

## Soluble M3 proteins of murine gammaherpesviruses 68 and 72 expressed in *Escherichia coli*: analysis of chemokine-binding properties

R. MATÚŠKOVÁ, P. PANČÍK, I. ŠTIBRÁNIOVÁ, P. BELVONČÍKOVÁ, I. REŽUCHOVÁ, M. KÚDELOVÁ\*

Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 05 Bratislava, Slovak Republic

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**Summary.** – M3 protein of murine gammaherpesvirus 68 (MHV-68) was identified as a viral chemokine-binding protein 3 (vCKBP-3) capable to bind a broad spectrum of chemokines and their receptors. During both acute and latent infection MHV-68 M3 protein provides a selective advantage for the virus by inhibiting the antiviral and inflammatory response. A unique mutation Asp307Gly was identified in the M3 protein of murine gammaherpesvirus 72 (MHV-72), localized near chemokine-binding domain. Study on chemokine-binding properties of MHV-72 M3 protein purified from medium of infected cells implied reduced binding to some chemokines when compared to MHV-68 M3 protein. It was suggested that the mutation in the M3 protein might be involved in the attenuation of immune response to infection with MHV-72. Recently, *Escherichia coli* cells were used to prepare native recombinant M3 proteins of murine gammaherpesviruses 68 and 72 (Pančík *et al.*, 2013). In this study, we assessed the chemokine-binding properties of three M3 proteins prepared in *E. coli* Rosetta-gami 2 (DE3) cells, the full length M3 protein of both MHV-68 and MHV-72 and MHV-68 M3 protein truncated in the signal sequence (the first 24 aa). They all displayed binding activity to human chemokines CCL5 (RANTES), CXCL8 (IL-8), and CCL3 (MIP-1 $\alpha$ ). The truncated MHV-68 M3 protein had more than twenty times reduced binding activity to CCL5, but only about five and three times reduced binding to CXCL8 and CCL3 when compared to its full length counterpart. Binding of the full length MHV-72 M3 protein to all chemokines was reduced when compared to MHV-68 M3 protein. Its binding to CCL5 and CCL3 was reduced over ten and seven times. However, its binding to CXCL8 was only slightly reduced (64.8 vs 91.8%). These data implied the significance of the signal sequence and also of a single mutation (at aa 307) for efficient M3 protein binding to some chemokines.

**Keywords:** murine gammaherpesvirus; chemokine-binding protein; M3 protein, signal sequence

### Introduction

Herpesviruses and poxviruses encode viral protein homologs of chemokines, members of a complex family of cytokines that control the recruitment of cells to

the sites of infection and inflammation. They also contain viral homologs of chemokine receptors (vCKRs) and viral chemokine-binding proteins (vCKBPs) (Lalani *et al.*, 2000). A large number of herpesvirus-encoded CKRs with sequence similarity to cellular CKRs have been identified and characterized. Only two of these homologs have been identified in the poxviruses swinepox and capripox, but no functional data are available up to date. In contrast to herpesviruses, poxviruses are known to encode soluble vCKBPs that are secreted in large amounts from infected cells and bind chemokines in solution. Chemokines play a crucial role in host defense. They are involved in both innate and adaptive immunity (Luster, 2002), and thus many infectious agents have

\*Corresponding author. E-mail: virukude@savba.sk; phone: +421-2-59302-434.

**Abbreviations:** GAGs = glycoaminoglycans; MHV-68 = murine gammaherpesvirus 68; MHV-72 = murine gammaherpesvirus 72; vCKBP = viral chemokine-binding protein; vCKR = viral homolog of the chemokine receptor

developed strategies to manipulate the host chemokine network. Chemokines are classified into four subfamilies based on the arrangement of cysteine residues at their N-terminus: CXC, CC, C, and CX<sub>3</sub>C (Zlotnik and Yoshie, 2000). The specific effects of chemokines on target cells are mediated by the members of a family of seven-transmembrane-spanning G-protein coupled receptors. As predominantly basic, positively charged molecules, chemokines also interact with the negatively charged glycosaminoglycans (GAGs) expressed on the endothelial cell surface (Proudfoot *et al.*, 2003).

