Prokaryotic Expression of Immunogenic Polypeptides of the Large Phosphoprotein (pp150) of Human Cytomegalovirus

By BIRGIT-CHRISTINE SCHOLL, JUTTA VON HINTZENSTERN, BETTINA BORISCH,1 BERND TRAUPE, MICHAEL BRÖKER2 AND GERHARD JAHN*

Institut für Klinische und Molekulare Virologie, Loschgestrasse 7, D-8520 Erlangen, 1Institute of Pathology, University of Würzburg, D-8700 Würzburg and 2Research Laboratories, Behringwerke AG, D-3550 Marburg, F.R.G.

(Accepted 26 February 1988)

SUMMARY

The large phosphorylated matrix protein pp150 of human cytomegalovirus (HCMV) is the polypeptide most frequently reactive in immunoblotting analyses with human antisera when compared with other viral proteins. Several defined regions of pp150 were expressed as β-galactosidase fusion proteins and these were tested for their immunoreactivity with human sera and their immunogenicity. One antigenic region could be expressed in large amounts and was found to carry immunodominant epitopes, as shown by immunoblotting and ELISA. A rabbit antiserum raised against recombinant pp150 antigens produced in bacteria proved to be useful for immunofluorescence and immunohistochemistry studies of HCMV-infected cells and tissues. The results suggest that this anti-pp150 serum will help to elucidate the process of virus assembly and antigen detection in infected cells.

INTRODUCTION

Human cytomegalovirus (HCMV) infections play an important role as a source of human disease. The virus is a major pathogen in immunosuppressed individuals and a common infectious cause of congenital abnormalities. Conventional methods of HCMV serodiagnosis await improvement for a number of reasons. They are based on poorly defined viral antigens, and serological tests only give indirect evidence of HCMV infection. For the diagnosis of an active HCMV infection, viral isolation is usually required; however, the slow growth of HCMV in cell cultures causes this to be time-consuming.

HCMV has at least 25 structural proteins (Stinski, 1976; Gibson, 1983; Kim et al., 1976; Nowak et al., 1984b). Studies on immunogenic proteins of purified virions have been done by immunoprecipitation and immunoblotting (Schmitz et al., 1980; Pereira et al., 1982, 1983; Landini et al., 1985, 1986; Zaia et al., 1986). Predominantly, five structural proteins are recognized by Western blot assays. These are two phosphoproteins of 65K and 150K, designated pp65 and pp150, the major glycoprotein with an apparent M, of 58K and two other proteins of 38K and 28K (Landini et al., 1985, 1986; Jahn et al., 1987a). The coding sequences of the immunogenic proteins have been determined recently (Nowak et al., 1984a; Pande et al., 1984; Rüger et al., 1987; Mach et al., 1986; Cranage et al., 1986; Jahn et al., 1987b). Polypeptide pp150 is of particular interest since its sequence does not show any significant homology to open reading frames (ORFs) of other members of the herpesvirus group. In contrast, some other herpesvirus genes encoding structural proteins such as the major capsid protein and the major glycoprotein are well conserved with respect to DNA and protein sequences (Kouzarides et al., 1987; Davison & Scott, 1986). For this reason, pp150 represents a specific structural HCMV protein without a homologous counterpart in other herpesviruses and it can therefore serve to distinguish between HCMV and other herpesviruses in isolation and differentiation procedures.
Of all the HCMV polypeptides recognized by human sera, the phosphorylated matrix protein pp150 appears to be the most immunogenic. The immune reaction against this polypeptide persists longer than those against other HCMV polypeptides after convalescence (Landini et al., 1985). Polypeptide pp150 is recognized by IgG and IgM antibodies in HCMV-positive human sera. This is remarkable, since IgM reactivity seems to be directed toward only a restricted number of HCMV polypeptides (Landini et al., 1985, 1986).

