Expression of the human cytomegalovirus 65K tegument phosphoprotein in insect cells by baculovirus vectors


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The gene encoding the 65K tegument phosphoprotein (pp65) of human cytomegalovirus (HCMV) was cloned into pAc373 to construct a recombinant baculovirus (Acpp65-3) expressing pp65 in insect Sf9 cells. A baculovirus that carried a fragment of the gene, corresponding to the first 442 amino acids of pp65, was also developed, using vector pVL941 (Acpp65-2). Recombinant proteins migrating in SDS-polyacrylamide gels with an M_r of either 65K (Acpp65-3) or 56K (Acpp65-2) were detected in cytoplasmic and nuclear extracts of infected Sf9 cells. The 56K and 65K proteins were recognized in immunoblots by monoclonal antibodies (MAbs) 28-77 and 28-19, which are specific for pp65. The insect cell-expressed antigens were also analysed on Western blots using MAbs 4D11, 7D2, 8E3, 7B4 and 8E10, which recognize the HCMV antigen GP66 in immunoblots. The truncated pp65 antigen of Acpp65-2 was reactive with MAbs 4D11, 7D2, 8E3 and 7B4 mapped in the region of pp65, comprising amino acids 1 to 442, and also that GP66 and pp65 represent the same HCMV antigen. Immunoblot analysis of human sera from individuals seropositive for HCMV showed that the recombinant pp65 products were as antigenic as the native 65K phosphoprotein produced in HCMV-infected human embryonic fibroblasts.

Human cytomegalovirus (HCMV) causes serious disease in immunocompromised individuals and in patients receiving immunosuppressive therapy. The virus is also one of the most common known causes of congenital mental retardation, blindness and deafness. The HCMV particle contains at least 33 structural proteins, several of which are glycosylated or phosphorylated (Kim et al., 1976). The most abundant of these virus-encoded proteins is the 65K tegument phosphoprotein (pp65) (Nowak et al., 1984; Ruger et al., 1987), which probably corresponds to the major viral polypeptide GP66 described by Kim et al. (1983). This phosphoprotein has also been designated the lower matrix protein (Gibson, 1983), pp64 (Gibson; 1983; Clark et al., 1984; Forman et al., 1985; Pande et al., 1984) and ICP27 (Geballe et al., 1986). Numerous reports have described pp65 as one of the HCMV proteins most frequently recognized on Western blots by human sera from individuals seropositive for the virus (Landini et al., 1985; Jahn et al., 1987; Klages et al., 1989; Landini & LaPlaca, 1991).

In an effort to elucidate the role of pp65 in HCMV infections and to construct biological reagents for the detection of antibodies against this antigen, we expressed the phosphoprotein in Spodoptera frugiperda Sf9 cells by using baculovirus vectors. The nucleotide sequence of the pp65 gene of HCMV strain AD-169 (Ruger et al., 1987) was used to design oligonucleotide primers for PCR. The pair of primers B66-S1 (5' ATGCCTGGATCCATGGAGTCGCGCGGTCGCCGTTGT 3') and 66-A4 (5' GCCGAGGATGCTGATTTGCGTTTG 3') was designed to amplify a 1328 bp DNA fragment carrying the portion of the gene encoding the first 442 amino acids (1326 bp). Primers B66-S1 and B66-A0 (5' CCCGGGATCCATAGAGTCGTCCTAAGCGCGT 3') were used to amplify a 1719 bp DNA fragment containing the entire pp65 gene (1686 bp). PCR was done in 100 μl final volume containing 50 ng of HCMV (Towne) DNA, using the standard protocol of Saiki (1989). The 1328 bp and 1719 bp DNA fragments were cloned into the BamHI site of baculovirus transfer vectors pVL941 and pAc373, respectively (Summers & Smith, 1987; Luckow & Summers, 1989). The resulting plasmids were used to develop baculoviruses Acpp65-2 (1328 bp pp65 insert) and Acpp65-3 (1719 bp pp65 insert) according to the method of Summers & Smith (1987). The recombinant baculoviruses were then plaque-purified four times and used to propagate viral stocks.

