The analysis of polypeptides in the nuclei and cytoplasm of cells infected with murine herpesvirus 72

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Twenty-six polypeptides in murine herpesvirus isolate 72 (MHV-72) were identified and the synthesis and accumulation of 21 virus-specific polypeptides in Vero cells during the course of productive infection with MHV-72 were examined. Five of the infected cell polypeptides accumulated within the nuclei and nine accumulated within the cytoplasm of MHV-72-infected cells. Seven polypeptides were identified within the nuclei and cytoplasm in equivalent amounts. The major capsid protein was shown to have an $M_r$ of 161K. Thirteen virus-specific polypeptides were solubilized with Nonidet P-40 or radioimmunoprecipitation assay buffer and immunoprecipitated with rabbit and mouse immune sera. Analysis of the polypeptides of MHV-72 indicate a closer resemblance between MHV-72 and the gammaherpesviruses herpesvirus saimiri and Epstein–Barr virus than with the alpha herpesvirus herpes simplex virus type 1.

Introduction

Five murine herpesvirus isolates recovered from small free-living rodents Clethrionomys glareolus (MHV-60, MHV-68 and MHV-72) and Apodemus flavicollis (MHV-76 and MHV-78) have been described (Blaskovic et al., 1980). Electron microscopic studies of infected rabbit embryo fibroblasts (REF) provided morphological evidence that they belong to the Herpesviridae family (Ciampor et al., 1981). All five isolates were shown to be antigenically related (Svobodova et al., 1982b) and to induce c.p.e. in various epithelial and fibroblastoid cell lines (Svobodova et al., 1982a) as is characteristic of herpesvirus infection.

Limited sequence analysis of the MHV-68 genome has shown that this virus is closely related to the gamma herpesviruses of primates, Epstein–Barr virus and herpesvirus saimiri in terms of both gene content and organization (Efstathiou et al., 1990a, b).

Studies on the pathogenesis of MHV-68 infection demonstrated that both newborn and 5- to 10-day-old outbred laboratory mice that were infected orally or intranasally developed a severe exudative pneumonia with viral spread through the blood stream (Rajcani et al., 1985; Blaskovic et al., 1984). Virus antigen was detected in many different cell types of infected animals including alveolar epithelium, heart muscle, kidney, adrenal gland, spleen and lymphocytes (Rajcani et al., 1985; Sunil-Chandra et al., 1992a). MHV-68 establishes a latent infection in mouse B lymphocytes in vivo and is biologically similar to Epstein–Barr virus and other members of the B cell lymphotropic gammaherpesvirus 1 subgroup (Sunil-Chandra et al., 1992b). These observations suggest that murine gammaherpesvirus is likely to be a useful animal model for understanding the virological and immunological processes that occur in a natural, permissive gammaherpesvirus infection (Sunil-Chandra et al., 1992b).

We have previously described the synthesis of 28 virus-specific polypeptides during productive infection of tissue culture cells with six isolates of MHV. The time course of virus polypeptide synthesis in MHV-infected REF cells resembled that occurring in herpesvirus saimiri-infected cells more than that of herpes simplex virus type 1-infected cells (Reichel et al., 1991). We now report findings from an analysis of the polypeptides of MHV-72, a comparison of how virus-specific polypeptides accumulate in cytoplasmic and nuclear fractions of MHV-72-infected Vero cells and a study of MHV-72 virus-specific polypeptide solubility. Comparisons were made between the virus-specific polypeptides of MHV-68 and MHV-72. The role of macrophages in BALB/c mice following prolonged infection with MHV-72 is currently under investigation.

Methods

Cells and viruses. Vero cells were grown at 37 °C as monolayers in Eagle's basal medium (BEM) containing 5 to 10% inactivated bovine serum (IBS), glutamine (3 g per 100 ml) and antibiotics (100 units of
Fig. 1. Electrophoretic analysis of polypeptides present in the nuclear (lanes 1, 3, 5, 7, 9 and 11) and cytoplasmic (lanes 2, 4, 6, 8, 10 and 12) fractions of MHV-72-infected (lanes 1, 2, 9 and 10) or mock-infected (lanes 7, 8 and 13) Vero cells. The cells were labelled with $^14$C-amino acids from 46 h to 48 h (a) or 70 h to 72 h (b) after infection or mock infection. Incubation was continued without labelling in maintenance medium for 6 h (lanes 3 and 4) and 22 h (lanes 5, 6, 11 and 12) chase intervals. Ms of polypeptides are given on the left.

penicillin and 100 µg of streptomycin per ml), in Roux or Muller tissue-culture flasks. MHV-72 (Blaskovic et al., 1980) was used throughout.

