Identification of an 85 kDa phosphoprotein as an immunodominant protein specific for human herpesvirus 7-infected cells

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The reactivity of human cord blood sera was directed most frequently in Western blot assays to a protein with an apparent molecular mass of 85 kDa that belongs to the p85 complex, a family of antigenically related proteins identified previously in our laboratory with the aid of two MAbs. We show that the 85 kDa protein is phosphorylated. As antibodies present in the human sera were directed in part to proteins carrying cross-reactive epitopes between human herpesvirus 6 (HHV-6) and 7 (HHV-7), it is remarkable that reactivity to the 85 kDa phosphoprotein was maintained after preabsorption of the sera with HHV-6 antigen, but abolished after preabsorption with HHV-7 antigen. Therefore, the 85 kDa phosphoprotein may be considered a major determinant of the human immune response to HHV-7, discriminating HHV-6 from HHV-7 infection.

Introduction

Human herpesvirus 7 (HHV-7) has been isolated from healthy individuals (Frenkel et al., 1990; Wyatt & Frenkel, 1992; Black et al., 1993; Hidaka et al., 1993; Yoshikawa et al., 1993) and from individuals affected by chronic fatigue syndrome (Berneman et al., 1992b). It is a T lymphotropic virus (Frenkel et al., 1990; Berneman et al., 1992b) and CD4 appears to be a component of the virus receptor (Lusso et al., 1994). HHV-7 is shed in the saliva and is highly prevalent among humans, seroconversion occurring between 1-4 years of age (Wyatt et al., 1991; Clark et al., 1993; Yoshikawa et al., 1993). Seroconversion to HHV-7 was reported to occur in cases of exanthem subitum (Tanaka et al., 1994; Ueda et al., 1994; Asano et al., 1995). Our laboratory identified a minimum of twenty proteins specific for HHV-7-infected cells by means of polyvalent rabbit and mouse sera. Four MAbs were also derived. Two of them (MAbs 5E1 and 3B1) were directed to a complex of at least five antigenically related proteins (87/85, 70, 61 and 57 kDa) designated a p85 complex, and two were directed to proteins with molecular masses of 121 kDa (MAb 3H12) and 51 kDa (MAb 2C1), respectively (Foà-Tomasi et al., 1994).

Recently, it has become increasingly evident that despite the initial observation that HHV-7 and human herpesvirus 6 (HHV-6) are antigenically distinct (Frenkel et al., 1990), they also share common epitopes. The evidence is as follows: MAbs raised to HHV-6 show a weak cross-reactivity to HHV-7 (Wyatt et al., 1991; Berneman et al., 1992a). Conversely, some MAbs and polyvalent sera raised to HHV-7 show a weak cross-reactivity to HHV-6 (Foà-Tomasi et al., 1994). A number of human CD4+ T cell clones can proliferate in response to either HHV-6B or HHV-7 (Yasukawa et al., 1993). Sequence analysis in progress in some laboratories reveals the extent of amino acid homologies to be as high as 75% in some genes [G. Dominguez, P. Pellett (Center for Disease Control, Atlanta, USA) and J. Nicholas (John Hopkins Oncology Center, Baltimore, USA); personal communication]. In some cases seroconversion to HHV-7 is accompanied by a simultaneous rise in antibody titre to HHV-6, or vice versa (Tanaka et al., 1994; Ueda et al., 1994; Asano et al., 1995). The key implication emerging from these findings is that human antibodies to HHV-7 (or HHV-6) cannot necessarily be interpreted as an index of a past infection with the homologous virus.

The objective of this work was to identify and preliminarily characterize HHV-7-infected cell proteins that elicit a strong immune response in humans. We report that one HHV-7 immunodominant protein is an 85 kDa protein belonging to the p85 complex. This is a family of antigenically related proteins identified pre-
viously in our laboratory with the use of two MAbs (Foà-Tomasi et al., 1994). We show that the 85 kDa protein is phosphorylated and have designated it phosphoprotein 85 (pp85). Inasmuch as reactivity to pp85 is maintained after preabsorption of sera with HHV-6 antigen, this protein can be used to differentiate HHV-7 from HHV-6 infection.

