Acta virologica **57**: 59 – 68, 2013 doi:10.4149/av_2013_01_59

Purification of recombinant M3 proteins of murine gammaherpesviruses 68 and 72 expressed in *Escherichia coli*

P. PANČÍK[1], V. BAUEROVÁ-HLINKOVÁ[2*], M. KÚDELOVÁ[1*]

[1]Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 05 Bratislava, Slovak Republic; [2]Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 845 51 Bratislava, Slovak Republic

Received December 17, 2012; accepted February 14, 2013

Summary. – M3 proteins of 44 kDa of murine gammaherpesviruses 68 and 72 (MHV-68, MHV-72) were identified as herpesvirus vCKBP-3, soluble inhibitors of the host chemokine network providing a selective advantage for the virus by inhibiting the antiviral and inflammatory response during both acute and latent infection. The MHV-72 M3 protein was found to contain a single mutation (Asp307Gly) near its chemokine-binding domain and differ from that of MHV-68 in the ability to bind some human chemokines. In this study, we optimized the expression of his-tagged M3 proteins of MHV-68 and MHV-72 in *Escherichia coli* and their purification by Ni-NTA chromatography under both denaturing and native conditions. The integrity of the N-terminus of the MHV-72 M3 protein was verified by partial sequencing. The results showed that *E. coli* is useful for the preparation of native, recombinant M3 proteins of murine gammaherpesviruses in sufficient quantity and purity for further biological studies.

Keywords: M3 protein; murine gammaherpesvirus 68; murine gammaherpesvirus 72; expression; E. coli; Ni-NTA chromatography

Introduction

Large DNA viruses, such as herpesviruses and poxviruses, encode viral protein homologs of chemokines, members of a complex family of cytokines that control the recruitment of cells to sites of infection and inflammation. They also contain viral homologs of chemokine receptors and viral chemokine-binding proteins (vCKBPs) (Lalani *et al*., 2000). M3 protein (M_r = 44 kDa) of a murine gammaherpesvirus, also known as vCKBP-3, is a soluble, chemokine-binding inhibitor encoded by a herpesvirus and was the first of its type to be reported. It prevents chemokine-induced signal transduction *in vitro* (Parry *et al*., 2000; van Berkel *et al*., 2000) and it is able to block the interaction of all four chemokine subfamilies with their cellular receptors with high affinity. It also blocks the induction of intracellular signaling. M3 has also been shown to block the interactions of chemokines with glycosaminoglycans (GAGs) (Webb *et al*., 2003). Like other viral proteins with chemokine-binding abilities, M3 is a unique protein product which has no sequence similarity to either its host´s chemokines or chemokine receptors (Virgin *et al*., 1997).

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 developed strategies to manipulate the host chemokine network. They are classified into four subfamilies on the basis of the arrangement of cysteine residues at their N-terminus: CXC, CC, C, and CX3C (Zlotnik and Yoshie, 2000). The specific effects of chemokines on target cells are mediated by the members of a family of 7 transmembrane-spanning G-protein coupled receptors. As predominantly basic, positively charged molecules, chemokines also interact with the negatively charged GAGs expressed on the endothelial cell surface.

*Corresponding authors. E-mail: virukude@savba.sk, vladena.bauerova@savba.sk; phone: +421-2-59302434.

Abbreviations: GAG(s) = glycosaminoglycan(s); MHV-68 = murine gammaherpesvirus 68; MHV-72 = murine gammaherpesvirus 72; SB-14 = sulphobetaine 3–14; vCKBP = viral chemokine-binding protein

These interactions are involved in chemokine transport and presentation (Proudfoot *et al.*, 2003).

Murine gammaherpesvirus 68 (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 because it is an attractive experimental model for gammaherpesvirus infection (Rajčáni and Kúdelová, 2007).

MHV-68 M3 protein is encoded by one of the early-late lytic genes which are expressed continually during lytic infection, during the establishment of latent infection, and during latency *in vivo* (Ebrahimi *et al.*, 2003; Simas *et al.*, 1999). The role that M3 plays in the establishment of latency is still uncertain; one possibility is that lytic infection of macrophages and dendritic cells may indirectly protect latently infected B cells (Marques *et al.*, 2003; Flaño *et al.*, 2000). For example, 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 MHV-68 (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 and macrophage control trafficking in the CNS during MHV-68 infection has been demonstrated (van Berkel *et al.,* 2002). M3-deficient mutant of MHV-68 was unable to establish latent infection (Bridgeman *et al.,* 2001). The absence of the M3 gene also alters the host response to viral infection 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 of M3 protein bound to the CC chemokine CCL2 (monocyte chemoattractant protein 1) and described the binding epitopes of vCKBP recognized by the chemokine and also by the cellular receptor CCR2. This model of the M3 protein suggested how this viral protein is able to bind chemokines despite having no amino acid homology to the host´s chemokine receptors.

In addition to the MHV-68 virus, the biological properties of the related murine gammaherpesvirus 72 (MHV-72) (clone h3.7) have also been characterized (Mistríková *et al.,* 2000; Rašlová *et al.*, 2001) and also the differences in the sequences of some genes have been identified (Mačáková *et al.*, 2003; Valovičová *et al.*, 2006; Belvončíková *et al.*, 2008a). Normal murine mammary gland (NMuMG) cells infected with MHV-72 exhibit a cytopathic effect up to 48 hours later than similar cells infected with MHV-68 (H. Rašlová, personal communication). Studies on the long-term infection of mice (Balb/c as well as CB17 wild-type) revealed that mice infected with MHV-72 displayed a malignancy development rate either a similar to or higher than that of mice infected with MHV-68 (Mistríková *et al.*, 2000; Oda *et al.*, 2005). More recently, a portion (22,899 bp) of the MHV-72 genome sequence encompassing 19 genes was determined, analyzed and compared with that of MHV-68 (Halásová *et al.*, 2011). MHV-72 appears to be less pathogenic and more oncogenic than MHV-68 (Nash *et al.*, 2001). MHV-72 is very likely to be a strain of murine gammaherpesvirus, but its classification is still not clear. MHV-68 remains the only virus classified as a MuHV-4 species.

In our previous study we showed that MHV-72 also has a M3 gene but with an amino acid mutation at position 920 nt, Asp307Gly (Belvončíková *et al.*, 2008a). This mutation is located near the chemokine-binding domain and was suggested to be involved in attenuating the immune response to MHV-72 infection. A study of the binding activities of the MHV-72 M3 protein, which was released into the media of infected BHK-21 cells, against five human chemokines (CCL3, CCL5, CCL11, CCL2, and CXCL8) showed that MHV-72 M3 exhibited significantly lower binding activity (11.1% to 20%) against CCL5 and CXCL8 than MHV-68 M3 but had comparable activity against CCL11, CCL2, and CCL3.