Recently, a soluble vCKBP was identified, designated as M3 protein, hvCKBP or vCKBP-3 encoded by murine gammaherpesvirus 68 (MHV-68), with no sequence similarity to cellular chemokine-binding receptors and cytokine binding protein II (vCKBP-II) encoded by poxvirus (Carfi *et al.* 1999, van Berkel *et al.*, 2000). This protein ( $M_r = 44$  kDa) is secreted from infected cells in large amounts, binds chemokines of the four subfamilies (CC, CXC, C and CX<sub>3</sub>C) with high affinity and blocks their interaction with CKRs. This is unusual because cellular CKRs display binding specificity and the poxvirus vCKBP-II is specific for CC chemokines. M3 protein prevents chemokine-induced signal transduction *in vitro* (Parry *et al.*, 2000; van Berkel *et al.*, 2000) and it also blocks the induction of intracellular signaling. It has also been shown to block the interactions of chemokines with GAGs (Webb *et al.*, 2003). MHV-68 is a natural pathogen of murid rodents belonging to the species Murid herpesvirus 4 (MuHV-4) (the genus *Rhadinovirus*, the subfamily *Gammaherpesvirinae*) (van Regenmortel *et al.*, 2000). It is genetically related to the primate gammaherpesviruses – Saimiriine herpesvirus 2, Human herpesvirus 8, and Human herpesvirus 4 (Virgin *et al.*, 1997). These viruses are able to establish latent, life-long infections and are often associated with various types of malignancies, such as Kaposi's sarcoma and B-cell lymphomas (Sunil-Chandra *et al.*, 1992; Kúdelová and Rajčáni, 2009). MHV-68 is intensively studied in many laboratories since it is an attractive experimental model for gammaherpesvirus infection (Rajčáni and Kúdelová, 2007).

MHV-68 M3 protein, encoded by early-late lytic gene, is expressed continually during lytic infection, during the establishment of latent infection, and during latency *in vivo* (Simas *et al.*, 1999; Ebrahimi *et al.*, 2003). M3 protein is predicted to play a role in the establishment of latency by indirect protection of latently infected B cells during lytic infection of macrophages and dendritic cells (Flaño *et al.*, 2000; Marques *et al.*, 2003). M3 protein produced by cells infected with MHV-68 may alter the migration of CCR7-expressing T, B, or dendritic cells towards local gradients of CCL19 and CCL21, thus potentially delaying the initiation of a specific immune response against the virus (Jensen *et al.*, 2003). M3 protein may also significantly inhibit the chemokine effector functions, thereby inhibiting the antiviral activity of recruited leukocytes and thus allowing the virus

to replicate at higher levels in the presence of cells serving as potential targets for the establishment of latency (Sarawar *et al.*, 2002). In support of this, upregulation of lymphocyte trafficking and the control of macrophage trafficking in the CNS during MHV-68 infection has been demonstrated (van Berkel *et al.*, 2002). The host response to viral infection with M3 del mutant MHV-68 was shown altered in both the lungs and the germinal reaction center in the spleen, suggesting M3 gene to be responsible for host-specific differences (Hughes *et al.*, 2011). Alexander *et al.* (2002) first reported the crystal structure model of M3 protein bound to the CC chemokine CCL2 (monocyte chemoattractant protein 1) and described the binding sites of this vCKBP recognized by the chemokine and also by the cellular receptor CCR2.

In addition to the MHV-68 virus, the only virus classified as a MuHV-4 species to date, murine gammaherpesvirus 72 (MHV-72) (clone h3.7) is very likely a strain of murine gammaherpesvirus. Its biological properties related to MHV-68 have also been characterized (Mistrišková *et al.*, 2000; Rašlová *et al.*, 2001) and the differences in genome sequence encompassing 19 genes (22,899 bp) have been identified (Mačáková *et al.*, 2003; Valovičová *et al.*, 2006; Belvončíková *et al.*, 2008; Halášová *et al.*, 2011). MHV-72 appears to be less pathogenic and more oncogenic than MHV-68 (Nash *et al.*, 2001), displaying delayed cytopathic effect in infected normal murine mammary gland cells (up to 48 hours) and higher malignancy development rate in long-term infected mice in comparison with MHV-68 (Mistrišková *et al.*, 2000). Besides MHV-72, several other murine herpesviruses were found occurring throughout some mouse species, which display similar pathogenesis of acute and latent infection manifested in their hosts. Among these is the Wood mouse herpesvirus isolated from wood mouse in the UK, the full genome sequence of which was recently identified by Hughes *et al.* (2010). Biological properties of some other murine herpesviruses (MHV-60, MHV-78, MHV-76, MHV-Šumava, MHV-4556) isolated from bank vole and wood mice in Slovakia and Bohemia (Blaškovič *et al.*, 1980; Kožuch *et al.*, 1993; Blaškovičová *et al.*, 2007; Halášová *et al.*, 2011; Kúdelová *et al.*, 2012) are also studied (reviewed by Wagnerová *et al.*, 2015).

