further explored.

Materials and Methods

Virus and cells. Primary CEF were cultured in DMEM supplemented with 10% FCS and were allowed to attach overnight. Cells were then incubated with multi-passage CEF-associated MDV strain RB1B (kindly provided by Prof. Zhi-Zhong Cui at Shandong Agricultural University) for 2 hrs. Following incubation, the virus inoculums on the cells were replaced with DMEM supplemented with 2% FCS, and the cultures were incubated for 5 days to allow for plaque formation. Consistent and uniform plaques were observed and counted using an Olympus microscope, and images were captured using the DP Controller software. Simultaneously, indirect immunofluorescent assay was used to verify plaque formation with mouse anti-pp24 antibody diluted 1:100 (Ding *et al*., 2008).

Preparation of cell-free MDV. CEF-associated MDV from the passages that contained 2 x 10^4 PFU were used in this study. Cell-free MDV was extracted from infected CEF cultures according to the method described in a widely cited report (Lee *et al*., 2001) and was used immediately.

Preparation of CEF proteins. Monolayers of primary CEF were pelleted at 800 x g for 5 mins and washed twice with Dulbecco′s Phosphate-Buffered Saline (Invitrogen). The cells were then lysed by sonication at 4°C for 15 secs. The pellet was collected by centrifugation and resuspended in RSB-NP-40 (1.5 mmol/l $MgCl_2$, 10 mmol/l tris-HCl, 10 mmol/l NaCl, and 1% Nonidet P-40). Soluble membrane protein extracts were obtained by centrifugation at 12,000 x g for 15 mins at 4°C, and the amount of proteins was determined by BCA protein assay kit (Lin *et al*., 2007).

Western blot analysis of CEF proteins for virus receptors. Briefly, cellular proteins of primary CEF were subjected to 10% SDS-PAGE and blotting. The blot was blocked overnight at 4°C using a 5% nonfat milk solution in PBS and subsequently washed twice with PBS containing 0.05% Tween 20 (PBST). The blot was then incubated with cell-free MDV freshly extracted from 2 x 10^4 PFU of cell-associated MDV in PBS with 5% low-fat milk for 6 hrs at 37°C (Das *et al*., 2009). After being washed three times with PBS, the blot was incubated with polyclonal antibody against glycoprotein gH (kindly provided by Prof. Klaus Osterrieder at Free University of Berlin) for 2 hrs at 37°C. For detection, an HRP conjugated goat anti-rabbit IgG ECL antibody, and ECL substrate were used. The experiment was performed in triplicate.

Western blot analysis of CEF proteins with antibodies to potential receptors. Cellular proteins from CEF infected with 100 PFU of MDV were collected at 20, 40, and 120 mins post infection (p.i.). The blot was incubated with antibodies targeting HSP70, HSP90, CD54, and CD44 (Sigma), and subsequently incubated with a goat anti-rabbit IgG ECL antibody. All experiments were performed in triplicate.

Western blot analysis of co-immunoprecipitated receptor. Briefly, MDV-infected CEF were harvested 20 mins p.i. in PBS-EDTA and then were lysed in pre-chilled RIPA buffer (1 ml/10^7 cells) for 1 hr at 4°C with rocking, centrifuged for 20 mins at 12,000 x g to remove cell debris and the supernatant was obtained. Protein A agarose beads with pre-clear were added into cell lysate and were incubated at 4°C rocking for 10 mins. After centrifugation at 10,000 x g, the supernatant was incubated with gH or HSP70 antibodies for 3 hrs on a rocker. The mixture was centrifuged at 10,000 x g for 15 secs, then the supernatant was carefully discarded, followed by the washing of beads twice with 1 ml RIPA buffer and then 3 times with 1 ml PBS to remove detergents. Finally, beads were resuspended in 60 µl loading buffer, boiled at 95°C for 5 mins and centrifuged at 10,000 x g for 15 secs. The blot was incubated with anti-gH antibody and subsequently incubated with a goat anti-rabbit IgG ECL.

Plaque inhibition assay. To confirm that the HSP70 molecule is involved in the entry of MDV into CEF, cells were incubated with medium containing a polyclonal antibody against HSP70 (simultaneous tests using HSP90, CD44 and CD54 antibodies were also performed) at concentrations of 50, 25, 5, 1, and 0 µg/ml in the presence of 100 PFU of cell-free MDV at 4°C for 1 hr. The infected cells were subsequently washed three times, after which

fresh medium was added, and the infection was allowed to proceed for 5 days at 37°C. After infection, cells were observed under an inverted microscope for plaque formation, as described earlier. All experiments were performed in triplicate.