These results prompted us to elaborate an efficient *E. coli* expression system using the pET-26b(+) vector, suitable for producing sufficient amounts of recombinant M3 proteins of MHV-68 and MHV-72 to assess their chemokine-binding properties as well as the changes in these properties caused by the Asp307Gly mutation in the MHV-72 M3 protein. Then, we established the conditions for optimal purification of these proteins in both native and denatured form using the Ni-NTA chromatography.

Materials and Methods

Viruses. Two murine gammaherpesviruses (MHV-68 and MHV-72) originally isolated from the bank vole, *Myodes glareolus* (Blaškovič *et al.*, 1980), were kindly provided by Prof. Mistríková (Faculty of Natural Sciences, Comenius University, Bratislava, Slovakia). Both viruses were twice-plaque purified 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).

Cells. The NMuMG cell line and BHK-21 fibroblasts were maintained in DMEM (GIBCO) supplemented with 10% (v/v) FCS, 2 mmol/l glutamine (Invitrogen) and penicillin-streptomycin-amphotericin (Cambrex) at 37°C. *E. coli* BL21 (DE3) and Rosetta (DE3) cells were obtained from Novagen. *E. coli* JM109 cells were obtained from Promega.

PCR amplification of M3 genes. To amplify the genomic region corresponding to ORF M3 of MHV-68 (Acc. No. AF105037) and MHV-72 (Acc. No. DQ378056), recombinant plasmids pGEM-T-M3 MHV68 and pGEM-T-M3 MHV72, prepared previously by Belvončíková *et al.* (2008a) were used as templates. 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 at the C-terminal end (6×His-tag, italic) of the M3 protein were used (5′-CCC **CAT ATG** GCC TTC CTA TCC ACA TCT GTG C-3′ and 5′-GCG **CTC GAG** TCA *ATG ATG ATG ATG ATG ATG* ATC CCC AAA ATA CTC CAG CCT G-3′). The PCR mixture contained about 10 ng of pGEM-T-M3 MHV-68 DNA or pGEM-T-M3 MHV-72 DNA as a template, 0.3 mmol/l dNTPs, 1.5 mmol/l $MgCl_2$, 0.3 mmol/l of each primer, and 1 U of *Pfu* DNA polymerase (Promega) with proof-reading activity to prevent incorporation of single mutations. The PCR reaction was performed using a Mastercycler personal (Eppendorf) as follows: initial denaturation at 95°C for 3 min, 35 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 2.5 min, and finally one cycle at 72°C for 10 min.

Cloning and sequencing of M3 genes. The PCR product containing the genes of either MHV-68 M3 or MHV-72 M3 (1,251 bp) was further purified using Wizard® SV Gel and PCR Clean-Up System (Promega) and cloned into expression plasmid pET-26b(+) (Novagen) *via Xho*I/*Nde*I restriction sites. *E. coli* JM109 competent cells were transformed with ligation reaction mixture according to the manufacturer´s instructions. Two clones with the corresponding ORF M3 were selected: P26-M3his/68 and P26-M3his/72. For the P26-M3his/72 recombinant plasmid, the presence of the virus-specific single nucleotide mutation was checked by *Bgl*I digestion (Fig. 1). Cloned inserts of both plasmids were also sequenced using a commercial sequencing service (BITCET, Faculty of Natural Sciences, Comenius University, Bratislava, Slovakia), which confirmed the correct ORF of both cloned inserts. For sequencing, the two universal primers T7F (20-mer) and T7R (19-mer) were used.

Expression of M3 proteins. E. coli competent cells (BL21 (DE3) or Rosetta (DE3)) were transformed with expression plasmids P26-M3his/68 and P26-M3his/72. Single colonies were picked and inoculated into 10 ml of LB medium (Sigma-Aldrich) containing 30 μg/ml kanamycin and grown at 37°C overnight to make a starter culture. For the Rosetta (DE3)) cells, 34 μg/ml chloramphenicol (Sigma-Aldrich) was also added to the cultivation media. M3 was expressed on both the small and large scale. For small scale M3 expression, 50 ml of LB media with antibiotics was inoculated with 0.5 ml of starter culture and the cells were grown at 37°C until A_{600} = 0.6. For large scale expression, 0.5 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, and 0.4% (w/v) glycerol) with antibiotics was used. Cells were grown to the same density as stated previously. The expression of M3 was induced by adding IPTG to a final concentration of 0.5 mmol/l and the protein was produced at 37°C for 2 hrs, or at 22°C for 4 hrs. *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, 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 and MHV-72 M3 proteins with 6×His-tags on their C-termini were purified under either denaturing or native conditions, using Ni-NTA agarose (Qiagen) according to the manufacturer´s instructions with minor modifications. For purification of M3 proteins (MHV-68 and MHV-72) under denaturing conditions, 5 ml of cells were sedimented at 8,000 g and lysed by resuspenion in 1 ml of 7 mol/l urea. 100 μl of agarose was then added to bind his-tagged M3. M3 protein was purified with 150 μl of elution buffer into several fractions. Each step of the purification procedure was analyzed on 12.5% SDS-PAGE and stained using Coomassie Brilliant Blue R-250 (Santa Cruz). For purification under native conditions, two different lysis buffers were used: Buffer A (100 mmol/l NaH_2PO_4, 10 mmol/l Tris-Cl (pH = 8.0), 300 mmol/l NaCl) and Buffer B (25 mmol/l NaH_2PO_4, 2.5 mmol/l Tris-Cl (pH = 8.0), 100 mmol/l NaCl, 10% (w/v) glycerol, 0.2% (w/v) sulphobetaine 3-14 (SB-14, Sigma-Aldrich). Protease Inhibitor Cocktail Set III, EDTA-Free (Calbiochem) was added in a dilution 1:200 to both buffers to prevent undesired protein degradation. Before purification, the cells were weighted and resuspended in an appropriate volume of either A or B lysis buffer, supplemented with 0.5 mg/ml of lysozyme, and incubated at 4°C for 30 min. This cell suspension was then either sonicated (9 × 15 s at 40% power) using a Bandelin Sonopuls (Bandelin electronic, GmbH) or disrupted by passing it twice through a French Press (20 MPa). The cell lysis mixture was then treated with 25 U Benzonase® Nuclease (Qiagen) for 10 min to remove nucleic acids followed by centrifugation at 13,000 x g at 4°C for 30 min. The M3 protein was separated from the lysate using an IMAC column with ≈ 0.6 ml of agarose. After a short, 4°C incubation, non-specifically bound proteins were washed away using two rinsings of 1 ml either Buffer A or B with 25 mmol/l imidazole. Finally, M3 protein was eluted from the column into three fractions by repeated washing with 200 μl of either Buffer A or B with 250 mmol/l imidazol. To improve the solubility of the M3 protein under native conditions, a detergent solubility kit (Jena Bioscience, Germany) was used.