In a previous study, we showed that M3 gene of MHV-72 has codon-changing mutation at nucleotide position 920 (Asp307Gly) located near the chemokine-binding domain (Belvončíková *et al.*, 2008). This mutation was suggested to be involved in attenuating the immune response to MHV-72 infection. A study of the binding affinities of the MHV-72 M3 protein purified from the media of infected BHK-21 cells to five human chemokines (CCL3, CCL5, CCL11, CCL2, and CXCL8) showed that MHV-72 M3 protein exhibited significantly lower binding affinity to CCL5 and CXCL8 (only 11.1% and 20%) than MHV-68 M3 protein, but had comparable affinity to CCL11, CCL2, and CCL3. On the other hand, the amount of MHV-72 M3 protein bound to

both human CCL11 and CCL2 reached about 90% of that detected for MHV-68 M3 protein. In our first study, we prepared recombinant His-tagged M3 proteins of MHV-68 and MHV-72 using the pET-26b(+) expression system. We optimized the expression of M3 proteins of MHV-68 and MHV-72 in *E. coli* BL21 (DE3) and Rosetta (DE3) cells as well as their purification by Ni-NTA chromatography under both denaturing and native conditions (Pančík *et al.*, 2013).

In this study, we determined chemokine-binding affinities of three recombinant M3 proteins: two of MHV-68 (the full length or truncated in the signal sequence) and the full length MHV-72, prepared in *E. coli* Rosetta-gami 2 (DE3) cells, to human chemokines CCL5, CXCL8, and CCL3. We described the changes in these properties of M3 protein caused by the absence of signal sequence and the presence of Asp307Gly mutation in this protein, which is unique for the MHV-72.

### Materials and Methods

**Cells.** Normal murine mammary gland (NMG) (ATCC CRL-1636) and Baby hamster kidney 21 (BHK-21) fibroblasts (ATCC CCL-10) cell lines were maintained in DMEM (GIBCO) supplemented with 10% (v/v) FCS, 2 mmol/l glutamine (Invitrogen) and penicillin-streptomycin-amphotericin (100 mg/ml) (Cambrex) at 37°C. *E. coli* Rosetta-gami2 (DE3) cells were obtained from Novagen. *E. coli* JM109 cells were obtained from Promega.

**Viruses.** Two murine gammaherpesviruses (MHV-68 and MHV-72) originally isolated from the bank vole, *Myodes glareolus* (Blaškovič *et al.*, 1980), kindly provided by Prof. Mistríková (Faculty of Natural Sciences, Comenius University, Bratislava, Slovakia) were plaque purified twice to obtain clones f2.6 (MHV-68) and h3.7 (MHV-72) (Kúdelová *et al.*, 2012). Viral DNA was isolated and purified from virions as previously described (Rašlová *et al.*, 2000).

**Recombinant expression plasmids with the full length M3 genes of MHV-68 and MHV-72.** Two expression plasmids (P26-M3his/68 and P26-M3his/72) containing the full length ORF M3 of MHV-68 or MHV-72 with 6 triplets encoding histidines at the C-terminal end described previously by Pančík *et al.* (2013) were used.

**PCR amplification of truncated M3 gene of MHV-68.** To amplify the genomic region corresponding to ORF M3 of MHV-68 (Acc. No. AF105037) shortened by triplets of the first 24 (aa), recombinant plasmid pGEM-TM3 MHV-68 prepared previously by Belvončíková *et al.* (2008) was used as a template. Primers derived from the MHV-68 M3 sequence (Virgin *et al.*, 1997) flanked by *Nde*I and *Xho*I sites (bold, underlined) and 6 triplets encoding histidines (*in italic*) at 5' end (5'-ACA TAT GCA CCA TCA TCA TCA TCA TCT TAC TCT AGG TTT GGC ACC TGC T-3' and 5'-ACT CGA GTC TAC TAC TAA TGA TCC CCA AAA TAC TCC AGC CT-3') were used in PCR to amplify desired sequence as previously described Pančík *et al.* (2013).

**Cloning and sequencing of truncated MHV-68 M3 gene.** The PCR product containing truncated ORF  $\Delta$ ssM3 of MHV-68

(1 188 bp) was further purified and cloned into expression plasmid pET-26b(+) (Novagen) according to Pančík *et al.* (2013). A novel recombinant plasmid P26- $\Delta$ ssM3his/68 was sequenced using a commercial sequencing service (BITCET, Faculty of Natural Sciences, Comenius University, Bratislava, Slovakia), which confirmed the correct ORF of cloned insert. For sequencing, the two universal primers T7F (20-mer) and T7R (19-mer) were used.