Methods

Cells and viruses. HHV-7 (strain RK), kindly provided by N. Frenkel (Frenkel et al., 1990), and HHV-6B (strain Z29) (Lopez et al., 1988) were grown in cord blood mononuclear cells (CBMCs) with prior stimulation for 2 days with 5 μg/ml phytohaemagglutinin (PHA-P), and two further days with PHA-P and recombinant interleukin-2 (5 U/ml), as previously detailed for HHV-6 (Foà-Tomasi et al., 1991). The percentage of HHV-7-infected cells, monitored by indirect immunofluorescence assay (IFA) with MAB 5El (Foà-Tomasi et al., 1994), exceeded 50% in cultures employed for further experiments. Labelling conditions with [35S]methionine/[35S]cysteine, details for IFA, radioimmunoprecipitation assay (RIPA), denaturing electrophoresis and Western blot (WB) were as described previously (Foà-Tomasi et al., 1991). Labelling with [32P] was performed by incubating CBMCs in Dulbecco’s modified Eagle’s medium containing no inorganic phosphate and 500 μCi [32P]/ml.

Sera. Heparinized cord blood samples, obtained from the Department of Obstetrics, University of Bologna, Italy, were diluted 1:2 with RPMI 1640 and subjected to centrifugation on a Ficoll gradient for CBMC purification. The top layers of the gradients are hereafter designated cord blood sera. The final dilutions of sera in IFA, RIPA and WB were 1:20, 1:40 and 1:20, respectively. Monovalent human sera employed in the experiment shown in Fig. 4 were obtained from J. Black (Center for Disease Control, Atlanta, USA) and were assessed to react monospecifically with either HHV-6B or HHV-7 by three tests: IFA, ELISA and RIPA. About 200 sera were screened in order to find those that were monospecific.

Preabsorption of sera with HHV-6B and HHV-7 antigens. Replicate aliquots (5 μl) of a human cord blood serum were either absorbed or not absorbed for 1 h at 37 °C with 50 μl of antigen for absorption, consisting of a lysate from 1.5 x 10⁶ uninfected or HHV-6B- or HHV-7-infected CBMCs, made in 1% BSA in PBS and sonicated for 2 cycles of 30 s each. The sera were then cleared by centrifugation at 14000 r.p.m. for 10 min and subsequently reacted for RIPA with [35S]labelled antigen for precipitation; the antigen consisting of lysates of [35S]methionine/[35S]cysteine-labelled CBMCs either uninfected or infected with HHV-6B or HHV-7. The unabsorbed samples were incubated in parallel in the solution employed for CBMCs suspension.

Table 1. Summary results on reactivity of 44 cord blood sera positive to HHV-7-infected cells

<table>
<thead>
<tr>
<th>Assay</th>
<th>Positive</th>
<th>Characteristics</th>
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<tr>
<td>IFA</td>
<td>44(100%)</td>
<td>Mean geometrical titre 1:537</td>
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<tr>
<td>RIPA</td>
<td>40/44(91%)</td>
<td>Major immunoprecipitated proteins (121, 101, 87/85, 61, 52 kDa)</td>
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<tr>
<td>WB</td>
<td>30/44(68%)</td>
<td>24 sera (55%) reactive with pp85 singly (34%) or in combination (21%), 4 sera (9%) reactive with p121 singly or in combination</td>
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Results

Reactivity of cord blood sera to HHV-7-infected cell proteins

In a preliminary screening 82 human cord blood sera were analysed by IFA for reactivity to HHV-7- and HHV-6-infected CBMCs at a 1:20 dilution. One serum was found to be unreactive to both HHV-6- and HHV-7-, one was unreactive to HHV-7- but reactive to HHV-6-, and three were reactive with HHV-7- but unreactive with HHV-6-infected CBMCs.