HSP70 silencing assay. CEF monolayers were pre-incubated with different concentrations of quercetin (0, 75, 150, and 300 μmol/l) for 6 hrs, washed and then infected with 100 PFU of cell-free MDV for 5 days, after which the plaque inhibition rate was evaluated as described earlier. Alternatively, 200 pmoles of small interfering RNA (siRNA) targeted at HSP70 (Santa Cruz Biotechnology) with Lipofectamine-2000 (Invitrogen) complexes was added to each flask, and then mixed gently by rocking. The cells were incubated at 37°C in a CO_2 incubator for 56 hrs, and then infected with 100 PFU of MDV for 5 days.

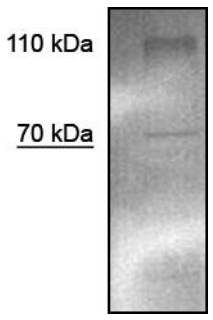

Fig. 1

Western blot analysis of cellular proteins for virus receptors

The blotted CEF proteins were incubated with the virus and the bound virus was detected with an antibody against MDV gH antibody (1: 10,000). A 70-kDa band is seen, showing the possible presence of receptors in CEF corresponding to glycoprotein gH.

Results and Discussion

Western blot analysis of CEF proteins for virus receptors was performed to identify cellular receptor proteins. The results demonstrated the presence of proteins corresponding to glycoprotein gH, with approximate molecular mass of 70 kDa. The result is shown in Fig. 1. Potential receptor protein with molecular weight of 70 kDa was found using cell-associated virus at same time (not shown here). Four candidate molecules, including heat shock proteins 70 and 90 (HSP70 and HSP90), intercellular adhesion molecule 1 (ICAM-1, also known as CD54) and CD44 were considered for further investigation. Heat-shock proteins HSP70 and HSP90 are highly conserved proteins that function as molecular chaperones. In virus biology, HSP70 and HSP90 have been implicated not only in the process of virus entry, but also in post-entry steps; notably, they were discovered to be a part of a receptor complex in monocytic cells involved in the entry of dengue viruses (Chavez-Salinas *et al*., 2008). Furthermore, HSP90 is a component of the cellular receptor complex of the infectious bursal disease virus (Lin *et al*., 2007), and HSP70 on Neuro2a cells is a putative receptor for the Japanese encephalitis virus that was targeted in vaccine designs (Ge *et al*., 2007; Das *et al*., 2009). Recent evidence has shown that the interaction between HSP70 and viral oncoprotein Meq plays a significant role in MDV oncogenesis, suggesting that HSP70 may be involved in virus pathogenicity (Zhao *et al*., 2009). CD54 is an endothelial- and leukocyte-associated trans-membrane protein that has long been known to play a role in stabilizing cell-cell interactions and facilitating leukocyte endothelial transmigration and has been characterized as a site for the cellular entry of human rhinovirus (Bella *et al*., 1998). The CD44 antigen is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and cell migration (Fujisaki *et al*., 1999).

To determine whether the expression of candidate molecules increases upon MDV entry, cellular proteins from CEF infected with 100 PFU of MDV were collected at 20, 40, and 120 mins p.i., and Western blot analysis was performed using antibodies targeting HSP70, HSP90, CD54, and CD44. The results observed using HSP70 antibody showed a specific band with a molecular mass of 70 kDa. This was confirmed by the expression of HSP70 in CEF, where its presence was detected at 20–40 mins p.i. Meanwhile, the band nearly disappeared at 120 mins p.i. (Fig. 2). Tests using the other antibodies described did not reveal significant bands, indicating that they are not involved in MDV entry (results not shown). Furthermore, the interaction between the MDV glycoprotein gH and HSP70 in the cell extract was confirmed through a co-immunoprecipitation assay. This experiment clearly showed detectable bands at 110 kDa when gH antibody was used in comparison with normal rabbit serum that was used as a negative control (Fig. 3). Thus, these co-immunoprecipitation experiments provided clear biochemical evidence of interaction between MDV gH and HSP70 in CEF.