**Expression of M3 proteins.** Competent *E. coli* Rosetta-gami2 (DE3) cells were transformed with expression plasmids P26-M3his/68, P26- $\Delta$ ssM3his/68, and P26-M3his/72. Single colonies were picked and inoculated into 10 ml of LB medium (Sigma-Aldrich) containing 30  $\mu$ g/ml kanamycin (Biolone), 34  $\mu$ g/ml chloramphenicol and 12.5  $\mu$ g/ml tetracycline (Sigma-Aldrich) grown at 37°C overnight to make a starter culture. For further purification, cells were inoculated into 1 l of Terrific Broth media (1.2% (w/v) peptone, 2.4% (w/v) yeast extract, 72 mmol/l  $K_2HPO_4$ , 17 mmol/l  $KH_2PO_4$ , 0.4% (w/v) glycerol) with antibiotics as above. Cells were grown to  $OD_{600} = 0.6$ . The expression of M3 protein was induced by adding IPTG to a final concentration of 0.5 mmol/l and the protein was produced at 37°C for 2 hr. *E. coli* cells were harvested by centrifugation at 6,000 x g at 4°C for 10 min, washed with 2 ml PBS, centrifuged at 6,000 x g again, and stored at -80°C. To check the expression of M3 protein, aliquots of *E. coli* cells were collected by centrifugation (8,000 x g for 3 min), resuspended in loading buffer and analyzed by SDS PAGE on 12.5% gel (Laemmli, 1970).

**Purification of M3 proteins.** The recombinant MHV-68 M3, MHV-68  $\Delta$ ssM3, and MHV-72 M3 proteins with 6xHis-tag on their C- or N-termini designated as M368HC,  $\Delta$ ssM368HN, and M372HC, respectively, were purified under native conditions using Ni-NTA agarose (Qiagen) according to the manufacturer's instructions with minor modifications. Before purification, the cells were weighted. 1g of wet cells was resuspended in 20 ml of lysis buffer (50 mmol/l  $NaH_2PO_4$ , 300 mmol/l NaCl, 10 mmol/l imidazole, 10% (w/v) glycerol, 0.2% (w/v) sulfobetaine-14) (SB-14, Sigma-Aldrich), 1 mg/ml lysozyme, protease Inhibitor Cocktail Set III, EDTA-Free (Calbiochem) (added at a dilution 1:200 to buffer to prevent undesired protein degradation) and incubated at 4°C for 30 min. Cells in the suspension were disrupted by French Press Cell Disruptor (Thermo Scientific) with two rounds at 20000 psi. The cell lysis mixture was then treated with 25 U Benzonase® Nuclease (Qiagen) for 10 min to remove nucleic acids followed by centrifugation at 18,500 x g at 4°C for 30 min. The supernatant was loaded onto a chromatography column with  $\approx$  0.6 ml of Ni-NTA agarose resin. After a short incubation at 4°C, non-specifically bound proteins were washed away using four rinses with 1 ml of lysis buffer without 25 mmol/l imidazole. Finally, each M3 protein was eluted from the column with the same buffer containing 250 mmol/l imidazole into three fractions. Each fraction was analyzed on 12.5% SDS-PAGE and stained using Coomassie Brilliant Blue R-250 (Santa Cruz) and the amount of the protein measured using Qubit 3.0 Fluorometer (Life Technologies).

**Western blot analysis.** Purified samples of M368HC,  $\Delta$ ssM368HN, M372HC proteins were separated on 12.5% SDS-PAGE and then identified using His-Tag® Monoclonal Antibody (Novagen) diluted

Table 1. Characteristics and chemokine-binding activities of recombinant M3 proteins prepared in *E. coli*

Name of recombinant protein	Protein length (aa)	His-tag anchor localization at protein terminal end	Binding activity to chemokine (%)		
			CCL5	CXCL8	CCL3
M368HC	406	C	2.1 <sup>d = 1:100</sup>	21.2 <sup>d = 1:5</sup>	87.6
$\Delta$ ssM368HN	382	N	88.3 <sup>d = 1:5</sup>	90	24.3
M372HC	406	C	95.6 <sup>d = 1:5</sup>	64.8	12.3

<sup>d = 1:100</sup> – sample diluted 100 times, <sup>d = 1:5</sup> – sample diluted 5 times.

in 2,5% (w/v) BSA in TBST buffer at the ratio 1:1000. The incubation was performed overnight at 4°C. Unbound antibody was washed away with TBST buffer three times for 15 min. Anti-mouse IgG HRP conjugate (Promega) diluted in TBST buffer at the ratio 1:2,500 was bound 1 hr at room temperature and then washed away as described earlier (Pančík *et al.*, 2013). Proteins on the PVDF membrane were visualized by staining with the chemiluminescent dye SuperSignal West Dura extended duration substrate (Pierce) and photographed using a Kodak Image Station 2000MM.

