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Detection of Cytomegalovirus Antibodies by an Enzyme-Linked Immunosorbent Assay Using Recombinant Polypeptides of the Large Phosphorylated Tegument Protein pp150

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Parts of the large phosphorylated tegument protein, pp150, of human cytomegalovirus (HCMV) were expressed in bacteria. The resulting fusion proteins were tested in a Western blot (immunoblot) assay for reactivity with a monoclonal antibody against pp150, with a polyspecific rabbit antiserum, and with human reconvalescent-phase sera. Those fusion proteins that performed well in the Western blot assay were used as antigens in enzyme-linked immunosorbent assays (ELISAs) for the detection of antibodies against HCMV. Five different recombinant β-galactosidase fusion proteins were evaluated by ELISA using 62 seropositive and 38 seronegative human serum samples. Of all the proteins tested, one peptide representing 162 amino acids of pp150 was superior to the others with regard to sensitivity and specificity. All sera known to be positive for antibodies against HCMV were identified by combining the results of the ELISAs with the different pp150 fusion proteins. Therefore, it appears that peptides from a single protein of HCMV might be sufficient to identify HCMV-seropositive individuals by recombinant ELISA.

Human cytomegalovirus (HCMV) has become one of the major pathogenic agents in immunosuppressed patients (5). Furthermore, it causes severe sequelae in newborns after primary infection of their mothers during pregnancy (1). As clinical symptoms of acute HCMV infection may be nonspecific or totally absent, reliable laboratory diagnosis is required. Virus isolation still is one of the most dependable methods for the diagnosis of acute infection. A number of recent studies focused on the development of new techniques for the detection of viral antigens or nucleic acids in patient material or in cell culture (2, 6, 17, 22). These techniques, in conjunction with virus isolation, have improved the laboratory diagnosis of acute HCMV disease considerably. However, isolation of HCMV may be time-consuming because of the slow replication of the virus in cell culture.

Rapid and reliable detection of HCMV-specific antibodies in patient sera is also important in certain settings, such as blood bank screening and testing of organ donors and recipients before transplantation. Serological testing is also an important diagnostic means of establishing the status of infection in women before or during pregnancy.

Currently available systems for HCMV serological testing are supplied with poorly defined antigen material from infected-cell culture, which usually is only partially purified. However, only a limited number of viral polypeptides have been shown to be reactive with patient sera in immunoblots and radioimmune precipitations (14, 15, 18, 23). Furthermore, a number of HCMV proteins show considerable sequence conservation in comparison with the homologous proteins of other herpesviruses (4). Therefore, the sensitivity and specificity of test systems such as enzyme-linked immunosorbent assays (ELISAs) can be substantially improved by using as antigens only those proteins that appear to be major targets of the humoral immune response and do not share conserved epitopes with other herpesvirus proteins. This is especially important with a complex virus such as HCMV.

One viral protein, the large phosphorylated tegument protein, pp150, has been shown to be most reliably detected by sera known to be antibody positive for HCMV (9, 12, 20). No sequence homology between this protein and the proteins of Epstein-Barr virus, varicella-zoster virus, and herpes simplex virus has been found.

This article describes the generation of a number of recombinant *Escherichia coli* fusion proteins from HCMV pp150 and the investigation of their immunoreactivities in immunoblots. The polypeptides found to be most reliably reactive in these assays were selected for purification. The purified material was used as an antigen for the generation of recombinant ELISAs for HCMV antibody detection. In an initial set of experiments, 100 human serum specimens were used to evaluate sensitivity and specificity.

MATERIALS AND METHODS

Expression vectors and expression cloning. Screening of an HCMV lambda gt11 cDNA library was done as previously described (8). Inserts of the recombinant lambda clones were excised with *Eco*RI and recloned in the bacterial expression vector pBD2 or pSEM (3, 11). The recombinant plasmids were partially sequenced. The data were compared with the nucleotide sequence published previously (8). Some clones were obtained by direct cloning from genomic DNA of the pp150 reading frame.

