Identification and Characterization of a 50K DNA-binding Protein of Guinea-pig Cytomegalovirus

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SUMMARY

In a previous study we showed that cells infected with guinea-pig cytomegalovirus (GPCMV) contain large amounts of a non-structural 50K nuclear protein that is detectable by immunoelectron microscopy using monoclonal antibodies. The present study shows that this 50K protein is a DNA-binding protein, as determined by single-stranded DNA affinity chromatography, and a phosphorylated protein as demonstrated by immunoprecipitation using [32P]orthophosphate-labelled cells. This protein binds both viral and host cellular double-stranded and single-stranded DNA, assayed by a simple method using DNA linked to a nylon membrane. Induction of the 50K protein in GPCMV-infected cells was highly dependent on viral DNA synthesis, which was detected by dot hybridization using a cloned GPCMV DNA probe. Synthesis of the 50K protein was significantly impaired when phosphonoacetic acid was added to the culture medium. Induction of the 50K protein was detected about 6 h before the appearance of the 76K viral matrix protein.

INTRODUCTION

Guinea-pigs infected with guinea-pig cytomegalovirus (GPCMV) have been used as models for congenital human cytomegalovirus (HCMV) infection because transplacental transmission of the virus to the foetus has been demonstrated (Choi & Hsiung, 1978; Kumar & Nankervis, 1978; Johnson & Connor, 1979; Griffith et al., 1986).

Virus-specific proteins synthesized in cells infected with GPCMV have not been as fully characterized as those synthesized by HCMV. In a previous study (Tsutsui et al., 1986), we prepared three groups of murine monoclonal antibodies (MAbs) which reacted with intranuclear inclusions, a matrix protein or a viral core protein of GPCMV. Antibodies that reacted with nuclear inclusions of GPCMV-infected cells immunoprecipitated an Mr 50K polypeptide of the infected cells. Tsutsui et al. (1986) showed by immunoelectron microscopy that these antibodies reacted with filamentous structures of the nuclear inclusions but not with virions or extracellular dense bodies. However, they failed to immunoprecipitate a 50K polypeptide from purified virions, suggesting that this 50K protein is non-structural.

Cells infected with a primate strain (Colburn) of CMV contain large amounts of an intranuclear 51K DNA-binding protein (Gibson et al., 1981; Gibson, 1983, 1984) which is phosphorylated. Characterizations of major virus-specific DNA-binding proteins have also been reported for herpes simplex virus types 1 and 2 (Bayliss et al., 1975; Purifoy & Powell, 1976; Powell et al., 1981; Ruyechan, 1983; Vaughan et al., 1985) and Marek's disease virus (Nakajima et al., 1986). Identification of virus-specific proteins, such as those binding DNA, in the nuclei of infected cells is important because of the possible regulatory roles of proteins in viral gene expression (Godowski & Knipe, 1983) and replication of viral DNA (Conley et al., 1981; Vaughan et al., 1985).

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This study further characterizes the non-structural 50K nuclear protein of GPCMV-infected cells.

**METHODS**

*Virus and cell cultures.* GPCMV, prototype strain 22122, was obtained from the American Type Culture Collection. Guinea-pig embryo (GPE) cells were grown in 10% foetal calf serum in Eagle’s MEM containing penicillin (100 units/ml) and streptomycin (50 μg/ml) as described previously (Tsutsui et al., 1986).

*Monoclonal antibodies and immunoprecipitation.* Preparation of MAbs specific to GPCMV-infected cells has been described previously (Tsutsui et al., 1986). MAb E-16 is specific for a 50K nuclear inclusion protein which is a non-structural protein; MAb A-29 is specific for a 76K viral matrix protein present in both cytoplasmic inclusions and extracellular dense virions. Forty-eight h after infection with GPCMV, GPE cells were labelled with [35S]methionine (50 μCi/ml; Amersham) for 2 h. Immunoprecipitation of the cell lysate and fractionated cell extracts was performed using MAbs as described previously (Tsutsui et al., 1986).