In the subsequent studies with HHV-7-infected CBMCs, 44 of the sera positive to both antigens were further analysed to determine their IFA titres and to
HHV-7 85 kDa immunodominant phosphoprotein

Fig. 2. (a) WB analysis of cord blood sera numbers 1, 3–6 reacted with lysates of HHV-7-infected (I) or uninfected (U) CBMCs, transferred to nitrocellulose. The WB were developed with anti-human IgG antibodies coupled to peroxidase. Squares and triangles mark the 85 kDa and 121 kDa proteins, respectively. Stars mark miscellaneous proteins detectable by the human sera in infected cell lysates. (b) RIPA with cord blood human serum number 1 (lanes 1, 2), or with MAb SE1 (lanes 3, 4) followed by WB analysis of the precipitated proteins with MAb SE1. Replicate amounts of a lysate made from HHV-7-infected (I) or uninfected (U) CBMCs were immunoprecipitated with the indicated antibodies. The immunoprecipitated proteins were first subjected to denaturing PAGE, transferred to a nitrocellulose sheet, and then allowed to react with MAb SE1. The reacting proteins were monitored with anti-IgG antibodies coupled to alkaline phosphatase (Immunoblot assay kit; Bio-Rad). The staining visualized the proteins of the p85 complex (85 kDa, 70 kDa doublet, 61 kDa; marked with squares) as well as the IgG molecules, marked by arrows on the right.

identify the immunoreactive proteins. The results of this series of experiments are summarized in Table 1. The IFA titres ranged from 1:80 to 1:2560 (mean geometrical titre 1:537). When analysed by RIPA, only 40 of the 44 sera (91%) were able to specifically immunoprecipitate proteins from HHV-7-infected CBMCs. The electrophoretic profiles of the proteins immunoprecipitated by the remaining four sera from infected and uninfected CBMCs could not be readily differentiated. The electrophoretic profiles of the proteins precipitated by the remaining four sera from infected and uninfected CBMCs were rather diverse, in that some proteins (121, 101, 87/85, 61 and 52 kDa; Fig. 1, marked with triangles) were detectable with almost any serum, although in variable amounts. Other proteins (e.g. those of 170, 110 and 70 kDa) were detectable with some but not all sera.

When analysed by WB, only 30 of the 44 sera (68%) were able to react specifically with HHV-7-infected CBMC proteins (Fig. 2a). The proteins recognized in this assay were more diverse, relative to those recognized in immunoprecipitation analysis. Specifically, 24 of the WB-positive sera (55% of the total sera examined) reacted with the protein with an apparent molecular mass of 85 kDa, singly (34%) or in combination (21%) with other proteins; four sera (9% of the total sera examined) reacted with the protein with an apparent molecular mass of 121 kDa, singly or in combination with the 85 kDa protein; two sera (7%) reacted with further proteins with apparent molecular masses 35 kDa and 32 kDa (Fig. 2a, lane 3) or 62 kDa (lane 5). Representative examples of the different results obtained by WB analysis are shown in Fig. 2(a). Lane 1 shows reactivity to a single 85 kDa protein (marked with a square). Lanes 3, 5 and 9 show reactivity to the 85 kDa protein in association with other proteins. Lanes 7 and 9 show reactivity to the 121 kDa protein (marked with triangles), singly (lane 7), or in combination with the 85 kDa protein (lane 9). Lanes 3 and 5 show reactivity to further proteins (marked with stars).