To determine whether cells infected with the recombinant viruses produced the appropriate proteins and to study the cellular location of the baculovirus-expressed (bpp65) antigens, Sf9 cells at 41 h post-infection were labelled with 25 μCi/ml Tran 35S-label (ICN) for 4 h.
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3. Immunoreactivity of recombinant proteins produced by Acpp65-2 (a) and Acpp65-3 (b) with the MAbs 4D11 (lanes 1), 7D2 (lanes 2), 8E3 (lanes 3), 7B4 (lanes 4) and 8E10 (lanes 5), which are known to react with GP66. As a control, the recombinant proteins were reacted with MAbs 7E2 (lanes 6), 8E9 (lanes 7) and 1B8 (lanes 8), which do not recognize native HCMV antigens in immunoblots. Lanes 9 show a nitrocellulose strip reacted with normal mouse serum instead of the primary antibody.

Fig. 3. Immunoreactivity of recombinant proteins produced by Acpp65-2 (a) and Acpp65-3 (b) with the MAbs 4D11 (lanes 1), 7D2 (lanes 2), 8E3 (lanes 3), 7B4 (lanes 4) and 8E10 (lanes 5), which are known to react with GP66. As a control, the recombinant proteins were reacted with MAbs 7E2 (lanes 6), 8E9 (lanes 7) and 1B8 (lanes 8), which do not recognize native HCMV antigens in immunoblots. Lanes 9 show a nitrocellulose strip reacted with normal mouse serum instead of the primary antibody.

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Fig. 1. Expression of pp65 products in insect cells. Infected Sf9 cells were labelled with Tras sulphhydrate-label and used to prepare cytoplasmic (lanes 1 to 4) and nuclear (lanes 5 to 8) extracts. Samples (5 x 10^6 cells) were run in gradient SDS-polyacrylamide gels, which were then dried and exposed to X-ray film. Lanes 1 and 5, uninfected cells; lanes 2 and 6, wild-type AcMNPV-infected cells; lanes 3 and 7, Acpp65-2-infected cells; lanes 4 and 8, Acpp65-3-infected cells.

Fig. 2. Immunoblot analysis of the recombinant proteins with MAbs 28-77 (a) and 28-19 (b), against pp65. Lanes 1, AcMNPV-infected cells; lanes 2, Acpp65-2-infected cells; lanes 3, Acpp65-3-infected cells.

(Summers & Smith, 1987) and used to prepare cytoplasmic and nuclear extracts according to the method of Miyamoto et al. (1985). The labelled proteins were then resolved by electrophoresis in 5 to 20% gradient SDS-polyacrylamide slab gels and detected by autoradiography (Kim et al., 1983). Cytoplasmic and nuclear extracts of cells infected with Acpp65-2 (Fig. 1 lanes 3 and 7) and Acpp65-3 (Fig. 1 lanes 4 and 8) each showed a novel band migrating with the expected M_r of 56K and 65K, respectively. These proteins were not found in cells infected with wild-type AcMNPV or in uninfected Sf9 cells (Fig. 1). A signal for the abundant baculovirus polyhedrin was not detected in the extracts of cells infected with wild-type Autographa californica nuclear polyhedrosis virus (AcMNPV), because polyhedrin is insoluble in the non-alkaline buffers used to prepare the cytoplasmic and nuclear fractions (Miyamoto et al., 1985; Summers & Smith, 1987). Bands representing various abundant baculovirus proteins were detected in both the cytoplasmic and nuclear fractions of cells infected with AcMNPV, Acpp65-2 or Acpp65-3 (Fig. 1). The presence of the recombinant proteins in the nuclear extracts indicated that insect cells are able to direct exogenous nuclear protein to the nucleus (Miyamoto et al., 1985; Kos et al., 1991).