*DNA affinity chromatography.* DNA affinity chromatography was performed essentially according to the method of Shiraki et al. (1982). Briefly, the radiolabelled cells, collected with a rubber policeman, were suspended in 20 mM-Tris-HCl pH 7.5, containing 2 mM-2-mercaptoethanol and 500 μg/ml bovine serum albumin (BSA). The cell suspension (5 × 10^7 cells) was intermittently sonicated with an ultrasonic disruptor (Kontes, Vineland, N.J., U.S.A.) for 3 min in iced water, then mixed with an equal volume of 20 mM-Tris–HCl pH 7.5, containing 3-4 mM-NaCl and 10 mM-EDTA and kept on ice for 40 min. After centrifugation at 30000 g for 20 min at 4 °C, the supernatant was dialysed overnight at 4 °C against column buffer composed of 20 mM-Tris-HCl pH 7.5, 50 mM-NaCl, 1 mM-EDTA, 2 mM-2-mercaptoethanol and 10% glycerol. The cell extract was centrifuged at 100000 g for 60 min at 4 °C and the supernatant was applied to a DNA affinity column. Single-stranded DNA (calf thymus) coupled to cellulose (DNA-cellulose) was equilibrated with the column buffer and packed in a plastic column with a 1 ml bed volume. In control experiments a column containing non-coupled cellulose was used. After applying the GPCMV-infected cell extract, the column was extensively washed with the column buffer, then bound proteins were eluted with the same buffer containing increasing concentrations of NaCl. The radioactivity of the eluted fractions was counted and the peak fractions were subjected to SDS–PAGE according to the method of Laemmli (1970). For immunoprecipitation the peak fractions were added to 5 volumes of extraction buffer (Blanton & Tevethia, 1981).

*DNA binding assay using DNA-linked nylon membrane.* GPCMV DNA and DNA of uninfected GPE cells (0-05 mg/ml each) were spotted (0-05 μg/spot) on nylon membranes (Hybond-N; Amersham) and linked to the membranes by u.v. irradiation according to the method of Church & Gilbert (1984). The membranes were then incubated in phosphate-buffered saline (PBS) containing 10% BSA and 0.5% Tween 20 overnight at 42 °C. After being washed briefly with PBS containing 0.5% Tween 20, the membranes were incubated at room temperature for 30 min in a solution containing 50 μg/ml of protein from the 0-45 m-NaCl eluate of the DNA affinity column (see above), after the NaCl concentration has been adjusted to 0.15 M. The membranes were washed with the column buffer (see above) containing 0.15 M-, 0.30 M- or 0.45 M-NaCl, and then incubated with MAb E-16 (1:200 dilution of ascites fluid). The spots were visualized after a series of reactions using an avidin-biotinylated horseradish peroxidase complex (ABC) kit (VECTASTAIN; Vector Laboratories, Burlingame, Cal., U.S.A.). The DNA-linked nylon membranes were also incubated with the GPCMV-infected or uninfected whole cell lysates, then incubated with MAb E-16, MAb A-29, or with MAb D-1, specific to murine CMV-infected cells, as a control antibody. Nylon membranes not linked to DNA were spotted to DNA with the GPCMV-infected or uninfected whole cell lysates and were also incubated with the MAbs.

*Immunofluorescence.* GPE cells plated on coverslips were either infected with GPCMV at an m.o.i. of 5 p.f.u./cell or left uninfected. The GPCMV-infected cells were cultured in the presence or absence of phosphonoacetic acid (PAA; 100 μg/ml). At appropriate times after infection, the monolayers were fixed and subjected to immunofluorescence tests using MAbs as described previously (Tsutsui et al., 1986).

*Dot hybridization for detection of GPCMV DNA in infected GPE cells.* GPE cells plated in 10 cm plastic dishes (Falcon) were infected with GPCMV at an m.o.i. of 5 p.f.u./cell. At different times, the cells were collected with a rubber policeman and their DNA was extracted according to the procedure of Blin & Stafford (1976). The concentration of total DNA in each sample was adjusted to 0.2 μg/ml, then 2 μl of each aliquot was spotted onto a nylon membrane (Hybond; Amersham). The DNA in each spot was denatured in an alkaline solution (0.5 M-NaOH, 1.5 mM-NaCl) for 1 min.