We next ascertained that the reactive 85 kDa protein is the same protein recognized by MAb SE1 (Foà-Tomasi et al., 1994) in a double immunoprecipitation-WB experiment shown in Fig. 2(b). Specifically, the proteins immunoprecipitated by human sera were separated by electrophoresis, transferred to a nitrocellulose sheet, and reacted with MAb SE1. As shown in Fig. 2(b) for serum number 1, the serum immunoprecipitated a series of
proteins that were able to react in WB with MAb 5E1 (lane 1), and were indistinguishable in electrophoretic mobility from those precipitated by MAb 5E1 (compare lanes 1 and 3). Surprisingly, whereas MAb 5E1 reacts by both RIPA and WB with a family of proteins, the human sera were able to immunoprecipitate the same proteins but reacted in WB with a single 85 kDa band (Fig. 2a, b; compare lanes 1). A likely explanation for this, as well as for the overall lower sensitivity observed with WB as compared to RIPA and IFA, is that human sera antibodies are raised to native proteins. Whereas antigens employed in RIPA and IFA are subjected to mild treatments (extraction in NP40/deoxycholate or acetone fixation), those employed in WB are subjected to drastic denaturation procedures (SDS and boiling). Differences in secondary reagents employed for the detection may further contribute to differences in sensitivity among the different assays.

Identification of proteins carrying HHV-6 and -7 cross-reactive and HHV-7-specific epitopes

First, we ascertained whether antibodies to cross-reactive epitopes were present in human sera. Preliminarily, we compared the IFA titres of six sera to HHV-6 and HHV-7, prior to and after preabsorption with homologous or heterologous antigen. Whereas absorption with one antigen virtually abolished the reactivity to homologous antigen (Fig. 3; lane 8 for HHV-6, lane 12 for HHV-7). The second concerns preabsorption with heterologous antigen. With both HHV-6 and HHV-7 this treatment virtually abolished the reactivity to homologous antigen (Fig. 3; lane 8 for HHV-6, lane 12 for HHV-7). The second concerns preabsorption with heterologous antigens. In this case the electrophoretic pattern of proteins precipitated from HHV-7 antigen (Fig. 3; lane 16) could be readily differentiated from that of proteins precipitated from HHV-7 antigen (lane 11). In lane 9 (Fig. 3; RIPA with HHV-7 antigen following preabsorption with HHV-6 antigen), the major bands were at 85 kDa, 70 kDa, 61 kDa and 52 kDa (marked with squares). Whereas the 85 kDa, 61 kDa and 52 kDa proteins were consistently observed after preabsorption, the 70 kDa protein was detectable with some but not all
sera. We have shown above that the 85 kDa protein is the same protein recognized by MAbs 5E1 and 3B1. It is likely that the 61 kDa protein is also a component of the p85 complex, although this remains to be proven. Inasmuch as the antibody reactivity to the 85 kDa, 61 kDa and 52 kDa proteins was abolished by sera preabsorption with HHV-7 antigen, but unaffected by preabsorption with HHV-6 antigen, these may be considered specific markers of HHV-7 infection. Conversely, in lane 11 (Fig. 3; RIPA with HHV-6 antigen) major bands at 101 kDa and a doublet of 73 kDa and 70 kDa were detected (marked with triangles). These may be considered to be specific markers of HHV-6 infection. In addition, the antibody reactivity to a subset of HHV-6 proteins (130, 119, 115, 110, 88, 62 and 46 kDa; Fig. 3, arrowheads in lane 5) was reduced or almost abolished following preabsorption with either antigen (compare lanes 8 and 11). Similarly, the antibody reactivity to a subset of HHV-7 proteins (121, 110 and 101 kDa; Fig. 3, arrows in lane 6) was reduced or almost abolished following sera preabsorption with either antigen (compare lanes 9 and 12). These may be considered examples of proteins carrying cross-reactive epitopes. Lastly, preabsorption with uninfected cell lysate sometimes enhanced the ability to precipitate from HHV-6 antigen a high molecular mass protein (apparently 170 kDa; Fig. 3, lane 5). The reason for this is unclear at the moment.