Western blot analysis. Purified M3 protein samples were separated on 12.5% SDS-PAGE. The gel was semi-dry blotted using an Immobilon-P PVDF membrane (Millipore), briefly immersed in pure methanol and then gel, membrane, and filter papers were all equilibrated in blotting buffer (25 mmol/l Tris, 40 mmol/l ε-aminocaproic acid, 20% (v/v) methanol). Semi-dry blotting was performed with a constant current of 0.8 mA/cm² at RT for 1 hr

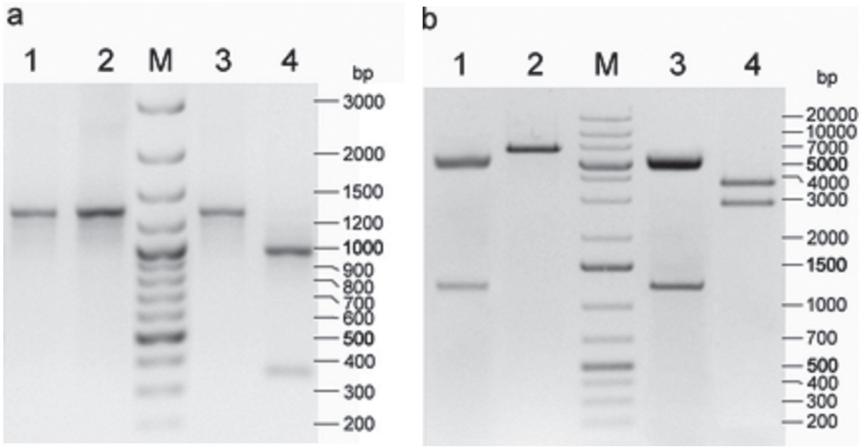

Fig. 1

Construction of vectors expressing M3 proteins of MHV-68 and MHV-72

Agarose gel electrophoresis. (a) PCR amplification of M3 genes of MHV-68 and MHV-72 from plasmids pGEM-T-M3 MHV-68 (lane 1) and pGEM-T-M3 MHV-72 (lane 3), respectively. Products of *Bgl*I digestion of MHV-68 M3 gene (lane 2) and MHV-72 M3 gene (lane 4). (b) Products of *Xho*I/*Nde*I digestion of P26-M3his/68 (lane 1) and P26-M3his/72 (lane 3). Products of *Bgl*I digestion of P26-M3his/68 (lane 2) and P26-M3his/72 (lane 4). DNA ladders (lane M).

(The Novex® Semi-Dry Blotter, Invitrogen). The PVDF membrane was blocked in 5% (w/v) BSA solution with a slow agitation at RT for 1 hr. It was subsequently treated with a His-Tag® monoclonal antibody (Novagen) diluted 1:1,000 in TBST with 2.5% (w/v) BSA at 4°C overnight. The membrane was then washed three times at RT for 15 min each with an appropriate volume of TBST to remove unbound antibody. The membrane was then treated with anti-mouse IgG HRP conjugate (Promega) diluted 1:2,500 in TBST at RT for 1 hr and then washed as described above. 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.

Protein sequencing. The M3 protein purified under native conditions was analyzed on 12.5% SDS-PAGE in a carbonate-bicarbonate buffer (10 mmol/l $NaHCO_3$, 3 mmol/l Na_2CO_3, 20% (v/v) methanol (pH = 9.9)) and then electroblotted in a Mini Trans-Blot® Cell Assembly (Bio-Rad) according to the manufacturer´s instructions. To identify the M3 protein, the membrane was stained with a solution containing 0.125% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid at RT for 1 min and then briefly washed with an appropriate volume of destaining solution containing 50% (v/v) methanol with 10% (v/v) acetic acid and then washed three times with distilled water for 5 min. The amino acid sequence of the first seven residues of MHV-72 M3 was determined by Edman degradation using a Procise Protein Sequencing System (Applied Biosystems) using the Pulsed Liquid PVDF cLC method and RP-HPLC analysis (Dr. Voburka, Institute of Organic Chemistry and Biochemistry, Academy of Sciences, Prague, Czech Republic).

Results

Construction of bacterial expression vectors for M3 genes

The sequences of M3 genes were amplified using pGEM-T-M3 MHV-68 and pGEM-T-M3 MHV-72 plasmids previously prepared by Belvončíková *et al.* (2008a), and primers containing *Xho*I and *Nde*I linker sequences (Fig. 1a, lanes 1, 3). The reverse primer included a sequence encoding six histidines at its 3´-end allowing their expressed proteins to be purified by IMAC. The PCR products of these M3 genes were cloned into a pET-26b(+) vector *via* the *Xho*I and *Nde*I restriction sites (Fig. 1b, lanes 1, 3) creating recombinant plasmids P26-M3his/68 and P26-M3his/72. The specificity of the MHV-72 M3 gene (both the PCR product and the recombinant plasmid) was verified by digestion with *Bgl*I, which cleaves a unique restriction site found only in the MHV-72 M3 gene. This yielded two fragments of 322 bp and 929 bp for the PCR product (Fig.1a, lanes 4), and 2,812 and 3,658 bp for the P26-M3his/72 recombinant plasmid (Fig. 1b, lanes 2, 4).

Expression of recombinant M3 proteins in E. coli

E. coli BL21 (DE3) or Rosetta (DE3) cells were transformed with a recombinant bacterial expression vectors (either P26-M3his/68 or P26-M3his/72). Expression of the M3 protein was induced with 0.5 mmol/l IPTG and evaluated

by SDS-PAGE. After induction at either 37°C for 2 hrs or at 22°C for 4 hrs, MHV-68 M3 and MHV-72 M3 proteins of the expected size (~44 kDa) were found in the lysates of transformed cells in comparable amounts (Fig. 2, lanes 2, 4). The amount of M3 protein expressed was independent of either the *E. coli* strain used (data not shown) or the virus from which the M3 gene was derived. Because of the enhanced codon usage of the *E. coli* Rosetta (DE3) strain, this strain was used in the following experiments.

Purification of recombinant M3 proteins by Ni-NTA chromatography

After determining that the M3 proteins of both murine gammaherpesviruses are expressed in *E. coli* cells, we purified these proteins from *E. coli* Rosetta (DE3) cells. Both proteins were purified under denaturing (in the presence of 7 mol/l urea, Fig. 3a) and native conditions (Fig. 3b). Under denaturing conditions, both proteins could be purified with comparable quality and efficiency (Fig. 3a).