Infection and labelling of cells. Confluent cell monolayers were infected at an m.o.i. of 10 TCID$_{50}$ per cell and kept for 60 min at 37 °C. After virus adsorption, the inoculum was removed and replaced with BEM supplemented with 5% IBS. For labelling, the medium was replaced with BEM/10 (containing 10-fold reduced levels of amino acids apart from arginine and supplemented with 1% IBS) containing 0.2 MBq/ml (2 h pulses) or 0.1 M bq/ml (48 h pulses) or $^14$C-labelled amino acid hydrolysate (UVVVR, specific activity 4 x $10^4$ MBq/g). For the pulse-chase protocol, monolayers were rinsed three times with prewarmed (37 °C) PBS and then incubated in maintenance medium for various chase intervals. At the end of the long-term pulse and pulse-chase labelling experiments, the cells were rinsed with ice-cold PBS, scraped off, pelleted and stored at −70 °C or divided into nuclear and cytoplasmic fractions and stored prior to analysis by PAGE.

SDS-PAGE. Samples were analysed on 8%, 10% and 12% polyacrylamide gels as described (Matis & Rajcani, 1980). For the Ms determinations the following proteins were used as standards: myosin, β-galactosidase, phosphorylase B, BSA, ovalbumin and carbonic anhydrase (Sigma).

Preparation of nuclear and cytoplasmic fractions. Detection of the major capsid protein. The method described by Imrie & Gibson (1985) was used. The cells were scraped off, collected by low-speed centrifugation (1500 g for 10 min at 4 °C), washed once with 0.5 ml PBS, and pelleted by low-speed centrifugation. Cells were then
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separated into cytoplasmic and nuclear fractions by using 0.5 ml 0.5% Nonidet P-40 in PBS for 5 min at 0 °C. The resulting nuclei were recovered by low-speed centrifugation, rinsed twice with PBS and then the nuclear and cytoplasmic fractions were analysed by SDS-PAGE. To detect the major capsid protein, nuclei were resuspended in 0.4 ml of PBS, and lysed by three cycles of freezing (−70 °C for 4 min) and thawing (37 °C for 2 min). After low-speed centrifugation, capsids were recovered from the clarified preparation by sedimentation through a 15 to 50% (w/w) sucrose density gradient (Beckman SW41 rotor, 40000 r.p.m. at 4 °C for 20 min). The gradients were fractionated into 0.3 ml samples and analysed by SDS-PAGE.

Solubilization of polypeptides. The labelled MHV-72-infected Vero cells were solubilized in RIPA buffer (0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 150 mM-NaCl, 1% mM-Tris–HCl pH 7.4, 1 mM-PMSF) for 60 min at 4 °C or in 1% Nonidet P-40 in PBS for 90 min at 4 °C. The cell debris was removed by low-speed centrifugation (1500 g, for 10 min at 4 °C) and the supernatant was layered on a 30% (w/w) sucrose cushion and centrifuged (70.1 Ti rotor at 33000 r.p.m. for 3 h). The supernatant and the pelleted fraction were analysed by SDS-PAGE.

Radioimmunoprecipitation. Radioimmunoprecipitation experiments were done according to Raucina et al. (1984).

Purification of MHV-72 virus particles. MHV-72 was inoculated at an m.o.i. of 10 TCID₉₀ per cell into cultures of Vero cells in Roux bottles. Three days post-infection extracellular virus (at a titre of 10⁶ TCID₉₀ per ml medium) was obtained and purified by a three-step procedure as described by Svobodova et al. (1982b). Enveloped, unenveloped and empty virus particles were present.