To confirm further that the 85 kDa, 61 kDa and 52 kDa proteins carry epitopes specific for HHV-7-infected cells, two human sera monospecific for HHV-7 and two sera monospecific for HHV-6 were employed in preabsorption and RIPA experiments. We expected the presence of antibodies to either one or the other virus to avoid ambiguities due to antibodies raised to cross-reactive epitopes of the heterologous virus. Fig. 4(a) shows the RIPA profile with sera positive for HHV-7 and negative for HHV-6 (lanes 2 and 3). Preabsorption with heterologous HHV-6 antigen allowed immunoprecipitation of the HHV-7 proteins of 85 kDa, 70 kDa, 61 kDa, 52 kDa (lane 6, squares), an ability which was abolished by preabsorption with homologous antigen (lane 9). Proteins which were shown in Fig. 3 to contain cross-reactive epitopes (e.g. the 121 kDa HHV-7 protein) appeared only slightly decreased in this preabsorption experiment. It is likely that sera which react monospecifically are rather poor in antibodies to cross-reactive epitopes. Remarkably, the proteins precipitated by the HHV-7 monovalent serum were absent in the immuno-precipitations with a serum negative for HHV-7 and
Fig. 5. Electrophoretic profile of proteins immunoprecipitated by MAb 3H12 and 5E1, and by a hyperimmune mouse serum described previously (Foà-Tomasi et al., 1994) from lysates of CBMCs infected with HHV-7 (lanes 2-7) or uninfected (lane 8), and labelled with \[^{35}S\]methionine/\[^{32}P\] or \[^{32}P\]. Lane 1 shows the positions of molecular mass markers.

positive for HHV-6 (Fig. 4b, lane 3). The profile of proteins immunoprecipitated by this latter serum from HHV-6 antigen again showed that the 101 kDa protein(s) was selectively present after heterologous preabsorption (lane 8, marked with a triangle) and absent after homologous preabsorption (lane 5). Altogether the results with monospecific sera substantiate those obtained with polyvalent sera, inasmuch as the HHV-7 85 kDa, 61 kDa and 52 kDa proteins and the HHV-6 101 kDa protein(s) were confirmed to carry epitopes specific for HHV-7-infected cells, respectively.

The 85 kDa protein is phosphorylated

The above results indicate that the 85 kDa protein recognized by MAb 5E1 and 3B1 is a major determinant of the human immune response to HHV-7 infection. In view of the observations (Yamamoto et al., 1990; Neipel et al., 1992) that a major HHV-6 immunogenic determinant is the 101 kDa protein homologous to human cytomegalovirus (HCMV) phosphoprotein 150, we ascertained that the proteins of the p85 complex are phosphorylated. Lysates of HHV-7-infected CBMCs were labelled with \[^{32}P\], or with \[^{35}S\]methionine/\[^{35}S\]cysteine as control, and immunoprecipitated with MAb 5E1. A hyperimmune polyvalent mouse serum, with a reactivity very similar to that of human sera (Foà-Tomasi et al., 1994), and MAb 3H12, directed to p121, were included. As shown in Fig. 5, MAb 5E1 (lane 5) and the polyvalent mouse serum (lane 7) immunoprecipitated a single protein with an apparent molecular mass of 85 kDa that was strongly labelled with \[^{32}P\] from lysates of HHV-7-infected cells. It is noteworthy that, whereas MAb 5E1 immunoprecipitated several proteins labelled with \[^{35}S\]methionine and \[^{35}S\]cysteine (Fig. 5, lane 4), consistent with previous results (Foà-Tomasi et al., 1994), the 85 kDa peptide was strongly labelled and the other peptides of the p85 complex were very weakly labelled in the precipitates from \[^{32}P\]-labelled cells. One further protein (64 kDa) immunoprecipitated by the mouse serum (Fig. 5, lane 7), was immunoprecipitated also from the uninfected cell lysate (lane 8), and therefore is not specific for HHV-7-infected cells. MAb 3H12, directed to p121, immunoprecipitated no \[^{32}P\]-labelled protein (lane 3). The results indicate that the 85 kDa protein of the p85 complex is a phosphoprotein. We have designated it pp85.