Antigen purification. Bacterial cells containing fusion proteins were washed once with phosphate-buffered saline (PBS), incubated with lysozyme (100 mg/ml) in 50 mM Tris-HCl–50 mM EDTA (pH 8.0), and then disrupted by sonication. Intact cells were removed by low-speed centrifugation (1,500 × *g*, 10 min, 4°C). The supernatant fraction was sedimented by ultracentrifugation (110,000 × *g*, 45 min, 4°C), and the resulting pellet was solubilized in 6 M guanidine

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hydrochloride overnight at 4°C. After ultracentrifugation (130,000 × *g*, 45 min, 4°C), the supernatant was purified by gel filtration through a Sephacryl S-200 column (5 by 100 cm) using buffer A (6 M guanidine hydrochloride, 10 mM β-mercaptoethanol, 10 mM Tris-HCl [pH 7.5]). Fractions containing the purified fusion protein were collected. To change the buffer conditions to buffer B (5 M urea, 10 mM β-mercaptoethanol, 10 mM Tris-HCl [pH 7.5]), gel filtration on Sephadex G-10 was performed. The fusion protein was further purified by ion-exchange chromatography (DEAE–Sepharose CL-6B) using a linear NaCl gradient (0 to 0.6 M) in buffer B. Fusion protein-containing fractions were collected and analyzed further by gel electrophoresis and ELISA.

Antibodies and antisera. For the primary screen of the lambda gt11 library, a monospecific rabbit antiserum was used; this serum was generated against the viral band of 150 kDa that was cut out of polyacrylamide gels. A polyspecific rabbit antiserum was generated by injecting preparations of extracellular HCMV particles from cell culture. The immunization of New Zealand White rabbits was done as described previously (9). The generation of a monoclonal antibody against one of the fusion proteins (XP1) was performed as described elsewhere (7).

Protein gel electrophoresis and immunoblotting. Gel electrophoresis and immunoblotting were done as described elsewhere (19).

ELISA. ELISA plates were coated with 0.1 to 0.2 μg of fusion protein per well. After adsorption overnight at room temperature, the reaction wells were rinsed twice with 50 mM Tris–50 mM EDTA buffer, pH 7.5, and incubated with serum samples at a dilution of 1:50 for 1 h. After two washes with PBS buffer, peroxidase-conjugated anti-human immunoglobulin G (IgG) antibodies were added in a dilution which had previously been established to be appropriate. Incubation was continued for one additional hour. After this further incubation, the substrate, tetramethylbenzidine, was added, and incubation was continued for 30 min at room temperature. The reaction was stopped with 0.5 N H_2SO_4. The optical density was determined at 430 nm and 548 nm. Dilutions of samples and conjugate were performed with a commercially available buffer (Enzygnost-Anti-CMV-IgG-POD; Behringwerke, Marburg, Germany).

RESULTS

Expression cloning of pp150. Parts of the DNA coding for the large basic phosphoprotein, pp150, of HCMV were cloned into procaryotic expression vectors. These vectors, pBD2 and pSEM, express heterologous peptides as parts of a fusion protein containing 375 amino acids from the bacterial β-galactosidase. The recombinant clones and their respective locations relative to the pp150 reading frame are shown in Fig. 1.

Expression clones pSeml1, pSeml2, and pSeml3 were generated by recloning the inserts from recombinant lambda phages into vector pSEM. These lambda clones were isolated by screening an HCMV lambda gt11 cDNA library with a polyclonal rabbit antiserum. This antiserum was raised against a band of 150 kDa that was cut out of a preparative polyacrylamide gel containing purified HCMV virions. By this procedure, those parts of pp150 that were immunogenic in animals were selected, thus providing some preselection of putatively highly reactive epitopes for assays with human sera.

The viral DNA inserts were excised from lambda gt11 by cleavage with *Eco*RI. The resulting fragments were ligated into the multiple-cloning site of pSEM. This site is located 3′ to the amino-terminal portion (amino acids 1 to 375) of the bacterial β-galactosidase. The viral DNA insert of clone pSeml1 contains nucleotides 1395 to 2533 of the HCMV pp150 gene (numbering according to reference 8). The viral insert of clone pSeml2 contains nucleotides 2167 to 2460. The viral insert of clone pSeml3 contains nucleotides 3209 to 3459.

