GLYCOPROTEIN gD OF MDV LACKS FUNCTIONS TYPICAL FOR α-HERPESVIRUS gD HOMOLOGUES

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Summary. – Glycoprotein D (gD) belongs to family of conserved structural proteins of α-herpesviruses. During productive infection of cells by herpes simplex virus 1 (HSV-1) gD has several important functions, is involved in virus penetration to and release from infected cells and is one of main targets of neutralizing antibodies. Similar functions are shared also by other α-herpesvirus gD homologues. Surprisingly, in previous studies it was found that MDV gD expression could not be detected during infection *in vitro* using immunological methods. In this study we have analyzed expression of MDV gD and its biological consequences. *In vitro* expression using rabbit reticulocyte lysate and/or overexpression in transfected cells showed that the second ATG codon is required for synthesis of mature, glycosylated gD. In addition, it was found that gD overexpression is neither toxic for transfected cells nor is involved in membrane fusion. After MDV infection of a proprietary cell line stably transfected with plasmid overexpressing MDV gD, no viral particles could be found in culture. On the other hand, cells overexpressing the MDV gD were sensitive to MDV infection in similar way as parental, non-transfected cells. From our study and results of other authors we propound the following conclusions: (i) MDV gD expression is blocked during *in vitro* infection at transcription level; (ii) MDV gD is lacking many important functions characteristic for other α-herpesvirus gD homologues; (iii) overexpression of single MDV gD does not result in production of mature infectious MDV particles.

Key words: MDV; cell-associated herpesvirus; gene expression; glycoprotein D

Introduction

Envelopes of herpesviruses embody several virus-encoded glycoproteins. Some of them are involved in primary steps of virus entry to cell. In the case of α-herpesviruses, initial interaction of virus with cell surface heparan sulfate proteoglycans is mediated by glycoprotein C (gC) and particularly also by glycoprotein B (gB) (Herold *et al.*, 1994). This interaction is followed by stable attachment of virus conferred by binding of gD and/or glycoprotein H + glycoprotein L (gH+gL) complex to specific cellular receptors that leads to fusion of viral envelope with host cell membrane. This interaction is interceded for gD through binding with herpesvirus entry mediator molecules. First one identified and characterized was HVEM, later designated HveA, member of the TNF/NGF receptor family (Montgomery *et al.*, 1996). Later, two additional surface proteins were identified that are capable to confer susceptibility of cells to herpesvirus infection. HveB was previously described as poliovirus receptor-related protein 2 and in addition to HSV-1 it also mediates entry of HSV-1 mutants unable to bind HveA, HSV-2, pseudorabies virus (PRV) but not bovine herpesvirus 1 (BHV-1) (Warner *et al.*, 1998). A human member of the immunoglobin superfamily, poliovirus receptor-related protein 1, HveC was shown to mediate entry of several a-herpesviruses including HSV-1, HSV-2, PRV and BHV-1 (Geraghty *et al.*, 1998).

α-herpesviruses gD homologous proteins have also other functions that are substantial during life cycle of the viruses and interfere with functions of infected organism. Particularly, HSV-1 gD is one of main targets of neutralizing antibodies (Cohen *et al.*, 1984; Para *et al.*, 1985) and its expression in *in vitro* transfected cells blocks penetration

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but not attachment of HSV-1 to the cells (Johnson and Spear, 1989).

Genes encoding α-herpesvirus gD homologous proteins were also identified within short unique genomic (U_S) regions of serologically related avian herpesviruses, MDV-1, MDV-2 and herpesvirus of turkeys (HVT) (Ross *et al.*, 1991; Zelník *et al.*, 1993; Jang *et al.*, 1996). Surprisingly, it was found that MDV and HVT do not express efficiently their gD genes during *in vitro* infection (Brunovskis and Velicer, 1995; Ono *et al.*, 1996). The only tissue indicated to express MDV gD are feather follicles of infected birds (L.J.N. Ross, unpublished results) where the enveloped particles are produced and released to environment (Calnek *et al.*, 1970). The reasons why these viruses cannot cause fully productive infection with release of infectious virus particles in other tissues or cells are not known. These findings led to speculations that low expression of MDV gD *in vitro* could result in inability of MDV to produce fully infectious cell-free viral particles.

Recent data from our and also other laboratories demonstrated that MDV and/or HVT gD expression is blocked at transcriptional level. Furthermore, several in-frame potential initiation ATG codons were identified at the 5'-ends of both MDV and HVT gD genes that might hamper efficient initiation of translation. The main goal of the presented study has been to analyze expression of MDV gD in infected and transfected cells and consequences of its overexpression in susceptible proprietary cells on biological properties of MDV.