To confirm that the HSP70 molecule is involved in the entry of MDV into CEF, plaque inhibition assay was performed. The percentage of plaque inhibition (i.e., the inhibition rate) was normalized to 100 PFU of cell-free MDV, which was assigned a value of 0%. The results showed potent antiviral activity; 50 μg of HSP70 antibody per ml almost completely inhibited the formation of plaques with an inhibition rate of 93 ± 5.2% (Fig. 4a, left panel). The results of the plaque inhibition assay using other antibodies described (including HSP90) up to a concentration of 50 μg/ml yielded different results (Fig. 4a, right panel), suggesting that the observations related to anti-HSP70 were specific.

To further investigate the involvement of HSP70 in the entry of the MDV into CEF, receptor reconstruction concept

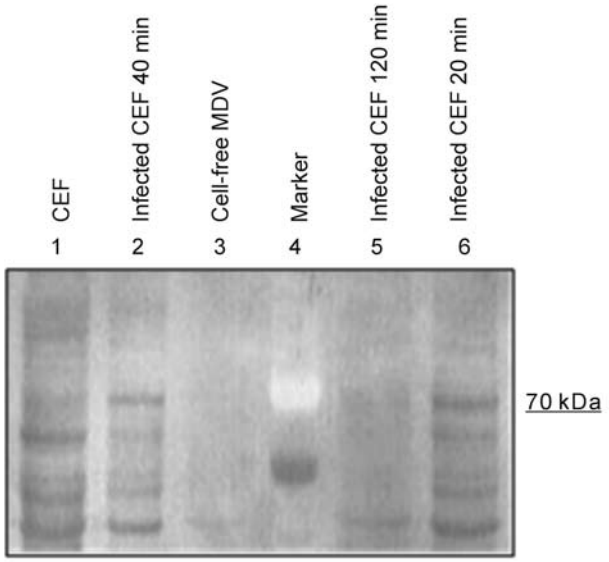

Fig. 2

Western blot analysis of CEF proteins with antibodies to potential receptors

Blotted proteins of MDV-infected CEF (20, 40, and 120 mins p.i.) were detected with an HSP70 antibody, see lanes 6, 2, and 5 in comparison to uninfected CEF (lane 1). Lane 3 shows 100 PFU of CEF-associated MDV; lane 4 is the protein marker.

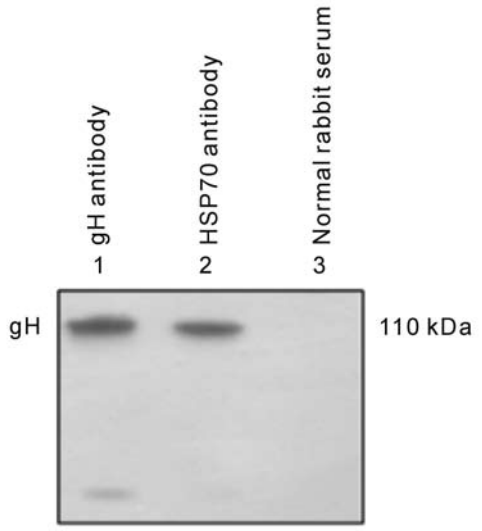

Fig. 3

Western blot analysis of co-immunoprecipitated receptor

Proteins from MDV-infected CEF were incubated with either antibodies against gH (lane 1) or HSP70 (lane 2) or normal rabbit serum (lane 3). The receptor molecules precipitated with corresponding antibodies were released and subjected to Western blot analysis for gH.

was implemented by the use of the compound quercetin. Quercetin is known to deplete HSP70 from the cell surface (Das *et al*., 2009). HSP70 knockdown and treatment with quercetin both reduced hepatitis C virus infectious particle production at nontoxic concentrations (Gonzalez *et al*., 2009). Our results showed a significant reduction in the number of plaques, with an inhibition rate of 91 ± 3.6% at a quercetin concentration of 300 μmol/l in comparison with control cells infected with MDV but not treated with quercetin (Fig. 4b). Furthermore, HSP70 knockdown assay demonstrated that the HSP70 silencing significantly interfered with the MDV entry into CEF, with an inhibition rate of 72 ± 2.7% (Fig. 4c). These results proved that HSP70 plays a critical role in the entry of MDV. To confirm that quercetin did not exert toxic effects on CEF, monolayers were exposed to a range of concentrations (100, 300, 500 μmol/l) of the compound for 24 hrs, and the cell viability was analyzed by the lactate dehydrogenase assay according to the manufacturer's instructions using a commercial cytotoxicity detection kit (Roche). There was no statistical difference between the viability of the control (untreated) cells and the viability of cells exposed to the quercetin. Quercetin did not exhibit cytotoxic effects at the concentrations tested.