As the expression of fusion protein from clone pSeml1 was low and proteolytic degradation in very early stages of fermentation and purification resulted in a low yield of recombinant protein, clones pSeml1V1 and pSeml1V2 were made by deleting the carboxy-terminal part of pSeml1. pSeml1V1 was generated by cutting pSeml1 with *Pst*I (one *Pst*I site from the polylinker of pSEM) and religating the ends. The resulting insert contains the viral sequence from nucleotides 1395 to 1734. pSeml1V2 was generated by cutting pSeml1 with *Sma*I and *Hin*dIII (the *Hin*dIII site from the polylinker of pSEM) and religating the ends. The resulting insert contains the viral sequence from nucleotides 1395 to 2037.

Constructs pSmaI and pXP1 were made by subcloning parts out of the *Eco*RI Y fragment of HCMV AD169. pSmaI was created by isolating a *Sma*I fragment and cloning it into the multiple-cloning site of pSEM. The insert contains the viral sequence from nucleotides 2038 to 2423. pXP1 was made by cutting with *Xho*II and *Pst*I and cloning the fragment into expression vector pBD2. The resulting viral insert contains the sequence from bases 2155 to 2641. Clones pSmaI and pXP1 cover the region of the HCMV genome that was deleted by shortening pSeml1 plus some additional portions of the sequence 3′ to that region (Fig. 1).

The fusion proteins were induced with IPTG (isopropyl-β-D-thiogalactopyranoside) and separated on a sodium dodecyl sulfate-polyacrylamide gel (Fig. 2).

Reactivity of recombinant proteins in immunoblots. From previous studies it was known that the fusion protein made from pXP1 was highly immunoreactive (21). A monoclonal antibody generated against this polypeptide performed well in detecting viral antigen in infected cells from cell culture as well as in paraffin-embedded tissue sections from infected organs (7). Figure 3A shows an immunoblot with this antibody and antigen from pXP1 as well as from a preparation of purified virions.

However, it was not clear whether this small antigen would be sufficient to replace the viral pp150 in serological assays. The latter protein had previously been shown to be the major antigen recognized by human reconvalescent-phase sera (9). In order to determine whether the natural antibody response would be directed predominantly against the XP1 fusion protein or whether other parts of pp150 would also be targets, polyclonal antisera against extracellular virus particles made in rabbits were tested. These sera were used in immunoblots with the recombinant fusion proteins and with a virion preparation. As shown in Fig. 3B, there were marked differences in the reactivities of individual fusion proteins with the polyspecific antisera. In this case, the proteins of clones pSmaI and pXP1 reacted well with the polyspecific antiserum (Fig. 3B, lanes 2 and 5). In virion preparations, the prominant immunoreactive bands represent the phosphorylated tegument proteins pp65 and pp150 (lane 7).

In the next set of experiments, we tested the reactivities of the recombinants with human sera in the immunoblot. In general, the fusion proteins of clones pSeml3 and pXP1