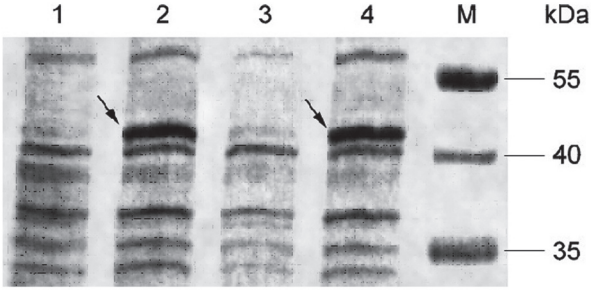

Fig. 2

Expression of M3 proteins in *E. coli*

E. coli Rosetta (DE3), 2 hrs after IPTG induction at 37°C, cell lysate SDS-PAGE. Cells transformed with P26-M3his/68, uninduced (lane 1) and induced (lane 2). Cells transformed with P26-M3his/72, uninduced (lane 3) and induced (lane 4). Protein size marker (lane M). The arrows indicate the 44 kDa M3 proteins.

To purify M3 protein under native conditions, it was necessary to optimize the purification conditions by altering the temperature and cultivation time of the cells, the way by which they were lysed, and the composition of the lysis buffer used.

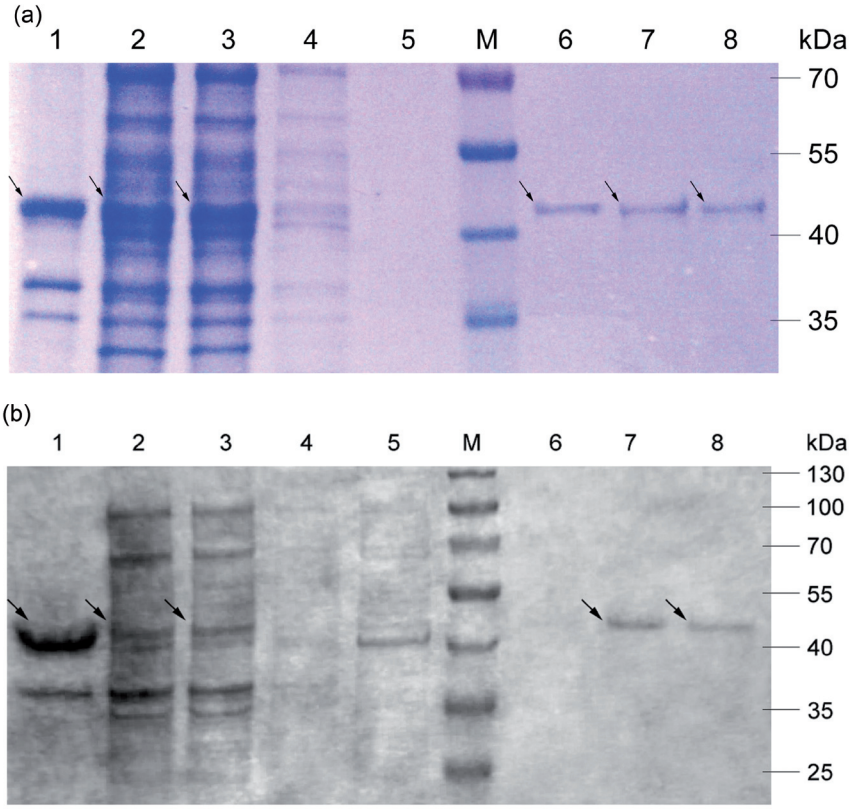

Fig. 3

Purification of recombinant MHV-72 M3 protein by Ni-NTA chromatography

(a) Denaturing conditions, cell lysate applied to the column. Bound insoluble (lane 1) and soluble (lane 2) proteins, unbound proteins (lane 3), non-specifically bound proteins (lanes 4, 5), E buffer eluate (lanes 6–8). (b) Native conditions, cell lysate applied to the column. Bound insoluble (lane 1) and soluble (lane 2) proteins, unbound proteins (lane 3), specifically eluted proteins (lanes 4, 5). Purified M3 protein applied to the column and specifically eluted (lanes 6–8). Protein size markers (lanes M). The arrows indicate the 44 kDa M3 protein.

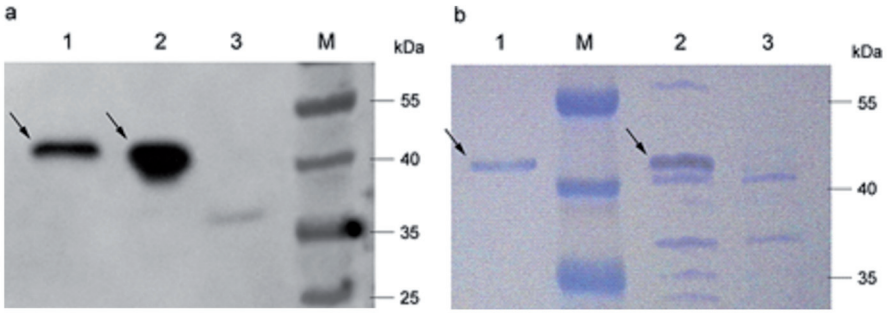

Fig. 4

Western blot analysis of recombinant MHV-72 M3 protein

SDS-PAGE. (a) Chemiluminiscent staining. IPTG induction, cell lysate purified under native (lane 1) and denaturing (lane 2) conditions. Uninduced cells (lane 3). (b) Coomassie Brilliant Blue staining. Purified M3 protein (lane 1), lysate of induced (lane 2) and uninduced (lane 3) cells. Protein size markers (lane M). The arrows indicate the 44 kDa M3 protein.

Moreover, several buffers were used to elute the M3 protein from the agarose column. The highest amounts of native M3 protein were obtained under the following conditions: cultivation at 37°C until A_{600} = 0.6, induction with 0.5 mmol/l IPTG at 37°C for 2 hrs, lysis of cells using a French press, a lysis buffer containing 100 mmol/l NaCl, 25 mmol/l phosphate buffer, 10% (w/v) glycerol, 0.2% (w/v) SB-14 along with 0.5 mg/ml lysozyme, and a washing buffer containing 25 mmol/l imidazole (Fig. 3b). The presence of SB-14, chosen from several tested reagents, was crucial for obtaining a detectable soluble amount of M3 protein. Despite all these optimizations, the yield of protein under these conditions was quite low, around 0.5–2 mg/l.

The purification of both M3 proteins was much more efficient under denaturing conditions (Fig. 3a), most likely because of M3′s lower solubility under native conditions. A substantial amount of M3 protein appears to stay in the insoluble fraction under both native and denaturing conditions; the denaturing conditions most likely recover more of it (Fig. 3b, lane 1).