Our approach was to express epitopes of pp150 in Escherichia coli and to test the recombinant proteins for their antigenic potential. Hybrid polypeptides consisting of defined stretches of pp150 were expressed in large amounts by means of a high level expression system (Bröker, 1986). These HCMV antigens were used to establish a recombinant ELISA for the detection of HCMV antibodies. Secondly, a high titre antiserum directed against the bacterially produced parts of pp150 was raised in rabbits. We show in this report that antiserum obtained in this way specifically detects pp150 antigen in infected cells by immunofluorescence as well as by immunohistochemistry in fixed sections of infected organs and tissues. This antiserum may help us to understand more about the tropism of this virus, the antigen localization within infected cells, organs and tissues, and the process of HCMV morphogenesis in infected cells.

METHODS

Viruses, cell culture and virion purification. HCMV AD169 was provided by U. Krech, St Gallen, Switzerland. Isolates of wild-type HCMV were prepared from the urine of infected patients. Propagation of the virus in human foreskin fibroblasts (HFF) followed standard methods. Purification of HCMV particles from the supernatant of infected cells was done by centrifugal separation in glycerol–tartrate gradients essentially as described by Talbot & Almeida (1977) and modified by Irmiere & Gibson (1983).

Human and rabbit antisera. Individual HCMV antibody-positive sera were obtained from our diagnostic laboratories. Antisera to HCMV proteins were raised in New Zealand white rabbits. Extracts of induced E. coli BMH7118 (Messing et al., 1977) harbouring the recombinant β-galactosidase (β-gal)–pp150 fusion protein were suspended in 1.5 ml phosphate-buffered saline (PBS) containing 4 μl DNase (1 mg/ml) and 10 μl lysozyme (0.1 mg/ml). After incubation for 30 min on ice, the lysate was sonicated for 30 s, centrifuged at high speed, washed three times with PBS and resuspended in increasing concentrations of urea up to 8 M. The supernatant of the last fraction was dialysed and taken for immunization of rabbits as described previously (Jahn et al., 1987a).

Protein gel electrophoresis and immunoblotting. Proteins were separated by SDS–PAGE essentially as described by Laemmli (1970). Samples were solubilized by heating for 3 min at 100 °C in SDS buffer (2% SDS, 10% 2-mercaptoethanol, 5% glycerol, 0.005% bromophenol blue, 50 mM-Tris–HCl pH 7.0). Resolving gels contained either 8-5% or 10% polyacrylamide with an acrylamide–methylenebisacrylamide ratio of 28 : 1 (‘high-bis’). For the immunoblotting experiment, proteins were electrophoretically transferred from acrylamide gels to nitrocellulose filters (Towbin 1979). After transfer, nitrocellulose sheets were blocked with NET buffer (150 mM-NaCl, 5 mM-EDTA, 50 mM-Tris–HCl pH 7.4, 0.25% gelatine, 0.05% Nonidet P40, 2% bovine serum albumin; BSA) and reacted with antibody. Staining was done with horseradish peroxidase-coupled Protein A (Sigma) and 4-chloro-1-naphthol and H2O2.

Expression of fusion proteins. Five ml of LB medium containing 100 μg ampicillin/ml was inoculated with 1 ml of an overnight culture of E. coli BMH7118 harbouring recombinant expression plasmids. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM at an OD600 of 1 and bacteria were grown for 3 to 5 h. Lambda gt11 β-gal fusion proteins were synthesized in E. coli strain Y1089 (Young & Davis, 1983). Overnight cultures of Y1089 were infected with recombinant phages at an m.o.i. of 2 to 3 for 20 min at room temperature in 0.1 ml of 10 mM-Tris–HCl pH 7.5, 20 mM-MgCl2. The infected cells were diluted with 3 ml of LB medium and grown at 32 °C to OD600 0.2 unit. After temperature shift up to 42 °C for 15 min induction was performed by addition of IPTG. After 2 h at 37 °C, cells were pelleted and resuspended in PAGE loading buffer.