**Determination of chemokine-binding activities of recombinant M3 proteins.** Anti-cytokine activities were detected for 200 ng recombinant M3 protein (20 ng/ul) and its aliquots diluted at ratios 1:5, 1:10, 1:50, and 1:100. Recombinant M3 protein was screened for affinity to human chemokines – CCL5 (RANTES), (CXCL8 (IL-8) CCL3 (MIP-1 $\alpha$ ), activities, using ELISA kits: Human CCL5/RANTES DuoSet (DY278, UK), CXCL8/IL-8 (Bender MedSystems, Austria), and Human CCL3/MIP-1 alpha DuoSet (DY270, MN, USA). For each assay, 50 pg of the recombinant cytokine in ELISA diluent (PBS supplemented with 1% BSA) were mixed with 10  $\mu$ l of recombinant protein M3, undiluted or diluted, and/or PBS as a negative control, to give a total volume of 100  $\mu$ l. Each mixture was incubated for 1.5 h at room temperature, with gentle shaking and then applied to the ELISA plates (100  $\mu$ l/well). Duplicate assays were performed for each cytokine and each sample was measured in duplicate per assay. A reduction in detectable levels of a particular cytokine, when compared with 50 pg of commercial recombinant cytokine as a control, was interpreted as an evidence of cytokine-binding activity of the sample. The results, expressed as mean  $\pm$  SEM of two independent experiments, constitute percentage reduction of OD reading compared with the control.

## Results

### Construction of bacterial expression vector with truncated M3 gene of MHV-68

The sequence of MHV-68 M3 ORF lacking the first 72 nts (24 aa) ( $\Delta$ ssM3) was amplified using pGEMT-M3 MHV-68 previously prepared by Belvončíková *et al.* (2008) as a template, and primers containing *Nde*I and *Xho*I linker sequences. The forward primer included a sequence encoding six histidines at its 5'-end, allowing the expressed protein to be purified by affinity chromatography. This PCR product was cloned into

a pET-26b(+) vector *via* the *Nde*I and *Xho*I restriction sites creating recombinant plasmid P26- $\Delta$ ssM3his/68. The presence of desired sequence in the plasmid P26- $\Delta$ ssM3his/68 was verified using a commercial sequencing service.

### Expression of recombinant M3 proteins in *E. coli*

The cells of *E. coli* Rosetta-gami 2 (DE3) strain were transformed with a recombinant bacterial expression vector (P26-M3his/68, P26- $\Delta$ ssM3his/68P26 or M3his/72) according to Pančík *et al.* (2013). Expression of each of the proteins M368HC,  $\Delta$ ssM368HN or M372HC (Table.1) was induced with 0.5 mmol/l IPTG and evaluated by SDS-PAGE. We confirmed that M3 proteins of the expected size (~ 44 kDa) could be found in the lysates of transformed cells including truncated MHV-68 M3 protein (Fig.1, lane 3). The amount of all M3 proteins expressed was comparable with M3 proteins prepared by Pančík *et al.* (2013) in *E. coli* BL21 (DE3) or Rosetta (DE3) cells strains (data not shown).

### Purification of recombinant M3 protein by Ni-NTA chromatography

To purify M368HC,  $\Delta$ ssM368HN or M372HC proteins under native conditions, we used purification conditions optimized by Pančík *et al.* (2013). All recombinant M3 proteins were detected by SDS PAGE and Western blot analysis using

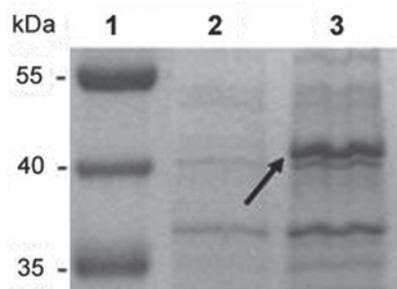


Fig. 1

**Expression of truncated M3 protein of MHV-68 in *E. coli***  
SDS-PAGE of *E. coli* Rosetta-gami2 (DE3) cell-lysate, 2 hr after IPTG induction at 37°C. PageRuler Prestained Protein Ladder (lane 1), cells transformed with P26- $\Delta$ ssM3his/68, uninduced (lane 2) and induced (lane 3). The arrow indicates the 42.4 kDa truncated M3 protein.