Western blot analysis of recombinant M3 proteins

Because of the rather low yield of M3 protein purified under native conditions, its presence was confirmed by Western blot analysis using a His-Tag® monoclonal antibody. Western blotting confirmed that pure MHV-72 M3 protein was present in both native and denaturing conditions (Fig. 4a, lanes 1,2). A larger amount of M3 protein was detected under denaturing conditions, in agreement with what was seen in SDS-PAGE (Fig. 4a, lane 2). Comparable results were obtained for M3 protein of MHV-68.

Partial sequencing of MHV-72 M3 protein

The MHV-72 M3 protein purified under native conditions (Fig. 4b, lane 1) was sequenced from its N-terminal end to verify its integrity. This sequencing showed that the first seven amino acids at the N-terminus of the purified protein (Ala, Phe, Leu, Ser, Thr, Ser, Val) matched those of the sequences of both MHV-68 M3 (Acc. No. AF105037) and MHV-72 M3 (Acc. No. DQ378056). In both cases, the first methionine was cleaved out by a cell enzymatic mechanism.

Discussion

The M3 gene is one of the latent-lytic infection associated genes whose function in pathogenesis of a murine gammaherpesvirus is relatively well understood. The M3 protein has been shown to be involved in the antiviral response by inhibiting chemokine-mediated calcium mobilization and chemotaxis in chemokine receptor bearing cells *in vitro* (van Berkel *et al.*, 2002). Because of M3 *in vitro* pan-chemokine binding activity, it was predicted to be an important immunomodulator, capable of altering the migration of T and B cells as well as dendritic cells, thus delaying the initiation of a specific immune response against the virus′s infection (Parry *et al.*, 2000; van Berkel *et al.*, 2000). *In vivo* studies confirmed that M3 does have a critical role in virulence and in the nature of the virally induced inflammatory response (Virgin *et al.*, 1999; van Berkel *et al.*, 1999, 2002; Usherwood *et al.*, 2000; Simas *et al.*, 1999). Jensen *et al.* (2003) discovered a direct correlation between M3 expression and the inhibition of lymphocyte and macrophage trafficking in the CNS. More recent studies on laboratory-bred wood mice (*Apodemus sylvaticus*) confirmed that the absence of the M3 gene causes attenuation of latent infection in both lung and spleen, which suggests that the M3 gene is responsible for host-specific differences in infection (Hughes *et al.*, 2010, 2011).

Studies on the biochemical properties of the M3 protein have also been sparked by the possibility that this unique

pan-chemokine antagonist could be used in the treatment of human diseases involving chemokines. The existence of a large number of chemokines suggests redundancy, and thus the therapeutic use of a broad chemokine antagonist such as M3 protein may be advantageous.

M3 protein is a viral protein, which has evolved to protect the virus from the immune system of the host; it is a potent immunomodulator. In previous studies, M3 was constitutively expressed in the pancreas of mice and was shown to be able to inhibit islet mononuclear infiltration and diabetes development in NOD mice after streptozocin treatment (Martin *et al.*, 2007, 2008). Unfortunately, constitutive over expression of M3 does not mimic its potential therapeutic use as a chemokine blocker. A doxycycline-dependent gene expression system was used to examine the usefulness of M3 as therapeutic agent (Gossen and Bujard, 1992; Pyo *et al.*, 2004; Lira *et al.*, 2009). M3 has also been used in an experimental animal model to overcome the cellular inflammatory responses in hepatocellular carcinoma lessions induced by a recombinant oncolytic vesicular stomatitis virus. M3 prolonged the theraputic effect of the oncolytic virus, improved animal survival, and led to a 50% cure rate (Wu *et al.*, 2008). All these examples show that M3 has the potential to be used in therapeutic applications where chemokines have been implicated. Additional examples include neurological traumas (Ransohoff, 2002), rheumatoid arthritis (McInnes and Schett, 2007), intimal hyperplasia associated with vessel reconstruction and the progression of atherosclerosis (Pyo *et al.*, 2004), transplant rejection, HIV progression, etc. (Lira *et al.*, 2009).

Alexander *et al.* (2002) used a recombinant baculovirus infecting insect Sf9 cells to produce enough M3 protein to determine the crystal structure of both M3 alone and bound to CC chemokine CCL2. They found that the M3 binding site is generated by antiparallel dimerization of its molecules and proposed that M3 interacts with the N-terminal region of chemokines, thereby interfering with the chemokine-receptor interaction and blocking the interaction of chemokines with GAGs. This interaction is required for the correct presentation of chemokines to passing leukocytes and for chemokine activity *in vivo* (Parry *et al.*, 2000; Proudfoot *et al.*, 2003). To date, studies on the binding activities of M3 to individual chemokines are still rare and consequently, information which might indicate possibilities for altering these activities is lacking.

All aforementioned experiments refer to M3 protein of MHV-68. In a previous study, we compared biological properties of M3 proteins derived from different murine gammaherpesviruses. We examined the binding activities of M3 proteins released into the media of BHK-21 cells infected with either MHV-68 or MHV-72 (clone h3.7) to five human chemokines. These two M3 proteins differed in only one mutation which is found near the chemokine-binding domain (Belvončíková *et al.*, 2008a). We found that the binding activity of MHV-72 M3 to CXCL8 and CCL5 is 11% and 20% lower, respectively, than that of MHV-68 M3 to the same proteins (Belvončíková *et al.*, 2008a). On the other hand, when the binding activities of recombinant MHV-68 M3 and MHV-72 M3 expressed in Sf9 insect cells using a baculovirus expression system were examined, it was found that the binding activity of MHV-72 M3 to at least four of the five chemokines tested was greater than that of MHV-68 M3 (Belvončíková, 2008b).

In order to study these differences more closely as well as to open up the possibility of directly affecting some of the binding activities of the M3 protein by site-directed mutagenesis, we used the easy-to-manipulate pET expression system to prepare both M3 proteins for expression in *E. coli*. This would allow the larger amounts of protein to be prepared relatively inexpensively and quickly. Although no information is available about the expression of recombinant M3 protein in bacteria, the properties of this protein, its relatively small size of 44 kDa, its solubility in cultivation media, its absence of posttranslational modifications, made this expression system an appropriate choice. To quickly and effectively purify the recombinant M3 protein using affinity chromatography, we introduced a 6×His-tag sequence at the C-terminal end of the protein, as is commonly done in pET vectors.