The 56K and 65K recombinant products were recognized in immunoblots by monoclonal antibodies (MAbs) 28-77 (Grefte et al., 1992) and 28-19, which are specific for pp65 and were a generous gift from Dr W. Britt, Birmingham, Ala., U.S.A. (Fig. 2). The insect-expressed HCMV antigens were further characterized by Western blot analysis by using MAbs 4D11, 7D2, 8E3, 7B4 and 8E10, which have been shown to react in immunoblots with epitopes of GP66 (Kim et al., 1983). The truncated protein produced by Acpp65-2 (bpp65-2) was reactive with the antibodies 4D11, 7D2, 7B4 and 8E10 (Fig. 3). The antigen expressed by a similar recombinant baculovirus (Acpp65-1) carrying the DNA fragment encoding the first 442 amino acids of pp65 was recognized in
immunoblots by MAbs 4D11, 7D2 and 8E10 (data not shown). The complete pp65 protein expressed by Acpp65-3 (bpp65-3) was immunoreactive with MAb 4D11 only (Fig. 3). The lack of reactivity of bpp65-3 with MAbs 7B4, 8E10 and 7D2 suggested that the structure of this protein is different from that of native pp65 and that the epitopes somehow were not available to the antibodies. Neither bpp65-2 nor bpp65-3 reacted with MAb 8E3 (Fig. 3 lanes 3), which was reported by Kim et al. (1983) to react with a nuclear HCMV antigen migrating in SDS–polyacrylamide gels with an $M_r$ of 66K. This result might imply that MAb 8E3 recognizes the phosphorylated tegument protein pp67 (Davis et al., 1984; Davis & Huang, 1985), which is known to comigrate with pp65 on SDS–PAGE. Alternatively, the antibody could be specific for an epitope of pp65 that was not conserved in the two bpp65 products. As expected, no reactions were observed in the control experiments with MAbs 7E2, 8E9 and 1B8, which do not recognize HCMV antigens on Western blots (Kim et al., 1983). MAb 4D11 was also immunoreactive by immunofluorescence with both recombinant antigens (Kim et al., 1983; data not shown).

To determine whether the recombinant proteins behave antigenically like the phosphoprotein made in human cells, we used bpp65-2 and bpp65-3 in immunoblot experiments with a panel of sera from individuals seropositive for HCMV. The sera of 11 seropositive and two seronegative individuals (determined by the Cytomegalia II test; Bio-Whittaker Bioproducts) were tested against native pp65 produced by MRC-5 cells infected with HCMV (Towne) and against each of the two baculovirus-expressed antigens. Six of the 11 positive sera reacted with native pp65 and the recombinants bpp65-2 and bpp65-3 (Fig. 4 lanes 3, 4, 8, 10, 11 and 12). In addition, weak positive immunoreactions were observed with two other human sera, which produced barely visible bands with Acpp65-2 (Fig. 4 lanes 1 and 2) and with Acpp65-3 (Fig. 4 lane 2) blots. The same sera produced either equivocal or negative results in the immunoblot with native pp65 antigen (Fig. 4c lanes 1 and 2). An equivocal result was obtained in the blot shown in Fig. 4(b lane 1); only a product with an apparent $M_r$ lower than that of bpp65-3 was found to be immunostained. No immunostaining was detected with three HCMV-positive sera (Fig. 4a, b and c lanes 5, 6 and 9) and the two seronegative controls (Fig. 4a, b and c lanes 7 and 13).

We have shown that the recombinant pp65 products expressed in insect cells infected with baculovirus Acpp65-2 or Acpp65-3 were as antigenic as the native 65K phosphoprotein produced in HCMV-infected human embryonic fibroblasts. Our results with native and recombinant pp65 confirm other studies in which the humoral immune response against pp65 was found to be highly variable and sometimes absent (Landini et al., 1985; Jahn et al., 1987; Klages et al., 1989; Plachter et al., 1990; Pande et al., 1991). Using the insect-expressed antigens, we were able to prove that the epitopes recognized by MAbs 4D11, 7D2, 8E10 and 7B4 mapped in the region of pp65, comprising amino acids 1 to 442, and that the phosphoprotein GP66 (Kim et al., 1983) and the 65K phosphoprotein are the same. This finding is of particular relevance because our laboratory has previously shown by using immunofluorescence and double-antibody sandwich ELISA that the MAbs above can detect GP66 in HCMV-infected human cells as early as 2 h post-infection (Kim et al., 1983). When used in

**Fig. 4.** Immunoblot demonstration of the reactivity of the pp65 antigens expressed by Acpp65-2 (a) and Acpp65-3 (b) with 11 human sera from individuals seropositive for HCMV (lanes 1 to 6 and 8 to 12 in a and b) and two sera from HCMV-seronegative individuals (lanes 7 and 13). The MRC-5+ HCMV(Towne) panel (c) shows the reactivity of HCMV antigens produced by MRC-5 cells infected with the Towne strain of HCMV with the same sera. In each panel, the blot in lane 14 was reacted with MAb 4D11. The secondary antibody was either goat horseradish peroxidase-conjugated anti-human IgG or anti-mouse IgG.
epidemiological studies, the bpp65 products could be helpful in determining whether the presence of antibodies against pp65 correlates with a particular stage of HCMV infection.

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References


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