In the present paper, the results of a Western blot analysis of CEF proteins for virus receptors, Western blot analysis of CEF proteins with antibodies to potential receptors, Western blot analysis of co-immunoprecipitated receptor, and inhibition of virus plaque formation support the conclusion that HSP70 is involved in the MDV entry into CEF. Further studies are necessary to prove the occurrence of a conformational change of HSP70 upon interaction with gH and to demonstrate the involvement of a putative ligand in the interaction of gH and HSP70. Our current research proves that CEF-associated MDV can replicate in the human tumor cell line HeLa and HeLa-associated MDV particles can re-infect CEF cells. HSP70 molecule may be involved in MDV replication in HeLa cells. A related study investigating the entry of oncogenic MDV into HeLa cells is currently in progress.

MDV is a commonly observed virus. The very virulent forms of this virus frequently cause acute explosive outbreaks in chickens, despite the availability of vaccines. Results in the present paper will facilitate the design of new antiviral agents that interfere with the viral entry into target cells once the receptor complex is defined. The work represents the first step in a series of studies of multifunctional HSP70 to enhance our understanding of the MDV infection process.

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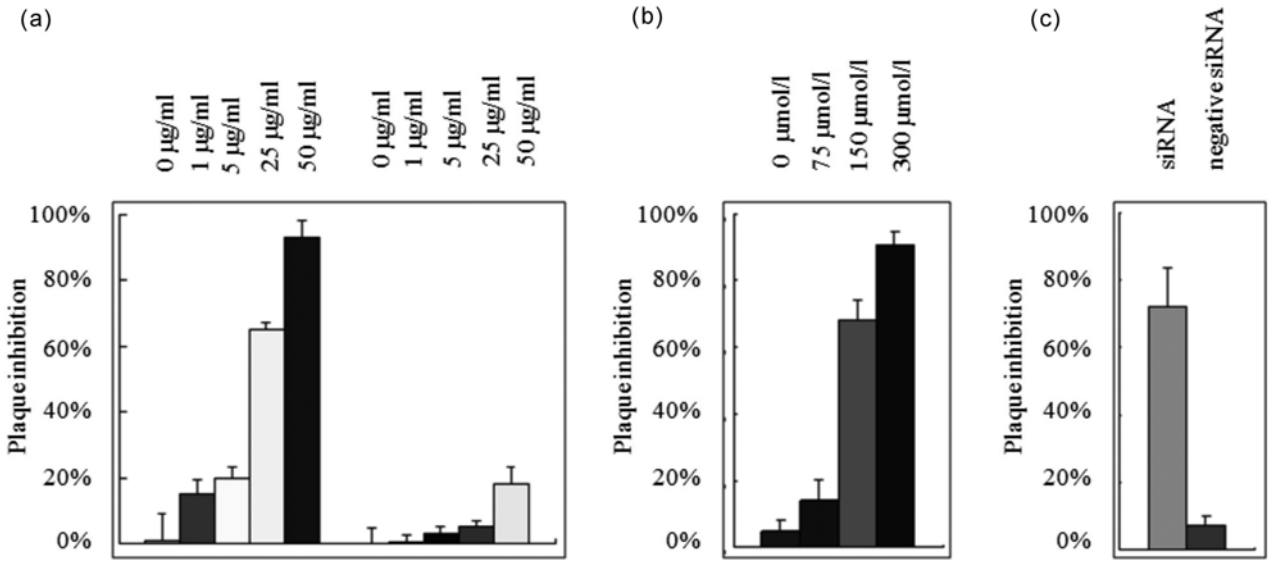

Fig. 4

Inhibition of virus plaque formation with HSP70 antibodies, quercetin and siRNA

(a) To examine the effect of HSP70 (left) and HSP90 (right) antibodies, MDV-infected CEF were incubated on day 1 p.i. with various concentrations of the two antibodies. After washing, fresh medium was added and plaques were counted on day 6 p.i. (b) To examine the effect of HSP70 depletion from the cell surface, CEF were incubated with various concentrations of quercetin for 6 hrs, washed, infected with the virus, supplied with fresh medium and 5 days later observed for plaque formation. (c) The plaque-forming inhibition assay was also used to study the effect of HSP70-siRNA or negative-siRNA treatment on plaque formation.

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