Materials and Methods

Cells, viruses, cells transfection. MDV-1 CVI988 strain that was previously adapted for propagation on a susceptible proprietary cell line (M. Geerligs, unpublished results) was used throughout this study.

For gD expression experiments, chicken embryo fibroblasts (CEFs) and/or the proprietary cell line were transiently transfected with individual plasmid constructs using LipofectAMINE reagent (Life Technologies, UK). In case of stable transfection ProFection Transfection System with calcium phosphate (Promega, USA) was used following instructions of the supplier. Stably transfected cells were selected for resistance with G418 (Life Technologies, UK) that was added to medium to final concentration of 600 μg/ml.

Construction of expression plasmids. Individual fragments of MDV DNA covering gD gene were cloned in pBK-CMV expression vector (Stratagene, USA) by conventional methods of molecular cloning (Sambrook *et al.*, 1989) and named pBKC-gDM1 to 4. Overview of 5'-ends mapping of these fragments is summarized in Fig. 2.

mRNA isolation and Northern blot analysis. mRNA from mock- and MDV-infected cells was isolated using QuickPrep mRNA Purification Kit (Pharmacia Biotech, Sweden). Approximately 2 μg of mRNA was separated in formaldehyde denaturing agarose gel, transferred to Hybond N^+ membrane (Amersham Bio-Tech, UK) and MDV gD-positive mRNAs were detected using MultiPrime (Amersham BioTech, UK) ^{32}P-labeled DNA probe (0.6 kbp *Mlu*I-*Bam*HI fragment of the MDV gD) following recommendations of the supplier and methods described by Sambrook *et al.* (1989).

In vitro expression studies. Synthetic transcripts were prepared from individual linearized plasmid templates using RNA Transcription Kit (Stratagene, USA). The transcripts were then used in *in vitro* translation experiments that were carried out using Nuclease-Treated Rabbit Reticulocyte Lysate (Promega, USA) according to recommendations of the supplier. To study processing of the synthesized polypeptides, Canine Microsomal Membranes (Promega, USA) were added to the *in vitro* translation reaction. The synthesized ^{35}S-labeled proteins were separated in 10% denaturing polyacrylamide gels (SDS-PAGE).

Western blot analysis. Cells from transiently and/or stably transfected cells were collected from cell culture plates and directly lysed in SDS-PAGE protein loading buffer. After electrophoretic separation, proteins were transferred to Hybond-C membrane (Amersham Life Science, UK) and polypeptides were detected using MDV gD-specific monoclonal antibody (MAb) DA7 (L.J.N. Ross, unpublished results) and BM Chemiluminescence Western Blotting Kit (Boehringer Mannheim, Germany) according to recommendations of the supplier.

Results and Discussion

Limited natural expression of MDV gD during in vitro infection

Initial attempts to identify and characterize MDV gD in *in vitro* infected cells were unsuccessful (Brunovskis and Velicer, 1995). Primary question to be addressed was at what level is MDV gD expression blocked. For this reason we decided to analyze MDV gD transcription in infected cells. Northern blot analysis was carried out on mRNA isolated from uninfected or MDV-infected cells. As it can be seen from Fig. 1, a major band corresponding to transcript of approximately 7.5 kb was specifically identified in infected cells. Despite the positive result from this experiment, it should be noted that to obtain positive signal approximately 2 μg of mRNA was loaded per lane and exposure times were prolonged to 3–5 days. These conditions allowed detection of less abundant transcripts. Indeed, when the same blot was hybridized with MDV gB probe, signal of similar intensity could be detected after overnight exposure (data not shown). The results match those of Tan and Velicer (1996) who also identified approximately 7.5 kb MDV gD-positive transcript in „overloaded" Northern blot analysis. It was also found that this transcript initiates far upstream of the MDV gD initiation codon, likely to embody MDV protein kinase and SORF4 genes at the 5'-end and gI and gE genes at the 3'-end of the transcript.