DNA sequencing and computer analyses. Lambda gt11 cDNA was subcloned in the M13mp19 phage vector (Yanisch-Perron et al., 1985). Sequencing was performed according to Sanger et al. (1977). Nucleotide sequence comparisons were carried out with a software package provided by the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

ELISA. Approximately 1 μg per well E. coli containing the β-gal–XP1 fusion protein or the pBD2-encoded β-gal portion was bound to microtitre plates (Nunc) overnight at 4 °C. All subsequent steps were performed at room temperature. Non-specific binding sites were blocked with 1% BSA in PBS. Serum samples were preincubated with 50 μg of induced E. coli harbouring vector DNA and added at dilutions from 1:40 to 1:5120. After 1 h, the wells were washed and horseradish peroxidase-coupled Protein A (Sigma) was added for 1 h. Substrate solution (Abbott) was applied. The reaction was stopped with 1 M-H2SO4 and absorbance values were measured at 495 nm. The recombinant ELISAs were compared with standard ELISAs (Behringwerke, Marburg, F.R.G.).
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Fig. 1. Immunoblot of HCMV proteins with individual human sera. Laboratory-adapted strain AD169 (lanes 2 to 6) and a recent clinical isolate (lane 7) were subjected to SDS–high-bis’ PAGE and electrotransferred onto nitrocellulose. The human antisera varied according to their complement fixation titres. The reaction was performed by using Protein A coupled to horseradish peroxidase, and 4-chloro-l-naphthol. The position of pp150 is indicated. Mr markers (lane 1): 205K, 116K, 97K, 66K, 45K and 29K (not visible).

Immunofluorescence and immunohistochemistry. For indirect immunofluorescence, HFF were grown on glass coverslips and methanol-fixed 24 to 48 h after infection with laboratory strain AD169 or recent clinical isolates. The F(ab')2 fraction of the XP1 antiserum was kindly provided by E. J. Kanzy (Ax et al., 1981). This IgG preparation was placed on the cells for 30 min at 37 °C at a dilution of 1 : 50; the cells were then washed for 10 min with PBS. Fluorescein-conjugated goat anti-rabbit gamma globulin serum (Sigma) was added for 30 min (1 : 40 dilution). The cells were washed with PBS and examined microscopically. Uninfected cells were prepared in the same way as controls. For immunohistochemistry, paraffin sections were dewaxed in xylene, rehydrated and preincubated with methanol–H2O2 to inhibit endogenous peroxidase activity. The procedure is described in detail elsewhere (Borisch et al., 1988). The incubation with the primary antibody, antiserum against the recombinant protein XP1, was carried out at a dilution of 1 : 100 in PBS for 1 h. Swine anti-rabbit antiserum (1 : 20 dilution in PBS) was used as the second antibody and a peroxidase–anti-peroxidase (PAP) complex was added. Each incubation step was followed by extensive washing in PBS.

RESULTS

Antigenic properties of the structural phosphoprotein pp150

Of all the HCMV polypeptides recognized by human sera, the 150K phosphorylated matrix protein appears to be the most extensively recognized structural polypeptide. It is reproducibly recognized by Western blot analyses with human sera from individuals of various clinical status. Fig. 1 demonstrates the reaction of human sera with proteins of purified HCMV particles from culture-adapted virus strain AD169. Polypeptides were separated by 8·5% (w/v) PAGE, blotted
onto nitrocellulose and probed with different human antisera. The most consistent signal corresponded to p150 (Fig. 1, lanes 2 to 7). Besides this structural protein, there were a few other virion constituents which were more variably immunoreactive. In Western blot assays, antibodies against pp65 as well as antibodies against gp58 and pp28 were also detectable. The protein patterns of the laboratory strain of HCMV, AD169, and recent clinical isolates have been found to be very similar (Jahn et al., 1987a). In lane 7 of Fig. 1 the reaction of HCMV-positive serum with polypeptides of a low passage clinical isolate is shown. The response of the serum to p150 of recent isolates was as strong as that to p150 of laboratory strain AD169.