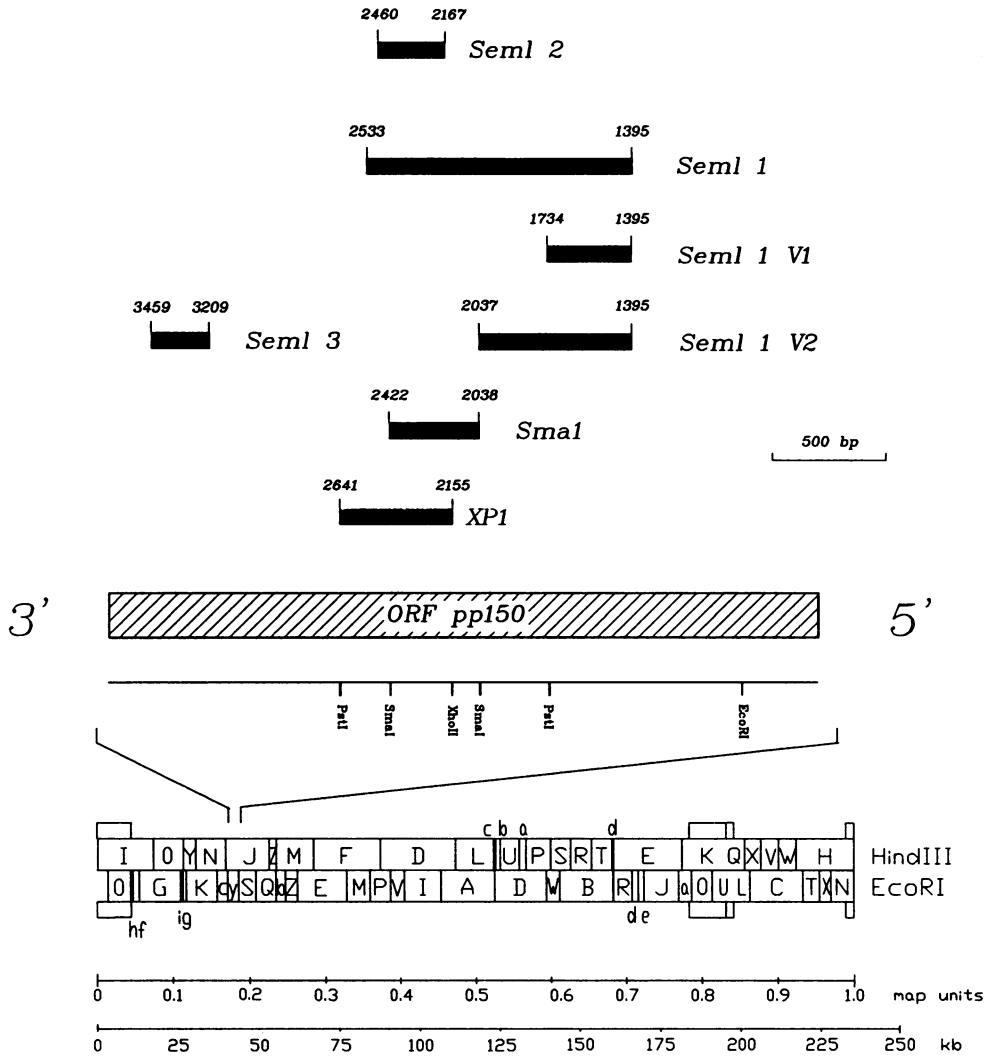

FIG. 1. Schematic representation of the genome of HCMV strain AD169 indicating the relative locations of the DNA fragments from the pp150 reading frame used for expression cloning. Restriction sites for the endonucleases *Eco*RI and *Hin*dIII are shown in the lower part of the figure. On the expanded scale, the restriction sites used for cloning are indicated. The hatched box gives the location of the open reading frame (ORF) coding for pp150. The black bars represent the viral inserts of the expression clones. The numbering above the bars gives the range of nucleotides contained in the clones (nucleotide numbers from the previously published sequence [8]).

reacted most consistently with all sera tested. One example is shown in Fig. 3C. With this HCMV antibody-positive serum, the fusion proteins from expression clones pSeml1V2 and pSmaI also showed clear reactivity.

The fusion proteins that showed the best performance in these pretests with the immunoblot system were selected for further purification and used as antigens for antibody detection in the ELISA system.

Reactivity of recombinant proteins in ELISAs. In order to test the applicability of the fusion proteins as antigens for a recombinant ELISA, a panel of 100 human serum specimens from blood donors was used. The sera were pretested with a commercially available ELISA (Enzygnost-Anti-CMV-IgG-POD). According to this test, which is based on cell culture-derived antigen, 62 of these serum samples were positive for IgG antibodies against HCMV and 38 were negative for HCMV antibodies.

The purified recombinant antigens were subjected to a very restrictive quality control procedure to exclude nonspecific reactivity caused by copurified contaminants. In separate ELISAs, the fusion proteins from recombinants pXP1, pSeml3, pSmaI, pSeml1V1, and pSEML1V2 were used for the detection of HCMV antibodies. The results were compared with the results obtained with the commercially available ELISA. In the cases with conflicting results, Western blot (immunoblot) analyses were performed. According to the results of Western blots carried out with infected-cell antigens, extracellular particles, and recombinant antigens, the results of ELISAs using particular recombinant antigens were considered false positive or false negative (Table 1). The blot results with this panel of sera agreed with the results seen in the commercially available ELISA. Sensitivity and specificity were calculated as described by Zweig and Robertson (25).