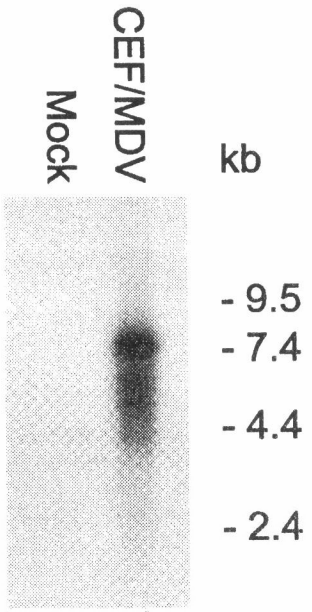

Fig. 1
Northern blot analysis of MDV gD expression in *in vitro* infected cells

In vitro expression (transcription/translation) of MDV gD

Sequence analysis of the MDV gD gene revealed that there are several ATG in frame codons at the 5'-end (Ross *et al.*, 1991). To study utilization of these ATG codons in translation initiation, there were constructed recombinant pBK-CMV plasmids carrying sequences of MDV gD open reading frame (ORF), where 5'-end proximal sequences of the gene were gradually removed (Fig. 2). The constructed plasmids were then used for *in vitro* expression experiments (*in vitro* transcription followed by *in vitro* translation). The results are demonstrated in Fig. 3. To study processing of synthesized polypeptides by signal peptide cleavage and glycosylation, microsomal membranes were added to the *in vitro* translation reaction (panel B in Fig. 3). By *in vitro* expression studies we demonstrated that the second ATG codon of MDV gD gene is sufficient to drive translation and processing of synthesized polypeptides by glycosylation. In addition, presence of additional ATG codons both upstream and/or downstream of the "real" initiation one does not affect translation efficiency and post-translational modification. *In vitro* expression studies were successfully used in our previous preliminary characterization of proteins encoded by the U_S region of HVT (Zelník *et al.*, 1994). Similar results were also obtained for HVT gD, that carries 5 potential translation initiation ATG codons at the 5'-end of the gene and where the forth one is utilized to produce glycosylated protein (data not shown).

Transient expression of MDV gD gene in transfected cells

Despite the fact that *in vitro* expression experiments offer fast results indicating translation „ability" of a given gene, proper studies on protein synthesis and function usually require native cell environment. We attempted to address several topics using transient MDV gD gene expression. The first one was to confirm results of *in vitro* transcription/translation assays. In these experiments the proprietary line or CEF cells were transfected with individual pBKC-gDM plasmid constructs using LipofectAMINE reagent. From transfected cells that were collected two days after transfection, protein lysates were prerared and were analyzed by Western blot analysis. The results of transient expression of MDV gD (Fig. 4)

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                                                              1->
AAA ATA TAT ATA GCG TCT CCT ATA ACT GTT ATA TTG GCA CCT TTT AGA

                                                    2->
GCT TCG GTA TGA ATA GAT ACA GAT ATG AAA GTA TTT TTT TTT AGA TAT
                                   Met Lys Val Phe Phe Phe Arg Tyr

                                                3->
ATC TCA TCC ACG AGA ATG ATT CTT ATA ATC TGT CTA CTT TTG GGA ATT
Ile Ser Ser Thr Arg Met Ile Leu Ile Ile Cys Leu Leu Leu Gly Ile

                                          4->
GGG GAC ATG TCC GCA ATG GGA CTT AAG AAA GAC AAT TCT CCG ATC ATT
Gly Asp Met Ser Ala Met Gly Leu Lys Lys Asp Asn Ser Pro Ile Ile
   ⇑
predicted signal peptide cleavage site
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Fig. 2
Mapping of the 5'-end of MDV gD DNA fragment cloned in PBK-CMV vector and further used throughout the study
ATG codons are shown in bold and vertical arrow indicates predicted signal peptide cleavage site.

were consistent with those obtained from *in vitro* expression studies. Efficient processing (glycosylation) of MDV gD requires the second ATG codon of the gD ORF. It should be also noted that the presence of additional upstream ATG that is in frame with the „real" translation initiation site does not interfere with expression of MDV gD (compare lanes 1 and 2 in Fig. 4.). The M_r of MDV gD detected in transient expression experiments (approximately 65 kDa) matches that of gD identified during MDV infection *in vivo* in feather follicles (L.J.N. Ross, unpublished results). This fact might indicate identical post-translational processing of MDV gD polypeptides during transient expression compared to its natural expression.

Construction of cell lines stably expressing MDV gD

From *in vitro* and transient expression experiments it became clear that pBKC-gDM2 construct carrying the second ATG codon but lacking the first one of the gD ORF enables synthesis of processed MDV gD. To further characterize effect of MDV gD overexpression in non-infected and/or MDV-infected cells we attempted to prepare a proprietary cell line that would stably express MDV gD and would be still susceptible for MDV infection. Following transfection with pBK-gDM2, the cells were treated with G418 to select positively transfected cells. After 3 weeks of selection 12 positive clones were picked and further grown. Four clones were randomly chosen and tested for MDV gD expression. All of them were found to be MDV gD-positive (data not shown). The growth properties and morphology of stably transfected cells were similar to those of non-transfected cells. In further experiments DM2A and DM2B clones were used. Successful stable transfection of the proprietary cell line overexpressing MDV gD also means that the protein is not toxic for cells. This fact contradicts cytotoxic effect of BHV-1 gD overexpression (Fehler *et al.*, 1992).