Jang *et al.* (2009) used a pET26b vector to express a 10 kDa recombinant human RIP-associated ICH-1 homologous protein with a death domain (RAIDD), which is involved in apoptosis, incorporating a C-terminal 6×His-tag in *E. coli* BL21 (DE3) cells. This protein was purified by affinity chromatography and gel filtration. Although it has been known that the majority of death domains (DDs) are not soluble under physiological conditions, RAIDD is soluble and it interacts tightly with a p53-induced protein with DD (PIDD) under physiological conditions. The amount of protein expressed using the pET26b vector was sufficient for a further crystallization study. Park *et al.* (2008) achieved strong overproduction of the 35 kDa HPr kinase/phosphorylase from *Leuconostoc* sp. after induction with 1 mmol/l IPTG in the same pET expression system and produced a functional protein. Feng *et al.* (2011) used the same pET vector and BL21 (DE3) strain of *E. coli* to express a 15 kDa recombinant VP0 protein of enterovirus 71, which was present mostly in inclusion bodies. However, they succeeded in solublizing and renaturating the protein which was then used for producing polyconal antibodies in guinea pigs. Taken together, it can be seen that the solubility of individual heterologous proteins produced by *E. coli* cells can vary greatly when using the pET26b vector expression system even under very similar conditions (Terpe, 2006).

In our initial experiments we selected two *E. coli* strains, BL21 (DE3) and Rossetta (DE3), in which to express the

recombinant M3 protein from both MHV-72 and MHV-68. We optimized both cultivation and induction conditions for the proper production of soluble M3 protein. We successfully purified both M3 proteins under denaturing conditions, achieving comparably efficient production of pure M3 from both viruses (about 10 mg/l of medium). The purification of native M3 protein needed to be widely modified since the yield of native protein purified according to manufacturer´s instructions was very low (below 0.1-0.2 mg/l) and was detectable only by chemiluminescent dye using an antibody conjugate against the poly-histidine anchor of recombinant protein. A lot of protein was present in the insoluble fraction; this often occurs when prokaryotic systems are used to express eukaryotic proteins (Martínez-Alonso *et al.*, 2009).

Although comparable amounts of protein were expressed in both the BL21 (DE3) and Rossetta (DE3) strains, in the next experiments, the Rossetta (DE3) strain was preferred because it is able to supply tRNAs for the naturally underrepresented codons (AGG, AGA, AUA, CUA, CCC, GGA) (Henaut and Danchin, 1996) encoded by the chloramphenicol-resistant plasmid pRARE, thus minimizing possible translation errors during the expression of heterologous proteins in *E. coli* (Scorer *et al.*, 1991). In the following experiments we also optimized both the growth conditions of the expression cells and the protein purification procedure. We found that the amount of M3 protein produced following induction was greater at 37°C for 2 hrs than at 22°C for 4 hrs, while the proportion of soluble M3 protein was roughly the same. To reduce undesirable protein aggregation, we added SB-14 and glycerol to the lysis buffer. We found that disruption of the cells using a French press was more efficient than using sonication (although it should be noted that the effect was strongly dependent on the volume of cell suspension; sonication worked just as well when used for small scale purification). We also lowered the salt concentration in the lysis buffer from 300 mmol/l to 100 mmol/l, and we carried out an additional column washing with buffer containing 25 mmol/l imidazole to remove additional contaminating proteins. With these changes, we were able to increase the yield of purified M3 proteins to a level high enough to analyze its biological properties. We were also able to verify identity, purity, and integrity of MHV-72 M3 protein by partial N-terminal sequencing. Although the yield of M3 protein purified under native conditions was lower than under denaturing ones (about 10 times) it was still sufficient for the further characterization of its biological properties. A better yield of native M3 protein might be achieved by trafficking the protein into the periplasm, thus avoiding its accumulation in cytoplasmic inclusion bodies, however some disadvantages of this method have been described by Chatel *et al.* (1999), including a not fully spliced signal sequence, incorporation of a portion of the protein into the cell membrane, and mis-folding of the protein. The huge list of possible modifiable parameters which might reduce inclusion body formation includes gene dosage, promoter strength, mRNA stability, protein and genetic engineering and over expression of folding modulators (Martínez-Alonzo *et al.*, 2009). It should be mentioned that sometimes the use of other bacteria (e.g. Bacilli) or baculovirus-mediated expression of protein is the only way to overcome this problem (Zhou *et al.*, 2011; Terpe, 2006). Additional changes to the expression and purification procedure which might increase the yield of soluble protein are being investigated.

In this study, we prepared an expression system which is suitable for producing recombinant M3 protein (vCKBP of murine gammaherpesvirus) in *E. coli,* allowing its chemokine binding properties to be studied. The use of this effective, inexpensive, and fast expression system should allow us to discover how a codon changing mutation changes these binding properties. This would then contribute to a greater understanding of this protein and how to utilize its unique properties in gene therapy.

Acknowledgements. This work was supported by the grants Nos. #2/0091/13 and #2/0131/10 from the Scientific Grant Agency of Ministry of Education of Slovak Republic and Slovak Academy of Sciences. The authors thank Dr. Zdeněk Voburka for N-terminal amino acid sequencing of M3 protein and Dr. Gabriela Ondrovičová and Dr. Jacob Bauer for the help with Western blot analysis and helpful discussions.

References

Alexander JM, Nelson CA, van Berkel V, Lau EK, Studts JM, Brett TJ, Speck SH, Handel TM, Virgin HW, Fremont DH (2002): Structural basis of chemokine sequestration by a herpesvirus decoy receptor. Cell 111, 343–356. http://dx.doi.org/10.1016/S0092-8674(02)01007-3

Belvončíková P, Kráľová A, Kúdelová M, Hajnická V, Režuchová I, Vančová I (2008a): Chemokine-binding activities of M3 protein encoded by Murine gammaherpesvirus 72. Acta Virol. 52, 91–97.

Belvončíková P (2008b): M3 protein of Murine herpesvirus-72. (PhD Thesis).

Blaškovič D, Stančeková M, Svobodová J, Mistríková J (1980): Isolation of five strains of herpesviruses from two species of free living small rodents. Acta Virol. 24, 468.

Bridgeman A, Stevenson PG, Simas JP, Efstathiou S (2001): A secreted chemokine binding protein encoded by murine gammaherpesvirus-68 is necessary for establishment of a normal latent load. J. Exp. Med. 194, 301–312. http://dx.doi.org/10.1084/jem.194.3.301

Chatel JM, Adel-Patient K, Créminon C, Wal JM (1999): Expression of a lipocalin in prokaryote and eukaryote cells: quantification and structural characterization of recombinant bovine beta-lactoglobulin. Protein Expr. Purif. 16, 70–75. http://dx.doi.org/10.1006/prep.1999.1055

Ebrahimi B, Dutia BM, Roberts KL, Garcia-Ramirez JJ, Dickinson P, Stewart JP, Ghazal P, Roy DJ, Nash AA (2003): Transcriptome profile of murine gammaherpesvirus-68 lytic infection. J. Gen. Virol. 84, 99–109. http://dx.doi.org/10.1099/vir.0.18639-0

Feng YF, Liu QW, Ku ZQ, Wen JJ, Shan H, Huang Z (2011): Expression of VP0 protein of enterovirus 71 in Escherichia coli and generation of the corresponding polyclonal antibodies in guinea pigs. Xi bao yu fen zi mian yi xue za zhi 27, 535–538.