According to the criteria used, the fusion protein from clone pXP1 performed best in the ELISA. This protein contains only 162 of the 1,048 amino acids of the native protein. In contrast to the other fusion proteins tested, each

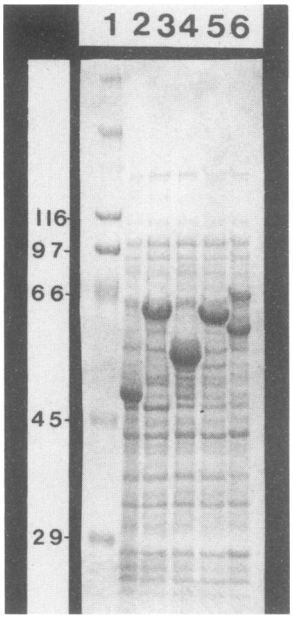

FIG. 2. Polyacrylamide gel analysis of lysates from bacterial clones containing the recombinant proteins. Lane 1, molecular weight markers (given in thousands at left); lane 2, lysate containing 375 amino acids of β-galactosidase expressed from the cloning vector pSEM; lane 3, lysate from *E. coli* containing fusion protein from expression clone pXP1; lane 4, lysate from *E. coli* containing fusion protein from expression clone pSeml3; lane 5, lysate from *E. coli* containing fusion protein from expression clone pSmaI; lane 6, lysate from *E. coli* containing fusion protein from expression clone pSeml1V2. Staining was done with Coomassie brilliant blue.

HCMV-positive serum of the panel was detected with the XP1 protein. However, the signal levels found could not always be correlated to the signal levels seen with the conventional ELISA based on cell culture-derived viral antigen.

In addition, the XP1 antigen has also been analyzed with a panel of 35 IgM-positive sera, preabsorbed for rheumatoid factor (24). Both rheumatoid factor-treated and untreated sera showed that XP1 is also very suitable as an antigen for IgM detection (data not shown).

The overall reactivity of the fusion protein from clone pSeml3 can be compared to that of XP1, although the test specificity with this protein was definitely lower. Similar results were seen by using the fusion protein from pSmaI as the coating antigen. This clone overlaps about 60% with pXP1, thus indicating again the presence of an immunodominant epitope for the elicitation of a humoral immune response residing in this region of pp150.

Of the two remaining fusion proteins, only the one made from clone pSeml1V2 performed well with the human sera tested in the ELISA. However, the test using this antigen showed lower specificity than those using XP1, SmaI, and Seml3.

DISCUSSION

A number of studies using immunoblot analyses have demonstrated that only a few virion proteins of HCMV are immunoreactive with human sera (9, 12–15). Recent publications have focused on the reactivity of human sera with recombinant peptides from HCMV proteins in immunoblots (12, 13, 16, 20, 21). The results of these studies have shown that it is possible to identify human sera containing antibodies against HCMV by using recombinant peptides from only a few viral proteins. However, for routine testing, the immunoblot technique is not the method of first choice. The most commonly used test for HCMV serodiagnostics is the ELISA. The hitherto commercially available test kits are supplied either with extracellular virus particles from cell culture or with infected-cell antigens as coating antigens. These antigens are poorly defined and contain cellular proteins. This may reduce the sensitivity and specificity of these test systems. In addition, overrepresentation of certain virion constituents such as the dense-body protein pp65, which accumulates in cell culture under certain conditions (10), may also influence the sensitivity and specificity of the ELISA. Furthermore, the presence of proteins with significant homology to other herpesvirus proteins such as the major capsid protein may lead to cross-reactions in ELISAs. Finally, the use of poorly defined viral antigens from cell culture might result in substantial variations in antigen quality from one lot to another.