MDV infection of gD-expressing cells

DM2A and DM2B cell lines stably expressing MDV gD were infected with MDV-1 CVI988 strain adapted for growth in the susceptible proprietary cell line. Monitoring of virus release to medium was accomplished by ELISA, PCR and virus titration of obtained supernatants used as inocula to infect fresh non-transfected cells. Transfected cells and supernatants (medium) were collected for analysis either 2 or 5 days post infection. There were not found any infectious viral particles in supernatants of infected cells. ELISA and PCR methods were also employed to monitor presence of viral particles in supernatants obtained from infected transfected and/or non-transfected cells. However, both these methods confirmed the results of the titration, i.e. the absence of viral particles in the supernatants.

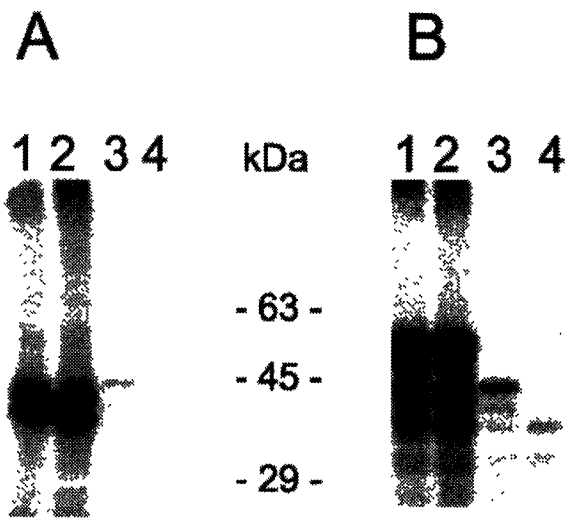

Fig. 3
Autoradiogram of ^{35}S-labeled MDV gD polypeptides translated *in vitro* using rabbit reticulocyte lysate
Polypeptides synthesized in the absence (A) and presence (B) of microsomal membranes. Numbers above the lanes correspond to individual pBK-gDM constructs.

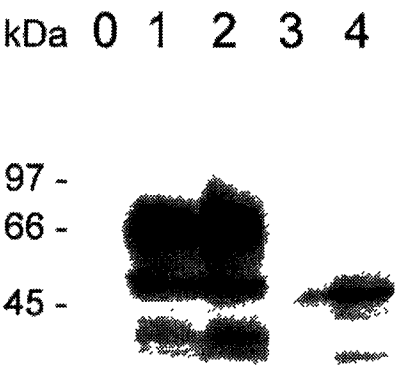

Fig. 4
Western blot analysis of MDV gD expression in transiently transfected cells
Numbers above the lanes correspond to individual pBK-gDM constructs, 0 indicates non-transfected cells.

MDV-1 CVI988 infection studies on established cell lines demonstrated that overexpression of MDV gD does result in increased virus release from cells to medium. In addition, it also suggests that there are required other factors besides gD (e.g. specific for feather follicle epithelium) that provide the means for production of mature infectious MDV particles and their release to environment.

Our data are further theoretically supported by recent studies on BHV-1 mutant viruses, in which passaging of gD-deleted BHV-1 can result in production of fully infectious viral particles demonstrating that gD deletion can be in BHV-1 model compensated by mutation in other virus-encoded glycoprotein, gH (Schröder *et al.*, 1997). Similarly, when HSV-2 gD expression cassette was inserted to genome of varicella-zoster virus (VZV) that is also cell-associated virus and lacks gD gene homologue, there was not observed increase of recombinant VZV-gD2 virulence (Heineman *et al.*, 1995).

It should be also noted that MDV gD-expressing cells were susceptible for infection with MDV in similar manner compared to non-transfected cells (same plaque morphology and virus growth kinetics). This result indicates that presence of MDV gD in cells does not intercede with MDV infection unlike interference of HSV-1 gD overexpression in cells with infection by the virus (Johnson and Spear, 1989).

The results presented in this paper clearly show that MDV gD is lacking many properties and functions characteristic for other a-herpesvirus gD homologues. In addition, Anderson *et al.* (1998) found that MDV gD is not essential for oncogenicity and horizontal transmission of MDV. The real function of MDV gD during infection, if there is any, remains to be elucidated.

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