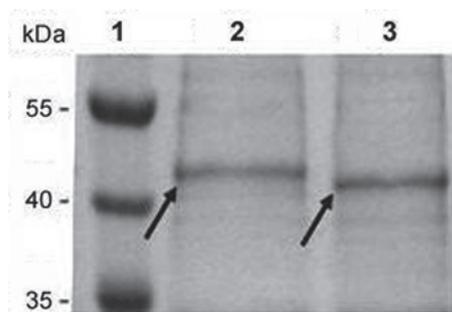


Fig. 2

#### Purification of native recombinant MHV-68 M3 proteins by Ni-NTA chromatography

SDS-PAGE of M3 proteins expressed in *E. coli*, strain Rosetta-gami 2 (DE3), and purified by affinity chromatography. PageRuler Protein Prestained Ladder (lane 1), the full-length M368HC protein (lane 2) (44.8 kDa) and truncated  $\Delta$ ssM368HN protein (lane 3) (42.4 kDa). The arrows indicate M3 proteins.

a His-Tag<sup>®</sup> monoclonal antibody (data not shown), which confirmed that we obtained all recombinant M3 proteins in quality and amount allowing analyses of their biological properties. As shown in Fig. 2, the full length and truncated M3 protein of MHV-68 exhibited different mobility in SDS PAGE (lanes 2 and 3).

#### Analysis of chemokine-binding activity of recombinant M3 proteins

The ELISA assay of the chemokine-binding properties of the full length of MHV-68, the MHV-68 M3 truncated by the signal sequence and the full length MHV-72 M3 protein showed that they all were able to bind each of the chemokines (CCL5, CXCL8, and CCL3) tested (Table 1; Fig. 3). The strongest affinity displayed the full length MHV-68 M3 protein to CCL5, when as few as 2 ng of protein bound this

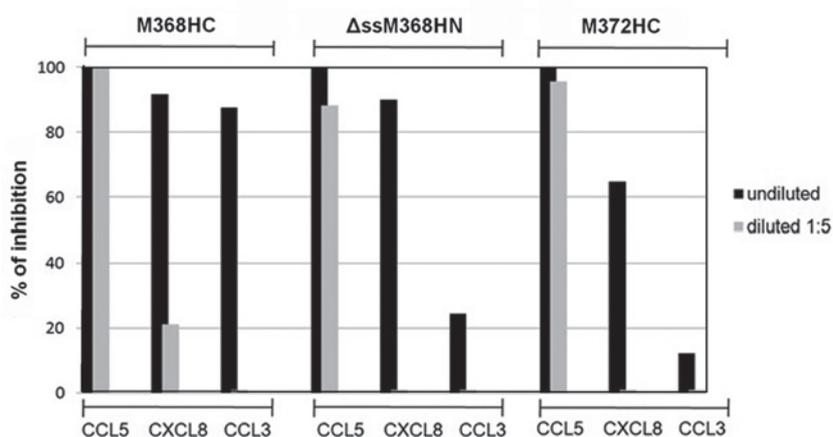


Fig. 3

Binding activity of the full-length (M368HC, M372HC) and truncated ( $\Delta$ ssM368HN) recombinant M3 proteins to CCL5, CXCL8, and CCL3 chemokine

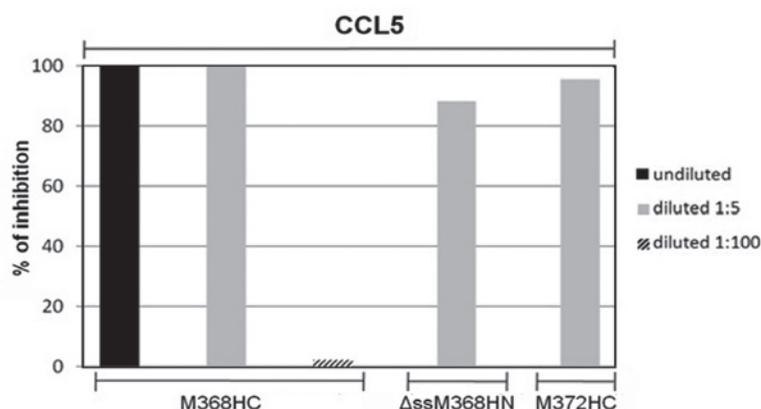


Fig. 4

Comparison of binding activities of the full-length (M368HC, M372HC) and truncated ( $\Delta$ ssM368HN) recombinant M3 proteins to CCL5 chemokine.

cytokine (Table 1; Fig. 4). In comparison to the full length MHV-68 M3 protein, truncated MHV-68 M3 protein had significantly, more than twenty times, reduced binding activity to CCL5. The truncation of MHV-68 M3 protein caused only about five and three times reduced activity to CXCL8 and CCL3, when compared to its full length counterpart. It should be noted that both proteins, five times diluted and undiluted, displayed binding activity to CXCL8 and CCL3 (Table 1). The full length MHV-72 M3 protein as compared to MHV-68 M3 had only slightly reduced binding to CXCL8 (64.8 vs 91.8%), but about seven times reduced binding to CCL3 (12.3 vs 87.6%). Binding of five times diluted full length MHV-72 M3 protein to CCL5 was comparable with that of MHV-68.