For preparation of a virus DNA probe, GPCMV virions were collected from culture fluid according to the method of Isom et al. (1984). Virus DNA was extracted from virions by the method of Stinski et al. (1979). After digestion with HindIII, fragments of virus DNA were cloned in cosmid vector Homer IV (Chia et al., 1982). A cloned DNA fragment corresponding to the HindIII C fragment of Gao & Isom (1984) was used as a probe for viral DNA after labelling in a multiple priming DNA-labelling system (Amersham) using [32P]dCTP. This virus probe hybridized with GPCMV DNA but not with cellular DNA.
RESULTS

The 50K protein identified by MAbs is a DNA-binding protein

We have previously prepared MAbs specific for a 50K polypeptide of GPCMV-infected cells (Tsutsui et al., 1986). By immunofluorescence and immunoelectron microscopy the 50K protein was shown to be localized in nuclear inclusions of the infected cells but not in intracytoplasmic or extracellular virions (Tsutsui et al., 1986). Since the 50K protein is predominantly localized in the nucleus, its DNA-binding capacity was investigated by DNA affinity chromatography. A radiolabelled whole cell lysate prepared from GPCMV-infected cells was applied to a DNA-cellulose column in a buffer containing 0.05 M-NaCl. The column was washed and bound proteins were eluted with buffer containing increasing concentrations of NaCl (0.15 M, 0.45 M and 1.0 M) (Fig. 1). A significant amount of radioactivity was recovered in the eluate at 0.45 M-NaCl from the DNA-cellulose, whereas almost no radioactivity was eluted at the same salt concentration from the non-coupled cellulose column. After separation of the eluted fractions by SDS-PAGE, a 50K protein band was observed with the 0.45 M-NaCl eluate from the DNA-cellulose column; a 105K band and several minor bands were also observed (Fig. 1, lane 4). The 0.45 M-NaCl eluate was immunoprecipitated using MAb E-16 and then separated by SDS-PAGE. The 50K protein was precipitated from the eluted fraction (Fig. 1, lane 9). No

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**Fig. 1.** SDS–PAGE and autoradiography of the 50K protein prepared by DNA affinity chromatography. GPCMV-infected cells were labelled with [35S]methionine 48 h after infection. Cell lysates prepared as described in Methods were chromatographed on DNA-cellulose (lanes 1 to 5) or a non-coupled cellulose column (lanes 6 and 7). After extensive washing, polypeptides bound to the columns were eluted with increasing NaCl concentrations (lane 2, 0.05 M; lane 3, 0.15 M; lanes 4, 7 and 9, 0.45 M; lane 5, 1.0 M). Whole cell lysates are shown in lanes 1, 6 and 8. The whole cell lysate and the fraction eluted with buffer containing 0.45 M-NaCl were immunoprecipitated using MAb E-16 (lanes 8 and 9 respectively). Samples were subjected to SDS–PAGE (10% polyacrylamide) and autoradiography. A 105K band and the 50K protein band are indicated.
Fig. 2. DNA-binding capacity of the 50K protein and the whole cell lysate using viral and cellular DNA-linked nylon membranes. GPCMV DNA (a) and uninfected cell DNA (b) were linked to nylon membranes by u.v. irradiation. The 50K protein eluted from the DNA affinity column with 0.45 M NaCl (as shown in Fig. 1) was adjusted to a NaCl concentration of 0.15 M (columns 1, 2 and 3). The DNA-linked nylon membrane was then incubated with the 50K protein solution. The membranes were washed with the column buffer at a NaCl concentration of 0.15 M (column 1), 0.3 M (column 2) and 0.45 M (column 3), then incubated with MAb E-16. Antigen–antibody reactions were visualized using an ABC kit. The DNA-linked nylon membranes were incubated with the GPCMV-infected whole cell lysate (columns 4, 5 and 6) or uninfected whole cell lysate (column 7). After washing with the buffer containing 0.15 M NaCl, the membranes were incubated with MAb E-16 (columns 4 and 7), MAb A-29, (column 5) or MAb D-1 (column 6). The DNA-linked nylon membrane was first incubated with the uninfected whole cell lysate, then with the GPCMV-infected whole cell lysate (column 8) and finally with MAb E-16. Nylon membranes not linked to DNA were spotted with GPCMV-infected whole cell lysate (c) or the uninfected whole cell lysate (d), then the membranes were incubated with MAb E-16 (column 9), with MAb A-29 (column 10) or with MAb D-1 (column 11).