Results

Some virus-specific polypeptides were rapidly transferred to the nucleus shortly after their synthesis. Other polypeptides accumulated slowly in the cytoplasmic or nuclear fractions of infected cells. Fig. 1 shows results from experiments conducted to examine the transfer of polypeptides to the nucleus in infected and mock-infected cells during and immediately after a 2 h labelling interval. The 42.5K and 16K virus-specific polypeptides were transferred rapidly and efficiently to the nuclear fraction. The majority of each of these polypeptides associated with the nuclear fraction by the end of the labelling interval. During subsequent 'chase' intervals of 6 h and 22 h a significant fraction of the intracellular pools of the 125K, 112K and 84K polypeptides appeared in the nuclear fraction.

The majority of the 138K, 79K, 66K, 50K, 48K, 46K, 38K, 35K and 32.5K polypeptides were associated with the cytoplasmic fraction by the end of the labelling interval and during subsequent 'chase' periods. The 161K, 74K, 55K, 44K, 40K, 34K and 28.5K polypeptides accumulated in the cytoplasmic and nuclear fractions in equivalent amounts (Fig. 1 b). Levels of the 46K and 44K virus-specific polypeptides decreased dramatically during the 'chase' periods.

To detect the solubilization of the virus-specific polypeptides, MHV-72-infected ¹⁴C-amino acid-labelled Vero cells were treated with 1% Nonidet P-40 or with RIPA buffer. After high-speed centrifugation of the cytoplasmic fraction, supernatant and sediment polypeptides were analysed by SDS-PAGE (Fig. 2a, b). Thirteen solubilized virus-specific polypeptides with

Fig. 2. Electrophoretic analysis of polypeptides of MHV-72-infected Vero cells treated with Nonidet P-40 (a) or RIPA buffer (b). The cells were labelled with ¹⁴C-amino acids 48 to 72 h post-infection and then treated with detergents and the cytoplasmic fraction was centrifuged through a 30% sucrose density gradient. Lane 1, Cytoplasmic fraction after treatment of infected cells with detergents. Sediment (lanes 2) and supernatant (lanes 3) after high-speed centrifugation of the cytoplasmic fraction of infected cells through a 30% sucrose density gradient. Mₛs of polypeptides are given on the right.
apparent $M_s$ of 138K, 112K, 107K, 79K, 74K, 70K, 66K, 50K, 46K, 42.5K, 40K, 34K and 32.5K were detected. The 161K, 125K and 62K polypeptides were not solubilized and were found in the sediment.

To prove the virus specificity of the solubilized polypeptides, they were immunoprecipitated with rabbit immune serum raised against MHV-72 and with mouse immune serum raised against a new MHV isolate 5682 (Kozuch et al., 1993). Thirteen polypeptides were precipitated with both immune sera and no precipitation was detected after treatment of solubilized polypeptides with preimmune rabbit and mouse sera (Fig. 3).

To identify the major capsid protein of MHV-72, nuclei of $^{14}$C-amino acid-labelled MHV-72-infected Vero cells were disintegrated as described in Methods. The supernatant, which contained nucleocapsids, was centrifuged in a 5 to 55% sucrose density gradient and samples that were taken from the bottom of the tube were analysed by PAGE (Fig. 4). The major capsid protein was shown to have an $M_s$ of 161K and was detected in fractions 6 and 7. Other capsid proteins were not detected.

PAGE and autoradiography of $^{14}$C-labelled denatured proteins from purified MHV-72 particles resolved at least 26 clearly discernible polypeptides ranging between 161K and 16K (Fig. 5, Table 1). As shown in Fig. 6, the polypeptide profile of MHV-72 closely resembled that of MHV-68. However, in the cells infected with MHV-68, polypeptide 46K is not synthesized.

Results are shown in Table 1. Thirty-one infected cell polypeptides (ICP), including 26 polypeptides of the virion, were detected. Five of the ICPs accumulated
within nuclei and nine within the cytoplasm of host cells. Seven polypeptides accumulated within the nuclei and the cytoplasm in equivalent amounts. Thirteen virus-specific polypeptides were solubilized after treatment of infected cells with Nonidet P-40 or RIPA buffer.