In this article, we describe for the first time the application of recombinant proteins from HCMV as coating antigens in recombinant ELISAs. Parts of the basic phosphoprotein pp150 were expressed in a high-level bacterial expression vector. This viral protein has been shown to be consistently detected by HCMV-positive human sera on Western blots (9). Recombinant proteins from pp150 were reactive in a similar way in immunoblots (12, 20, 21). When these proteins were used separately in ELISAs, it appeared that only one to three of these peptides might be needed as coating antigens to detect HCMV antibodies in all human sera found positive by a commercially available ELISA.

Undoubtedly, the best antigen of all of the recombinant pp150 clones tested for HCMV antibody detection is the XP1 protein. Although it only represents about 15% of the total pp150 protein sequence, this polypeptide exhibits the potential to detect antibodies in all HCMV-positive human sera so far tested. However, the signal levels determined with this recombinant protein in the ELISA did not always correlate with those found by the conventional ELISA. This reflects the individual concentrations of XP1-monospecific antibodies in the serum samples. For this first analysis of the feasibility of a recombinant ELISA for HCMV antibody detection, a panel of sera from healthy blood donors was used. If serum samples from particular clinical settings were used in a further evaluation of the recombinant test, the XP1 protein might not be sufficient to retrieve all samples positive for HCMV antibodies. It is presumed that one or two other peptides from pp150 will be necessary in a combined assay to reach a higher sensitivity than is currently achieved with commercially available ELISAs.

The relatively low specificity seen with some of the constructs cannot be ascribed to antibodies to *E. coli* proteins in the sera tested. Preabsorption of such sera with the recombinant fragment of β-galactosidase as well as with whole-cell lysate from the *E. coli* host strain did not result in a reduction of the signal in the ELISA. In addition, various amounts of contaminating *E. coli* proteins in the respective antigen preparations did not result in a change of the signal. Further elaboration of the assay design for particular recombinant antigens will therefore be necessary.

On the basis of the results of this study, it is not possible to say whether proteins other than pp150 encoded by HCMV might help in a recombinant test to detect specific antibodies in the very early phase of an acute HCMV infection. The