### Discussion

Gammaherpesviruses are predominantly host-specific, so the virus vs host immune system interaction plays a crucial role in the viral infection, particularly in the latency establishment and reactivation from latency (Speck and Ganem, 2010). It is very important for the proper function of the host immune system to effectively deliver the immunocompetent cells to the site of virus replication. Small soluble peptides of cytokine family, known as chemokines, are ligands that trigger many signaling cascades involved in innate immunity via binding to their receptors on surface of the immunocompetent cells (Luster, 2002; Proudfoot *et al.*, 2003). Many viruses developed strategies to overcome this signal transduction, which implies its significant role in the battle against viral infections (Alcami *et al.*, 2003). There are several pathogenic manifestations murine and human gammaherpesviruses have in common, which is the consequence of the genome homology. However, genome of murine herpesvirus 4 (MuHV-4) possesses some unique genes without known sequence homology to other viral or human genes. Out of these, M3 gene of MuHV-4 coding for the M3 protein (vCKBP of murine gammaherpesvirus) was the point of interest of last decade, pioneered by the work of Alexander *et al.* (2002). They used a recombinant baculovirus infecting insect cells Sf9 to produce enough M3 protein to determine the crystal structure of M3 both alone and bound to CC chemokine CCL2. M3 protein is a unique viral decoy receptor, which binds a broad spectrum of chemokines, thus destabilises chemokine network *in vitro* and *in vivo* (Parry *et al.*, 2000; van Berkel *et al.*, 2000; Jensen *et al.*, 2003). In spite of the high expression in lungs and spleen of experimentally infected laboratory mice (*Mus musculus*), its significant benefit for the virus replication or pathogenicity has not been successfully proved (Bridgeman *et al.*, 2001). This could imply stronger interplay of the virus replication on the immunity of its natural host (*Apodemus* spp. or *Myo-*

*des* spp.), which ought not to be fully compatible with the immune system of laboratory mice (Hughes *et al.*, 2011). The study of M3 protein alone could, however, be beneficial for human medicine. Because of its strong and specific binding of chemokines of all four subfamilies (Parry *et al.*, 2000; Belvončíková *et al.*, 2008), it can serve as a potential therapeutic tool for treating diseases linked to the deregulation of chemokine network, such as diabetes mellitus type I (Martin *et al.*, 2007, 2008; Lira *et al.*, 2009). A number of other autoimmune diseases leading to chronic inflammation could be treated with chemokine antagonists (Proudfoot *et al.*, 2003; Pyo *et al.*, 2004; Wu *et al.*, 2008).

All experiments mentioned above refer to M3 protein of MHV-68. However, data of the binding affinity to individual chemokine are still rare. In our previous study on MHV-72 (clone h3.7), Belvončíková *et al.* (2008) identified that M3 protein of MHV-72 differed from its MHV-68 counterpart in only a single amino acid mutation, which is located near the chemokine-binding domain. They found that MHV-72 M3 protein released into the media of infected BHK-21 cells bound only 11% and 20% of the amount of CCL5 and CXCL8 bound by MHV-68 M3 protein purified by the same manner. In a following study, Pančík *et al.* (2013) prepared a novel suitable expression system for production of recombinant M3 proteins in *E. coli* cells, allowing their chemokine-binding properties to be studied. The properties of M3 protein, its relatively small size of 44 kDa, its solubility in cultivation media, the absence of its posttranslational modifications, made this expression system an appropriate choice. Pančík *et al.* (2013) optimized both cultivation and induction conditions as well as purification procedure based on affinity chromatography for the proper production of soluble M3 protein. Expression of M3 protein in strain *E. coli* Rosetta (DE3) was effective, inexpensive, fast, and suitable for the continuation of the study of this protein.