Flaño E, Husain SM, Sample JT, Woodland DL, Blackman MA (2000): Latent murine gamma-herpesvirus infection is established in activated B cells, dendritic cells, and macrophages. J. Immunol. 165, 1074–1081.

Gossen M, Bujard H (1992): Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA 89, 5547–5551. http://dx.doi.org/10.1073/pnas.89.12.5547

Halásová Z, Valovičová M, Mačáková K, Pančík P, Belvončíková P, Režuchová I, Kúdelová M (2011): Partial genome sequence of murine gammaherpesvirus 72 and its analysis. Acta Virol. 55, 317–325. http://dx.doi.org/10.4149/av_2011_04_317

Henaut A, Danchin A (1996): Analysis and Predictions from Escherichia coli sequences. In Neidhardt F, Curtiss III R, Ingraham J, Lin E, Low B, Magasanik B, Reznikoff W, Riley M, Schaechter M, Umbarger H (Eds): Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology. Vol. 2, American Society for Microbiology, Washington DC, pp. 2047–2066.

Hughes DJ, Kipar A, Sample JT, Stewart JP (2010): Pathogenesis of a model gammaherpesvirus in a natural host. J. Virol. 84, 3949–3961. http://dx.doi.org/10.1128/JVI.02085-09

Hughes DJ, Kipar A, Leeming GH, Bennett E, Howarth D, Cummerson JA, Papuola-Pereira R, Flanagan BF, Sample JT, Stewart JP (2011): Chemokine binding protein M3 of Murine gammaherpesvirus 68 modulates the host response to infection in a natural host. PLoS Pathog. 7, e1001321. http://dx.doi.org/10.1371/journal.ppat.1001321

Jang TH, Park HH (2009): Purification, crystallization and preliminary x-ray crystallographic studies of RAIDD Death-Domain (DD). Int. J. Mol. Sci. 10, 2501–2509. http://dx.doi.org/10.3390/ijms10062501

Jensen KK, Chen SC, Hipkin RW, Wiekowski MT, Schwarz MA, Chou CC, Simas JP, Alcami A, Lira SA (2003): Disruption of CCL21-induced chemotaxis in vitro and in vivo by M3, a chemokine-binding protein encoded by murine gammaherpesvirus 68. J. Virol. 77, 624–630. http://dx.doi.org/10.1128/JVI.77.1.624-630.2003

Kúdelová M, Rajčáni J (2009): Gammaherpesviruses and oncogenesis. In Tao HE (Ed): DNA Tumor Viruses. Nova Science Publishers Inc., New York, pp. 193–231.

Kúdelová M, Halásová Z, Belvončíková P, Pančík P, Režuchová I, Valovičová M (2012): Partial genome analysis of murine gammaherpesvirus 4556. Acta Virol. 56, 177–186. http://dx.doi.org/10.4149/av_2012_03_177

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685. http://dx.doi.org/10.1038/227680a0

Lalani AS, Barrett JW, McFadden G (2000): Modulating chemokines: more lessons from viruses. Immunol. Today 21, 100–106. http://dx.doi.org/10.1016/S0167-5699(99)01556-X

Lira SA, Viejo-Borbolla A, Shang L, Martin AP (2009): The chemokine-binding protein M3 as a tool to understand the chemokine network in vivo. Methods Enzymol. 460, 193–207. http://dx.doi.org/10.1016/S0076-6879(09)05209-4

Luster AD (2002): The role of chemokines in linking innate and adaptive immunity. Curr. Opin. Immunol. 14, 129–135. http://dx.doi.org/10.1016/S0952-7915(01)00308-9

Mačáková K, Matis J, Režuchová I, Kúdela O, Rašlová H, Kúdelová M (2003): Murine gammaherpesvirus (MHV) M7 gene encoding glycoprotein 150 (gp150): difference in the sequence between 72 and 68 strains. Virus Genes 26, 89–95. http://dx.doi.org/10.1023/A:1022390407991

Marques S, Efstathiou S, Smith KG, Haury M, Simas JP (2003): Selective gene expression of latent murine gammaherpesvirus 68 in B lymphocytes. J. Virol. 77, 7308–7318. http://dx.doi.org/10.1128/JVI.77.13.7308-7318.2003

Martin AP, Alexander-Brett JM, Canasto-Chibuque C, Garin A, Bromberg JS, Fremont DH, Lira SA (2007): The chemokine binding protein M3 prevents diabetes induced by multiple low doses of streptozotocin. J. Immunol. 178, 4623–4631.

Martin AP, Grisotto MG, Canasto-Chibuque C, Kunkel SL, Bromberg JS, Furtado GC, Lira SA (2008): Islet expression of M3 uncovers a key role for chemokines in the development and recruitment of diabetogenic cells in NOD mice. Diabetes 57, 387–394. http://dx.doi.org/10.2337/db07-1309

Martínez-Alonso M, González-Montalbán N, García-Fruitós E, Villaverde A (2009): Learning about protein solubility from bacterial inclusion bodies. Microb. Cell Fact. 8, 4. http://dx.doi.org/10.1186/1475-2859-8-4

McInnes IB, Schett G (2007): Cytokines in the pathogenesis of rheumatoid arthritis. Nat. Rev. Immunol. 7, 429–442. http://dx.doi.org/10.1038/nri2094

Mistríková J, Rašlová H, Mrmusová M, Kúdelová M (2000): A murine gammaherpesvirus. Acta Virol. 44, 211–226.

Nash AA, Dutia BM, Stewart JP, Davison AJ (2001): Natural history of murine gamma-herpesvirus infection. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 356, 569–579. http://dx.doi.org/10.1098/rstb.2000.0779

Oda W, Mistríková J, Stančeková M, Dutia BM, Nash AA, Takahata H, Jin Z, Oka T, Hayashi K (2005): Analysis of genomic homology of murine gammaherpesvirus (MHV)-72 to MHV-68 and impact of MHV-72 on the survival and tumorigenesis in the MHV-72-infected CB17 scid/scid and CB17+/+ mice. Pathol. Int. 55, 558–568. http://dx.doi.org/10.1111/j.1440-1827.2005.01869.x

Park JY, Lee KW, Lee AR, Jeong WJ, Chun J, Lee JH, Kim JH (2008): Characterization of a bifunctional HPr kinase/phosphorylase from Leuconostoc mesenteroides SY1. J. Microbiol. Biotechnol. 18, 746–753.