immunoprecipitated band was observed in the 0.45 M eluate when normal ascites was used (data not shown). There was no immunoprecipitation, using MAb E-16, of a 50K polypeptide from a 0.45 M eluate from a DNA–cellulose column loaded with radiolabelled mock-infected cell polypeptides (data not shown). The 50K protein is therefore a major DNA-binding protein in GPCMV-infected cells.

The 50K protein binds both viral and cellular DNA

To investigate whether the 50K protein preferentially binds viral DNA or host cellular DNA, we designed a DNA binding assay in which protein bound to DNA attached to a nylon membrane was visually detected by specific antibodies using an avidin–biotin reaction system. As shown in Fig. 2, at 0.15 M NaCl, the 50K protein bound both viral and host cellular DNA (columns 1). This binding activity was markedly diminished when the nylon membrane was washed with buffer containing 0.3 M NaCl (columns 2), and completely disappeared after washing with the buffer containing 0.45 M NaCl (columns 3). This result corresponds to those obtained using the DNA affinity column. The 50K protein also bound to single-stranded viral and cellular DNA (data not shown). When using whole infected cell extract instead of the 0.45 M eluate, the 50K protein consistently bound preferentially to host cellular DNA rather than viral DNA (Fig. 2, columns 4). When the DNA-linked nylon membranes incubated with GPCMV-infected whole cell lysate were allowed to react with MAb A-29 (columns 5) or with MAb D-1, as a control antibody (columns 6), no spot was observed after a series of reactions using the ABC kit. No DNA-binding activity was detected when the DNA-linked nylon membrane was
The 50K protein is phosphorylated

Gibson et al. (1981) reported that the 51K DNA-binding protein of the Colburn strain of CMV was phosphorylated. The 50K protein of GPCMV is similar to this protein in many respects: it has a similar $M_r$, a nuclear localization, is not a viral structural protein and has DNA-binding capacity. We investigated whether the 50K DNA-binding protein of GPCMV is also phosphorylated. GPCMV-infected cells labelled with $[^{32}P]$orthophosphate (100 μCi/ml) from 24 to 48 h after infection were immunoprecipitated with MAb E-16 and the precipitate was separated by SDS–PAGE. As shown in Fig. 3, the 50K protein immunoprecipitated by the antibody was phosphorylated. No immunoprecipitated band was observed when radiolabelled mock-infected cells were used (Fig. 3, lane 3).
Fig. 4. Immunofluorescence photomicrographs of GPCMV-infected GPE cells. After adsorption with GPCMV, cells were incubated in the presence (b, e, h, k) or absence (a, c, d, f, g, i, j, l) of PAA (100 μg/ml) for 4 h (a and b), 10 h (c, d, e and f), 16 h (g and h), 24 h (j and k), 30 h (i) or 36 h (l). After fixation, the cells were incubated with MAbs E-16 (a, b, d, e, g, h, j and k), D-1 (c) or A-29 (f, i and l), then fluorescein isothiocyanate-labelled anti-mouse IgG. Photographs were taken under indentical conditions using Fuji Neopan F 400. Bar marker represents 100 μm.

*Induction of 50K protein in GPCMV-infected cells is dependent on viral DNA synthesis*

Using MAb E-16, faint dotted nuclear fluorescence was observed about 8 h after infection in both PAA-treated and untreated cells (Fig. 4d, e; also results not shown). This faint nuclear fluorescence was not observed when the cells infected with GPCMV for 10 h were allowed to react with MAb D-1 (Fig. 4c) and with MAb A-29 (Fig. 4f). The dotted nuclear fluorescence detected using MAb E-16 became larger and stronger in the cells not treated with PAA as
DNA-binding protein of GPCMV