In this study, we examined how a codon-changing mutation and deletion of the signal sequence of M3 protein change the chemokine-binding properties of this protein. This would contribute to a greater understanding of M3 protein biology and prospect to utilize its unique properties in gene therapy. We used conditions described by Pančík *et al.* (2013) for the production of recombinant M3 proteins in *E. coli* strain Rosetta (DE3), but we exploited the cells of *E. coli* strain – Rosetta-gami 2 (DE3) having non-reducing cytoplasm, which might enhance the formation of intramolecular disulphide bonds in recombinant M3 proteins produced. To prepare the full length M3 proteins of MHV-68 or MHV-72 in these cells, we used expression plasmids prepared in the previous study. We then constructed a novel expression plasmid containing MHV-68 M3 gene sequence lacking the codons for the first 24 aa of the protein to study the role of the signal sequence of M3 protein in binding activity to chemokines. We successfully purified three recombinant M3

proteins, two from MHV-68 and one from MHV-72, detectable by chemiluminescent dye using an antibody conjugate against the poly-histidine anchor of recombinant protein. As we expected, we obtained recombinant M3 proteins in amounts comparable with those produced before in Rosetta (DE3) cells. Similarly, the amount of purified proteins was independent on the localization of the His-tag anchor at the C- or N- terminal end of protein.

We have found that all recombinant M3 proteins prepared are able to bind each of chemokines tested but their binding activities against individual chemokine differed. The best binding we identified was of full length MHV-68 M3 protein to CCL5, when as few as only 2 ng of protein bound this chemokine. On the other hand, MHV-68 M3 protein truncated in the signal sequence had more than twenty times lower binding activity to CCL5, though 40 ng of protein still exhibited the binding. These results suggested a very important role of signal sequence of M3 protein in binding to CCL5. It is interesting that the same amount of the full length MHV-72 M3 protein (with a mutation at aa 307) displayed similar binding activity to CCL5 when compared to truncated MHV-68 M3 protein (95.6 vs 88.3%). The truncation of MHV-68 M3 protein caused about five times reduced affinity to CXCL8 as compared to its full length counterpart. However, binding of truncated MHV-68 M3 protein to CXCL8 was close (by a quarter lower) to that detected for full length MHV-72 M3 protein (64.8 vs 90.0%). Similarly, truncated MHV-68 M3 protein exhibited reduced binding to CCL3 (about three times) in comparison to the full length MHV-68 M3 protein (24.3 vs 87.6%), when both proteins displayed this binding only in undiluted form. Binding of MHV-72 M3 protein to CCL3 was two times weaker than that of truncated MHV-68 M3 protein (24.3 vs 12.3%).

Results of this study indicated differences among three recombinant M3 proteins in the binding affinity to each of three chemokines tested. We have found that the binding of the full length MHV-68 M3 protein to CCL5 is about hundred times stronger than to CCL3 and about ten times stronger than to CXCL8. Also, both “modified” proteins (without signal sequence or with a single mutation at aa 307) exhibited binding stronger by order to CCL5 than to CXCL8 and CCL3. All recombinant M3 proteins had the weakest binding to CCL3, when as much as 200 ng of protein was necessary to detect the binding. The latter observation was first described in a study on M3 protein released to the media by virus-infected BHK-21 cells (Belvončíková *et al.*, 2008). It should be mentioned that binding affinities of truncated MHV-68 M3 protein with His-tag anchor on its C-terminal end to CCL5, CXCL8, and CCL3 were comparable with those of truncated MHV-68 M3 protein with His-tag anchor on its N-terminal end studied by Pančík (2013). Thus, the localization of His-tag anchor in recombinant M3 protein had

no effect on its binding to chemokines tested in this study (Matúšková, personal communication).

To sum up, the binding to CCL5 and CXCL8 seems to be related to signal sequence, which is not generally recognized to play a role in protein folding important for its biological activity. We found that the mutation present in MHV-72 M3 protein reduces binding to all chemokine tested, CCL5, CXCL8, CCL3, when its impact on the binding to CCL3 seems to be the most important. Both, the lack of signal sequence and the mutation (unique for MHV-72) cause the largest reduction of M3 binding to CCL3.

Our study brought new and unique data on biological properties of M3 protein-vCKBP of murine gammaherpesvirus, opening up the possibility of directly affecting some of its binding activities to a broad spectrum of chemokines. This work is the ground for our subsequent experiments on the site-directed mutagenesis of M3 protein aiming to obtain a protein with modified chemokine-binding activity to a individual chemokine. Modulation of chemokine activity is emerging as a potential therapeutic strategy for regulating the immune response. The virus-encoded CKBPs with different binding specificity offer as a unique source of chemokine inhibitors. Exploration of optimized chemokine inhibitors of viruses, which interacted with the chemokine system for millions of years, might help to control an over-reactive inflammatory response in a number of human inflammatory diseases.

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