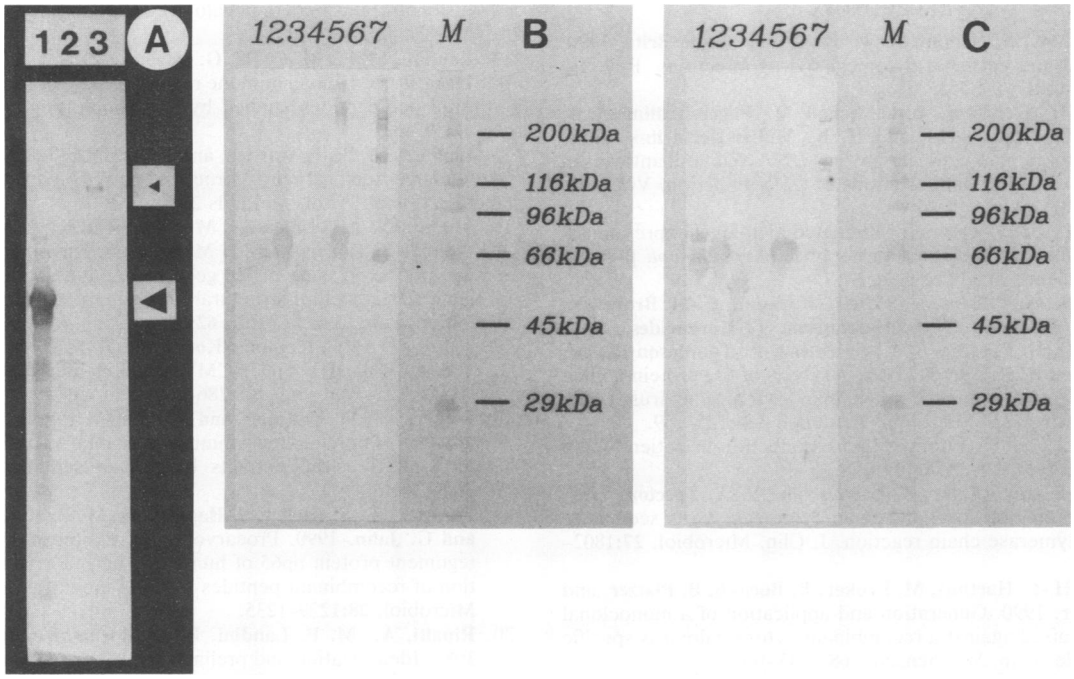

FIG. 3. (A) Immunoblot analysis of extracellular HCMV virion particles and recombinant fusion protein XP1 with a monoclonal antibody generated against XP1. Lane 1, *E. coli* lysate harboring fusion protein from clone pXP1; lane 2, *E. coli* lysate harboring fusion protein from cloning vector pSEM (negative control); lane 3, purified virions of HCMV from cell culture supernatants. The small arrowhead indicates the location of the viral pp150 band; the large arrowhead indicates the position of fusion protein XP1. (B) Immunoblot analysis of extracellular HCMV virion particles and the recombinant fusion proteins with a polyclonal rabbit antiserum generated against extracellular HCMV particles from cell culture supernatant. Lane 1, *E. coli* lysate harboring fusion protein from clone pSeml1V2; lane 2, *E. coli* lysate harboring fusion protein from clone pSmaI; lane 3, *E. coli* lysate harboring fusion protein from clone pSeml3; lane 4, *E. coli* lysate harboring protein from expression vector pSEM (negative control); lane 5, *E. coli* lysate harboring fusion protein from clone pXP1; lane 6, *E. coli* lysate harboring protein from cloning vector pBD2 (negative control); lane 7, purified virions of HCMV from cell culture supernatants; lane M, molecular mass markers. Numbers on the right indicate molecular masses. (C) Immunoblot analysis of a human serum specimen containing IgG and IgM antibodies against HCMV. Lane 1, *E. coli* lysate harboring fusion protein from clone pSeml1V2; lane 2, *E. coli* lysate harboring fusion protein from clone pSmaI; lane 3, *E. coli* lysate harboring fusion protein from clone pSeml3; lane 4, *E. coli* lysate harboring protein from expression vector pSEM (negative control); lane 5, *E. coli* lysate harboring fusion protein from clone pXP1; lane 6, *E. coli* lysate harboring protein from cloning vector pBD2 (negative control); lane 7, purified virions of HCMV from cell culture supernatants; lane M, molecular mass markers. Numbers on the right indicate molecular masses.

DNA binding protein p52 has been reported to be a major target of the humoral immune response in the early phase of active infection (20). Studies are under way to investigate the seroreactivity of particular immediate-early or early antigens of HCMV with patient sera.

TABLE 1. Reactivities of pp150-derived recombinant proteins in ELISAs[a]

Protein	No. of samples testing:				Sensitivity (%)	Specificity (%)
	Positive	Negative	False positive	False negative		
XP1	62	37	1	0	100	97
Seml3	61	26	12	1	98	63
SmaI	61	27	11	1	98	71
Seml1V1	28	30	8	34	45	79
Seml1V2	62	20	18	0	100	50

[a] ELISAs were performed as described in Materials and Methods. Individual cutoffs for the recombinant proteins were calculated as the mean value for all serum specimens defined as negative with the respective protein as the antigen plus three times the standard deviation. Number of HCMV antibody-positive serum specimens tested = 62; number of HCMV antibody-negative serum specimens tested = 38.

In conclusion, we have shown here that recombinant proteins from the large phosphorylated tegument protein, pp150, of HCMV can be used for the generation of a recombinant ELISA for antibodies against HCMV. By using only single recombinant polypeptides representing small portions of the viral protein in separate ELISAs and combining the results, the sensitivity of a conventional ELISA supplied with cell culture-derived antigen can be achieved. The specificity of the test system will have to be improved by optimization of the test design. More detailed analysis using a combination of the recombinant antigens from pp150 in ELISAs and evaluation of the results with other antibody test systems will show whether recombinants from the large tegument protein alone or in combination with peptides from other HCMV proteins will be necessary to design more-sensitive and more-specific ELISA systems for HCMV serological testing.

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