Fig. 5. DNA dot hybridization of GPCMV-infected cells using a GPCMV DNA probe. Total DNA, extracted from GPCMV-infected cells cultured for different times after infection (positions 1 to 9: 0, 4, 6, 8, 12, 16, 20, 24 and 36 h, respectively) was adjusted to 0.2 μg/ml and spotted onto nylon membrane. DNA hybridization was performed using a [32P]dCTP-labelled cloned GPCMV DNA fragment. Position 10 shows DNA extracted from uninfected cells as a negative control and position 11 shows the purified DNA fragment of GPCMV as a positive control.

infection time progressed from about 10 to 24 h after infection (Fig. 4d, g, i), whereas it became fainter in the PAA-treated cells (Fig. 4h, k).

Total DNA was extracted from cells infected with GPCMV at the same m.o.i. as above and subjected to DNA dot hybridization using a [32P]dCTP-labelled GPCMV cloned DNA fragment as a probe. A hybridized spot was detected 12 h after infection and it became larger and more intense as infection time progressed (Fig. 5). The 50K DNA-binding protein thus increased around the time of viral DNA synthesis.

GPE cells infected with GPCMV at the same m.o.i. as above were labelled with [35S]methionine at various times for 2 h and subjected to immunoprecipitation using either MAb E-16 (Fig. 6a) or MAb A-29 (Fig. 6b). The 50K band appeared faintly in cells labelled from 10 to 12 h after infection and became distinct in cells labelled from 14 to 16 h after infection (Fig. 6a, lane 3). With MAb A-29, a 76K band was recognized in cells labelled from 18 to 20 h after infection (Fig. 6b, lane 4). Synthesis of the 50K DNA-binding protein in GPCMV-infected cells was detectable about 6 h earlier than synthesis of the 76K viral matrix protein. Neither the 50K nor 76K proteins were synthesized in cells treated with cycloheximide (CH) for 10 h after infection and released for labelling for 2 h (Fig. 6, lanes 7), or in cells treated with PAA for 32 h and labelled for 2 h (Fig. 6, lanes 8). These results indicate that synthesis of the 50K DNA-binding protein of GPCMV seems to be dependent on viral DNA synthesis. The 76K matrix protein was detected in the cells by immunofluorescence more than 30 h after infection (Fig. 4i, l). The discrepancy between immunoprecipitation and immunofluorescence in the appearance of the matrix protein may be due to antigenic masking in situ in the early phase of the late infection.

DISCUSSION

In a previous study we prepared MAbs specific for a 50K protein localized in GPCMV-infected cell nuclei but not in virions or extracellular dense bodies revealed by immunoelectron microscopy (Tsutsui et al., 1986). The present study shows that the 50K protein is a DNA-binding, phosphorylated protein, probably belonging to the same class of proteins as the 51K DNA-binding protein of strain Colburn CMV (Gibson et al., 1981; Gibson, 1984) and the protein counterpart of HCMV (Gibson, 1983). Since viral DNA-binding proteins have been shown to have an important role in viral replication (Powell et al., 1981; Conley et al., 1981; Anders et al., 1986), we first tried to determine whether the 50K protein binds viral or cellular DNA and then examined the relationship between viral DNA synthesis and expression of the 50K DNA-binding protein of GPCMV.
Fig. 6. Fluorogram of immunoprecipitated GPCMV-infected cell extracts using MAb E-16 (a) or A-29 (b). GPE cells were infected (lanes 2 to 8) or mock-infected (lanes 1) with GPCMV and cultured for different times after infection (lanes 2 to 6: 10 to 12, 14 to 16, 18 to 20, 22 to 24 and 32 to 34 h, respectively). After extensive washing, cells were labelled with $^{35}$S]methionine in methionine-free medium containing 10% foetal calf serum for 2 h. For detection of immediate early proteins (lanes 7) (Blanton & Tevethia, 1981), cells were pretreated with CH (50 µg/ml) for 1 h and CH was also added during and after infection for 10 h. The cells were then released from the CH block and labelled with $^{35}$S]methionine for 2 h in the presence of actinomycin D (100 µg/ml) (lanes 7). Cells were also treated with PAA (lanes 8) for 34 h and labelled with the same isotope for the last 2 h. Bands of $M_r$ 50K and 76K are indicated.