The coding sequence for p150 has been determined recently (Jahn et al., 1987b). The gene for p150 was mapped to the overlap of HindIII fragments J and N between map positions 0.160 and 0.186 of HCMV strain AD169. Fig. 2 gives a schematic overview of the AD169 genome and the map position of the gene. The long ORF of 3144 bp, marked as an expanded line at the top of the figure, codes for a proline-rich basic protein. Computer analyses of putative polypeptide secondary structures indicate multiple /-pleated sheets in hydrophilic amino acid clusters. These properties offer an explanation for the highly antigenic potential of p150. Based on the sequence data, polypeptides of p150 were expressed in E. coli in order to study their antigenic potential and their advantages for use as diagnostic reagents.

Expression of polypeptides from p150 in E. coli using a phage vector

Three lambda gt11 clones harbouring cDNA sequences of p150 were tested for their recognition by HCMV human antisera. These cDNA clones had originally been identified by screening a gene library with an anti-p150 rabbit serum (Jahn et al., 1987b). The lambda gt11 cDNA library had been established with late RNA of HCMV-infected HFF cells (Mach et al., 1986). The viral DNA fragments from the p150 gene were recloned and sequenced. These clones, designated lambda 1, lambda 2 and lambda 3, were found to have viral inserts of 350 bp, 190 bp and 270 bp, respectively. These correspond to different parts of the ORF of p150 near the C-terminal and the internal part of p150 (Fig. 2). Upon induction with IPTG, the clones produced /-gal–p150 fusion proteins with apparent Mr values of about 120K to 130K, as determined by PAGE on 10% (w/v) gels (marked by the arrows in Fig. 3). The lambda gt11 /-gal of 116K can be seen in Fig. 3, lane 5. The fusion proteins were tested in immunoblotting experiments for their reactivity with HCMV antibody-positive human sera. Protein lysates of cells expressing fusion proteins were separated on 10% (w/v) polyacrylamide gels and electroblotted onto nitrocellulose filters. The blots were probed with 30 different antisera. Due
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Fig. 3. PAGE of protein extracts of *E. coli* cells infected with lambda gt11 or recombinant phages. Extracts were loaded on 10% gels, stained with Coomassie Brilliant Blue after electrophoresis (lanes 1 to 5) or electrotransferred onto nitrocellulose (lanes 6 to 9) followed by reaction with an HCMV-positive serum. Protein lysates of three recombinant cDNA clones lambda 1 (lanes 2 and 6), lambda 2 (lanes 3 and 7), lambda 3 (lanes 4 and 8) and a wild-type phage clone (lanes 5 and 9) are shown. Fusion proteins are marked 
, the position of the β-gal by <. *M*, markers (lane 1) as in legend of Fig. 1.

to the presence of antibodies specific for bacterial proteins, all sera were preincubated with induced total *E. coli* protein extract. An example of these experiments is shown in Fig. 3. The results proved that pp150 hybrid polypeptides representing different parts of the protein are recognized by HCMV-positive human sera.

Expression of polypeptides from pp150 with a high level expression vector

Screening of the lambda gt11 library with monospecific rabbit antisera showed that certain parts of the ORF of pp150 were preferentially present in the cDNA clones. It is possible that the missing sequences were not represented among the cDNA inserts of the recombinant clones or that corresponding fusion proteins were not reactive. For this reason the entire ORF was cloned piece by piece into the bacterial expression vector pBD2. While the lambda gt11 encodes the
total β-gal of 116K, this vector contains the DNA sequence for a truncated β-gal of only 375 amino acids (β-gal1–375), followed by a polylinker region which facilitates in-frame ligation of gene fragments to the amino terminus of β-gal. A number of our constructs, however, did not yield stable clones. A XhoI–PstI DNA fragment of the pp150 gene (Fig. 2) could be stably inserted into plasmid pBD2 cut with BamHI and PstI, giving rise to the clone XP1. *Escherichia coli* BMH7118 cells transformed with pXP1 directed highly efficient expression of the β-gal1–375–pp150555–705 (Jahn et al., 1987b) fusion protein upon induction of the lac promoter by IPTG. The recombinant clone expressed a hybrid protein of the expected size of about 63K (Fig. 4). The XP1 fusion protein was used to screen antibodies directed against pp150 in human sera, using Western blot assays. In order to reduce *E. coli*– and β-gal-specific antibodies, all sera were preincubated with total protein extracts from *E. coli* harbouring vector.
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Fig. 6. (a) Indirect immunofluorescence of HCMV-infected HFF 48 h post-infection using the rabbit IgG F(ab')2 preparation against the recombinant HCMV XP1 fusion protein. Bar marker represents 50 μm. (b) Immunohistochemistry with the rabbit antiserum raised against XP1. Kidney of a newborn with congenital HCMV infection; immunohistochemical detection of the viral protein in epithelial cells of renal tubules. Note the cytoplasmic distribution of the stained viral antigen. Bar marker represents 50 μm.