**Discussion**

We detected 26 polypeptides in MHV-72 and the synthesis and accumulation of 21 virus-specific polypeptides in Vero cells during the course of a productive
Table 1. Apparent Mr and localization of MHV-72 polypeptides

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Fig. 6. Polypeptide profiles of MHV-68- or MHV-72-infected cells. Cells were infected with MHV-68 (lanes 1 and 2) or MHV-72 (lanes 3 and 4) and labelled with 14C-amino acids 48 to 72 h (lane 3), 72 to 96 h (lanes 1 and 4) or 96 to 120 h (lane 2) post-infection. Mr’s of polypeptides are given on the right.

infection with MHV-72. These polypeptides range in Mr from 16K to 161K.

The following differences were detected in polypeptide profiles of MHV-72-infected Vero cells and REF (Reichel et al., 1991). The 161K, 138K, 125K, 84K, 46K, 40K, 34K, 28.5K and 16K major virus-specific polypeptides were the same in both cellular systems. The most striking differences were in polypeptides 79K and 50K. The 79K major polypeptide in infected Vero cells corresponded to a 74K major polypeptide in infected REF cells. The 50K polypeptide of infected Vero cells was minor and accumulated in the cytoplasmic fraction. The 50K polypeptide of infected REF cells was major and accumulated in the nuclear fraction (M. Reichel, J. Matis, M. Mistrikova & J. Lesso, unpublished).

Thirteen virus-specific polypeptides were solubilized with NP40 and were immunoprecipitated by the rabbit and mouse immune sera. Two control experiments were done. MHV-72-infected Vero cells were immunoprecipitated by preimmune rabbit and mouse sera (Fig. 3) and non-infected Vero cells were immunoprecipitated by immune rabbit and mouse sera (data not shown). Only a weak non-specific reaction was found to occur between preimmune rabbit serum and the 161K and 64K proteins of MHV-72. No other non-specific reactions were identified.

The 116K, 100K, 89K, 70K, 58K, 57K, 52K and 27.5K structural polypeptides were detected only in virions but not in the MHV-72-infected Vero cells. Similarly, the 107K and 70K polypeptides were detected among solubilized and immunoprecipitated polypeptides but not in the MHV-72-infected cells. These proteins could have been masked by cellular proteins because no inhibition of host-cell protein synthesis by MHV was observed (Reichel et al., 1991).

The genomes of gammaherpesviruses Epstein-Barr virus and herpesvirus saimiri encode a 140K membrane antigen (Baer et al., 1984) and a related 160K tegument protein (Cameron et al., 1987), respectively. No obvious homologue to these exists in either the alphaherpesviruses varicella-zoster virus and herpes simplex virus or the betaherpesvirus human cytomegalovirus. By analysis of short DNA sequences the genes for these proteins in the gammaherpesviruses bovine herpesvirus 4 (Bublot et
al., 1992) and MHV-68 (Efstathiou et al., 1990b) were found. Polypeptide profiles of MHV strains (Reichel et al., 1991 and this work) have shown that polypeptides 138K could be a counterpart to the 140K EBV membrane antigen or to the 160K herpesvirus saimiri major tegument protein. This is currently under investigation.

The MHV-72 125K polypeptide, which is not present in virions, was synthesized early after infection and accumulated in the nuclear fraction of infected cells. The MHV-72 16K structural polypeptide was synthesized late after infection and accumulated in the nuclear fraction (M. Reichel, J. Matis, M. Mistrikova & J. Lesso, unpublished). The MHV-72 125K and 16K polypeptides resemble the Epstein-Barr virus-associated 120K and 18K DNA-binding proteins (Kawanishi et al., 1981; Mueller-Lantzsch et al., 1979) and the 110K and 12K proteins of herpesvirus saimiri (Blair & Hones, 1983). The 16K polypeptide of MHV-72 is a major structural polypeptide. Polypeptides with a similar M, were detected in the gamma-2 herpesviruses bovine herpesvirus type 4 (16K) (Dubuisson et al., 1989) and alcelaphine herpesvirus type 1 (16-5K) (Seal et al., 1989).

We conclude that characteristics of the polypeptide profiles of MHV-72, as well as those of MHV-68, resemble the properties of the gammaherpesviruses herpesvirus saimiri and Epstein-Barr virus more closely than those of the alphaherpesvirus herpes simplex virus type 1.

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