We designed a simple DNA-binding assay system using nylon membranes: DNA dotted onto a nylon membrane was reacted first with DNA-binding proteins and subsequently with antibodies specific to the 50K DNA-binding protein; specifically bound antibody was then visualized using an avidin–biotin reaction system. With this in vitrō system, the 50K protein of
DNA-binding protein of GPCMV

GPCMV bound both viral and host cellular double-stranded and single-stranded DNA, in addition to the single-stranded calf thymus DNA used in the DNA affinity chromatography. Leinbach & Casto (1983) reported that the major DNA-binding protein (ICP8) of herpes simplex virus type 1 (HSV-1) was associated with both HSV-1 and cellular DNA in vivo; this was demonstrated by immunoprecipitation of infected cell nuclear fractions using specific antibodies and extraction of DNA from the immunoprecipitated fraction, followed by hybridization to 32P-labelled viral and cellular DNA. The present results, however, do not show whether the 50K protein binds to viral or host cellular DNA in vivo. Our previous results obtained by immunoelectron microscopy (Tsutsui et al., 1986) demonstrated that the 50K protein was localized not only in the nuclear inclusions but also throughout the infected cell nucleus, implying that the 50K protein binds both viral and cellular DNA in the infected cells. Puvion-Dutilleul et al. (1985) also showed by immunoelectron microscopy that HSV-1 ICP8 was preferentially associated with fibrillar material of electron-translucent viral inclusions and, to a lesser extent, with peripheral host chromatin. Nuclear distribution of the 50K protein of GPCMV is similar to that of ICP8, and both proteins are associated with filamentous structures of the chromatin. Until the exact correlation of nuclear localization of the 50K protein with known sites of viral DNA replication or packaging in the infected cells is demonstrated, it will not be possible to define the role of the protein in viral infection.

We examined in detail the relationship between induction of the 50K protein and viral DNA synthesis in the viral infection cycle. Expression of the 50K protein detected by immunofluorescence was observed at a low level from 8 h after infection when viral DNA synthesis was not detectable. From about 12 h after infection, when viral DNA synthesis became detectable by dot hybridization, expression of the 50K protein increased progressively with time. This increase in protein expression was completely suppressed when PAA was added to the culture medium. Time course experiments showed that synthesis of the 50K protein occurred about 6 h earlier than synthesis of the 76K viral matrix protein detected by immunoprecipitation using MAb A-29 (Fig. 6 and Tsutsui et al., 1986) and is almost in accordance with the time course of the viral DNA synthesis (Fig. 5). These results show that expression of the 50K protein is highly dependent on viral DNA synthesis, although direct evidence of any necessity for the protein in viral replication has not been obtained. Anders et al. (1986, 1987) reported another type of DNA-binding protein of strain Colburn CMV, with an Mr of 129K (DB129). The synthesis of the DB129 protein is not blocked in the presence of inhibitors of viral DNA replication; instead it continues to be made and accumulated to elevated levels. Therefore, this protein is a member of the early class of herpesvirus proteins (Anders et al., 1986), although it is localized in nuclear inclusions like the 50K DNA-binding protein (Anders et al., 1987).

Virus-specific proteins synthesized in cells after infection with herpesviruses have been categorized into three groups: immediate early, early and late (Honess & Roizman, 1974; Wathen & Stinski, 1982). Synthesis of these three groups of proteins is regulated in a cascade fashion. As described for the 51K DNA-binding protein of strain Colburn CMV by Gibson (1984), the 50K DNA-binding protein of GPCMV may belong to a specific group of proteins which appear in the late phase of early infection and are not defined in the three protein groups categorized above. The 50K DNA-binding protein was not expressed in cells studied for expression of the immediate early proteins. Furthermore, this protein began to be expressed about 6 h before induction of the 76K matrix protein, which belongs to the late protein class.

In conclusion, the 50K protein of GPCMV-infected cells is a DNA-binding, phosphorylated protein. It binds both viral and host cellular DNA in vitro. Induction of this protein is highly dependent on viral DNA synthesis. However, the function of the 50K DNA-binding protein in the virus infection cycle remains to be determined.

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