pBD2; this procedure proved to be sufficient to eliminate most of the non-specific reactions (Fig. 4, lane 5). As in the case of the lambda gt11 hybrid proteins, about 30 individual human anti-CMV sera were tested for their ability to recognize the various β-gal–pp150 fusion proteins. Western blots with *E. coli* extracts of IPTG-induced cultures as antigen were prepared and probed with human antisera. All the sera reacted intensely with the XP1 hybrid protein. Fig. 4 demonstrates the reaction of a human serum with this protein in a Western blot experiment. Depending on the microbial growth conditions, immunoreactive bands of smaller size were detected on the polyacrylamide gels; these are probably due to degradation of the XPI fusion protein with increasing growth time (Fig. 4).

Reactivity of the recombinant XP1 fusion protein in ELISA

The immunoblot results with the XP1 fusion protein demonstrated the usefulness of bacterially produced pp150 protein segments in this type of test. However, a more convenient assay for serodiagnosis would be preferable. Our purpose was to coat plastic microtitre plates with HCMV fusion protein and subsequently to test them in a standard ELISA procedure. Initial experiments demonstrated that the background level of *E. coli* antibodies in human sera could be reduced to very low values by preincubation with 50 μg of induced total *E. coli* extract without decreasing the sensitivity of the ELISA. The serum from an HCMV-negative control patient gave no reaction at all, indicating that the background binding was eliminated. Positive human sera varied according to their antibody titres as determined in standard ELISAs. The experiments clearly indicated a correlation between pp150 antibodies detected by the XP1 hybrid protein and those detected by the authentic polypeptide of whole virus preparations.
Detection of HCMV antigens in infected cell cultures and tissues

A high titre rabbit antiserum was raised against the fusion protein XP1 after enrichment as described in Methods. The rabbit was injected subcutaneously first with the XP1 fusion protein together with complete Freund's adjuvant. After monthly booster injections using Freund's incomplete adjuvant, the immune serum was tested for pp150 antibodies. Western blot experiments with purified virion proteins as antigens revealed a strong reaction with pp150 of the culture-adapted strain AD169 and with the viral pp150 of recent clinical isolates (Fig. 5). The reactivity of the XP1 antiserum with HCMV-infected cells was assayed. To avoid interaction of the antibodies with the HCMV-induced IgG Fc receptor in infected cells (Mackowiak & Marling-Cason, 1987), F(ab')2 fragments were prepared from the XP1 rabbit antiserum. HFF cells were infected with AD169 or recent clinical isolates and fixed with methanol at 14, 48 and 72 h post-infection. The monospecific antiserum identified a cytoplasmic antigen (Fig. 6a) which was present as early as 24 h post-infection. Only small amounts of fluorescence could be detected in the nucleus. The intensity of the fluorescence increased after 48 and 72 h, respectively.

In addition, this antiserum was used for detection of viral antigens in situ in paraffin-embedded tissue specimens using the PAP technique (Borisch et al., 1988). Similarly to the results of the immunofluorescence experiments, the viral antigen of pp150 was found to be localized predominantly within the cytoplasm (Fig. 6b).