Discussion

Two key features emerge from this study. (i) Analysis of the antibody reactivity of human cord blood sera led to the identification of immunodominant HHV-7-infected cell-specific proteins; these were differentiated into two groups, one group carrying epitopes cross-reactive between HHV-6 and HHV-7 and the other group carrying epitopes specific for HHV-7-infected cells. (ii) Of the immunodominant proteins carrying HHV-7-specific epitopes, the protein with an apparent molecular mass of 85 kDa is phosphorylated.

It should be noted that the vast majority of human cord blood sera examined contained antibodies reactive at the same time with HHV-7- and HHV-6-infected cell proteins. The high seroprevalence for HHV-7 is consistent with analyses published so far (Wyatt et al., 1991; Clark et al., 1993; Yoshikawa et al., 1993).

Proteins carrying HHV-7- or HHV-6-specific epitopes were differentiated from proteins carrying cross-reactive epitopes by preabsorption of the sera with homologous or heterologous antigens. Remarkably, overlapping results were obtained with polyvalent sera as well as with monospecific sera containing antibodies to either HHV-7 or HHV-6. Thus, following preabsorption of the sera with HHV-7 antigen, the residual reactivity to HHV-6 antigen was mainly directed to a 101 kDa protein.
Conceivably, this is the major HHV-6 immunogenic determinant described by Neipel et al. (1992) and by Yamamoto et al. (1990), reported to discriminate HHV-6 from HCMV infection. Other HHV-6B proteins have a very similar electrophoretic migration, e.g. glycoproteins B (Ellinger et al., 1993) and H (Liu et al., 1993a, b), but these are generally conserved glycoproteins in the Herpesviridae family and are likely to carry at least some common epitopes. Although the final identification of the 101 kDa protein(s) was beyond the purpose of the present work, our results indicate an ability to differentiate HHV-6 from HHV-7 infection. As far as reactivity to HHV-7-specific proteins was concerned, after preabsorption with HHV-6 antigen, the residual reactivity of the sera to HHV-7 antigen was reported that seroconversion to HHV-6 and HHV-7 occurred in the same age groups (Clark et al., 1993; Yoshikawa et al., 1993). The significance of the present finding that human antibodies are directed in part to proteins carrying cross-reactive epitopes stems from the consideration that detection of antibodies to HHV-6 or HHV-7 cannot be interpreted as bona fide evidence of a past infection with homologous virus, and highlights the need for identifying proteins carrying specific epitopes, which can be developed into specific reagents.

Current results show that a major immunodominant protein carrying HHV-7-specific epitopes is the 85 kDa protein that belongs to the family of antigenically related proteins identified previously in our laboratory as p85 complex (Foà-Tomasì et al., 1994). We show here that this protein is phosphorylated, and we have herein designated it pp85. A strong immune response to HCMV is directed to the tegument phosphoprotein pp150 (Jahn et al., 1987). Sera containing antibodies to HHV-6 react intensively to p101/p100, the homologue of HCMV pp150 (Neipel et al., 1992; Yamamoto et al., 1990; Pellett et al., 1993). Although circumstantial data are consistent with pp85 being a HHV-7-encoded protein, the final evidence requires mapping studies. Identification of the encoding gene will establish if pp85 is the homologue of HHV-6B p101 and of HCMV pp150. Taken together, the characteristics of pp85 described here make it an interesting protein worthy of being used as a specific marker of HHV-7 infection, discriminating HHV-6B from HHV-7 infection.

We thank J. Black and P.P. Pellett (Center for Disease Control, Atlanta, USA) for communication of unpublished results and gift of monovalent sera employed in Fig. 4. The work was supported by grants from: AIDS Project, Istituto Superiore di Sanità, Consiglio Nazionale delle Ricerche, Target Project in Genetic Engineering, Associazione Italiana per la Ricerca sul Cancro (AIRC), MURST.

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(Received 13 September 1995; Accepted 9 November 1995)