Parry CM, Simas JP, Smith VP, Stewart CA, Minson AC, Efstathiou S, Alcami A (2000): A broad spectrum secreted chemokine binding protein encoded by a herpesvirus. J. Exp. Med. 191, 573–578. http://dx.doi.org/10.1084/jem.191.3.573

Proudfoot AE, Handel TM, Johnson Z, Lau EK, LiWang P, Clark-Lewis I, Borlat F, Wells TN, Kosco-Vilbois MH (2003): Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. Proc. Natl. Acad. Sci. USA 100, 1885–1890. http://dx.doi.org/10.1073/pnas.0334864100

Pyo R, Jensen KK, Wiekowski MT, Manfra D, Alcami A, Taubman MB, Lira SA (2004): Inhibition of intimal hyperplasia in transgenic mice conditionally expressing the chemokine-binding protein M3. Am. J. Pathol. 164, 2289–2297. http://dx.doi.org/10.1016/S0002-9440(10)63785-6

Rajčáni J, Kúdelová M (2007): Murid herpesvirus 4 (MHV 4): an animal model for human gammaherpesvirus research. In Minarovits J, Gonczol E, Valyi-Nagy T (Eds): Latency Strategies of Herpesviruses. Springer Berlin Heidelberg, New York, pp. 102–136. http://dx.doi.org/10.1007/978-0-387-34127-9_5

Ransohoff RM (2002): Chemokines in neurological trauma models. Ann. NY Acad. Sci. 961, 346–349. http://dx.doi.org/10.1111/j.1749-6632.2002.tb03120.x

Rašlová H, Matis J, Režuchová I, Mačáková K, Berebbi M, Kúdelová M (2000): The bystander effect mediated by the new murine gammaherpesvirus 72-thymidine kinase/5′-fluoro-2′-deoxyuridine (MHV72-TK/5-FUdR) system in vitro. Antivir. Chem. Chemother. 11, 273–282.

Rašlová H, Berebbi M, Rajčáni J, Sarasin A, Matis J, Kúdelová M (2001): Susceptibility of mouse mammary glands to murine gammaherpesvirus 72 (MHV-72) infection: evidence of MHV-72 transmission via breast milk. Microb. Pathog. 31, 47–58. http://dx.doi.org/10.1006/mpat.2001.0441

Sarawar SR, Lee BJ, Anderson M, Teng YC, Zuberi R, Von Gesjen S (2002): Chemokine induction and leukocyte trafficking to the lungs during murine gammaherpesvirus 68 (MHV-68) infection. Virology 293, 54–62. http://dx.doi.org/10.1006/viro.2001.1221

Scorer CA, Carrier MJ, Rosenberger RF. Amino acid misincorporation during high-level expression of mouse epidermal growth factor in Escherichia coli (1991): Nucleic Acids Res. 19, 3511–3516. http://dx.doi.org/10.1093/nar/19.13.3511

Simas JP, Swann D, Bowden R, Efstathiou S (1999): Analysis of murine gammaherpesvirus-68 transcription during lytic and latent infection. J. Gen. Virol. 80, 75–82.

Sunil-Chandra NP, Efstathiou S, Arno J, Nash AA (1992): Virological and pathological features of mice infected with murine gamma-herpesvirus 68. J. Gen. Virol. 73, 2347–2356. http://dx.doi.org/10.1099/0022-1317-73-9-2347

Terpe K (2006): Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. Appl. Microbiol. Biotechnol. 72, 211–222. http://dx.doi.org/10.1007/s00253-006-0465-8

Usherwood EJ, Roy DJ, Ward K, Surman SL, Dutia BM, Blackman MA, Stewart JP, Woodland DL (2000): Control of gammaherpesvirus latency by latent antigen-specific CD8(+) T cells. J. Exp. Med. 192, 943–952. http://dx.doi.org/10.1084/jem.192.7.943

Valovičová M, Režuchová I, Mačáková K, Petrová P, Matis J, Kúdelová M (2006): Murine gammaherpesvirus (MHV) MK3 gene sequence diversity among 72, 4556, and 68 strains. Virus Genes 33, 51–58. http://dx.doi.org/10.1007/s11262-005-0038-4

van Berkel V, Preiter K, Virgin HW 4th, Speck SH (1999): Identification and initial characterization of the murine gammaherpesvirus 68 gene M3, encoding an abundantly secreted protein. J. Virol. 73, 4524-4529.

van Berkel V, Barrett J, Tiffany HL, Fremont DH, Murphy PM, McFadden G, Speck SH, Virgin HW 4th (2000): Identification of a gammaherpesvirus selective chemokine binding protein that inhibits chemokine action. J. Virol. 74, 6741–6747. http://dx.doi.org/10.1128/JVI.74.15.6741-6747.2000

van Berkel V, Levine B, Kapadia SB, Goldman JE, Speck SH, Virgin HW 4th (2002): Critical role for a high-affinity chemokine-binding protein in gammaherpesvirus-induced lethal meningitis. J. Clin. Invest. 109, 905–914.

van Regenmortel MHV, Fauquet CM, Bishop DHL (2000): Herpesvirus family. In Virus Taxonomy: Classification and Nomenclature of Viruses. 7th ICTV Report, Academic Press, San Diego, pp. 220–226.

Virgin HW 4th, Latreille P, Wamsley P, Hallsworth K, Weck KE, Dal Canto AJ, Speck SH (1997): Complete sequence and genomic analysis of murine gammaherpesvirus 68. J. Virol. 71, 5894–5904.

Virgin HW 4th, Presti RM, Li XY, Liu C, Speck SH (1999): Three distinct regions of the murine gammaherpesvirus 68 genome are transcriptionally active in latently infected mice. J. Virol. 73, 2321-2332.

Webb LM, Clark-Lewis I, Alcami A (2003): The gammaherpesvirus chemokine binding protein binds to the N terminus of CXCL8. J. Virol. 77, 8588–8592. http://dx.doi.org/10.1128/JVI.77.15.8588-8592.2003

Wu L, Huang TG, Meseck M, Altomonte J, Ebert O, Shinozaki K, García-Sastre A, Fallon J, Mandeli J, Woo SL (2008): rVSV(M Delta 51)-M3 is an effective and safe oncolytic virus for cancer therapy. Hum. Gene Ther. 19, 635–647. http://dx.doi.org/10.1089/hum.2007.163

Zhou M, Shi B, Wang P, Gu J, Li Z (2011): Large-scale production of functional recombinant CAR in a baculovirus- insect cell system. Acta Virol. 55, 93–99. http://dx.doi.org/10.4149/av_2011_02_93

Zlotnik A, Yoshie O (2000): Chemokines: a new classification system and their role in immunity. Immunity 12, 121–127. http://dx.doi.org/10.1016/S1074-7613(00)80165-X