DISCUSSION

Human cytomegalovirus consists of more than 25 structural proteins of which one was shown to be strongly reactive in a consistent manner in immunoblotting analyses. This paper describes the identification of essential antigenic regions, their prokaryotic expression, the immunoreactivity and the immunogenicity of the recombinant proteins. Initially, lambda gt11 β-gal–pp150 fusion proteins were used to investigate whether viral antigens produced in prokaryotic hosts are immunoreactive with HCMV-positive human sera. The expressed pp150 fragments are different in their computer-predicted antigenicity, varying between scarcely antigenic and highly antigenic. The computer program used (Wolf et al., 1988) superimposes the data for hydrophilicity (Hopp & Woods, 1981) upon secondary structure calculations (Chou & Fasman, 1974; Argos et al., 1978). However, it is not possible to predict the antigenic properties of pp150 fragments only on the basis of hydrophilicity and β-turn indices. The HCMV antibody-positive human sera so far tested recognized each of the different pp150 hybrid fragments, irrespective of their computer-predicted antigenicity values. As we intended to screen a large number of human sera for their reactivity with bacterially synthesized pp150 polypeptides, a gene fragment of pp150 was cloned into a high efficient expression vector. The recombinant vector directed the synthesis of a stable β-gal–pp150 hybrid protein. Roughly calculated, more than 20% of the induced total cell lysate corresponds to the pp150 recombinant protein. The shortened β-gal portion (β-gal–1–375) of the synthesized hybrid protein diminishes the possibility of partially hidden epitopes on the viral part of the fusion protein. In the same way, the number of β-gal epitopes is decreased, thus reducing the non-specific binding of human antibodies to fusion proteins. The recombinant protein is recognized specifically and reliably by all HCMV antibody-positive sera so far tested in Western blot experiments and ELISAs, independently of the clinical status of the individuals. The first results of a large scale efficacy study confirmed that the XP1 protein gives ELISA signals comparable to those obtained with commercial ELISAs, in which whole virus preparations are used as an antigen source. Although the antigenic potential of pp150 is undisputed, the question still remains as to whether pp150 alone, and a bacterially synthesized part of this polypeptide in particular, will meet all the necessary requirements for an antigen in a reliable standard diagnostic assay.

A partially purified preparation of XP1 hybrid protein was used to immunize rabbits. The high titre antiserum we obtained reacts specifically with pp150 of HCMV. This again proves that pp150 is a potential antigen in vivo. Preliminary experiments indicate the value of this antiserum. We used the XP1 antiserum for immunofluorescence and immunohistochemistry techniques, which clearly showed the usefulness of this monospecific antiserum to identify
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virus-specific antigens in situ. HCMV-infected cells were detected in tissue culture as well as in organs.

A serum produced in this way offers some advantages. In contrast to polyspecific sera directed against a multitude of polypeptides or whole particles, it reacts with antigenic sites of one well-characterized structural protein. This particular protein has no known counterpart in other herpesviruses. Other structural proteins such as the major capsid protein or glycoprotein B share homologous sequences and may result in cross-reactivity (Balachandran et al., 1987; Davison & Scott, 1986; Kouzarides et al., 1987; Snowden & Halliburton, 1985). Monoclonal antibodies on the other hand have the disadvantage of reacting with one epitope exclusively, which makes missing the target polypeptide more likely, especially in immunohistochemistry. The pp150 antiserum has been useful in detecting HCMV antigens in cell cultures, infected organs and infected tissues. The antiserum against a recombinant polypeptide of pp150 will be useful as a probe for investigation of the kinetics of HCMV infection and virus assembly.

We thank Bernhard Fleckenstein for help and support and Bodo Plachter for critical reading of the manuscript. We thank Michael Mach for providing the HCMV lambda gt11 library and we thank Hans Helmut Niller for help in sequencing. The technical assistance of Karin Wehrberger is gratefully appreciated. The work was supported by Deutsche Forschungsgemeinschaft.

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(Received 18 December 1987)