Identification of a DNA-binding protein of human herpesvirus 6, a putative DNA polymerase stimulatory factor

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A 41K early nuclear antigen (p41), expressed in human herpesvirus type 6 (HHV-6)-infected T cells, was cloned by screening a cDNA expression library with the anti-p41 monoclonal antibody (MAb) C5. When expressed in mammalian cells, the cloned p41 protein comigrated with the authentic p41 protein from HHV-6-infected cells and localized to the nucleus. HHV-6 p41 shares 44% sequence identity with the human cytomegalovirus (HCMV) DNA-binding protein, ICP36 (UL44 gene product); p41 binds to ssDNA with the same apparent affinity as ICP36. Since ICP36 has recently been shown to be an HCMV DNA polymerase-associated stimulatory factor, a similar function is suggested for p41.

Introduction

Human herpesvirus type 6 (HHV-6) is a T cell-tropic virus, which was first isolated in 1986 from patients with lymphoproliferative disorders and with AIDS (Sallahuddin et al., 1986). It was later shown that HHV-6 is widespread in the healthy adult population, with greater than 80% of individuals being seropositive. Initial infection occurs between 1 and 3 years of age, and it is now established that HHV-6 is the causative agent of roseola infantum (exanthem subitum), a benign rash and fever of early childhood (Levy et al., 1990; Yamanishi et al., 1988). Although the medical relevance of HHV-6 to other human diseases remains unknown, viral infection has been aetologically linked to approximately 12% of heterophile-negative infectious mononucleosis cases, and it may play a role in certain lymphoproliferative disorders (Ablashi et al., 1998a; Steeper et al., 1990). Importantly, HHV-6 has been strongly implicated as a cofactor in human immunodeficiency virus type 1 (HIV-1) infection, since increased replication of HIV-1 (as well as transcriptional stimulation of the HIV-1 long terminal repeat) is observed upon coinfection with HHV-6 in tissue culture (Ensoli et al., 1989; Horvat et al., 1989; Lusso et al., 1989). Another means by which HHV-6 may act as a cofactor in AIDS pathogenesis is to upregulate expression of CD4, the cell surface receptor utilized by HIV-1 (Lusso et al., 1991). In this way, HHV-6 infection may convert HIV-1 non-permissive (CD4+/CD8+) T cells to HIV-1 permissive (CD4+/CD8+) T cells.

HHV-6 has a dsDNA genome of approximately 170 kb, consisting of a unique long region flanked by 10 kb direct repeats (Martin et al., 1991). The genome is of sufficient length to encode more than 70 proteins. Partial sequence analysis has revealed that HHV-6 contains several genes that are conserved amongst all herpesviruses (e.g. DNA polymerase, glycoprotein H, major capsid protein and exonuclease), and that HHV-6 is most closely related to human cytomegalovirus (HCMV) (Josephs et al., 1991; Lawrence et al., 1990; Teo et al., 1991). In addition, the genomic organization of the unique long regions of HHV-6 and HCMV is largely collinear (Neipel et al., 1991).

To begin to characterize HHV-6 gene products, monoclonal antibodies (MAbs) have been raised against HHV-6-infected cells (Balachandran et al., 1989; Iyengar et al., 1991). One MAb, designated C5, recognizes an early viral antigen in the nuclei of infected cells. C5 stains cells infected with each of 14 virus isolates tested, suggesting that this early viral antigen is highly conserved. By Western blot analysis, C5 detects a major protein species of 41K (p41), and a minor species of 38K (Iyengar et al., 1991). In this study, we have identified the
gene encoding p41 by using the C5 MAb to screen a cDNA expression library constructed from HHV-6-infected cell RNA. The HHV-6 p41 nuclear protein expressed in vitro and in vivo was shown to be capable of binding DNA. It is predicted to be an important component of the viral DNA replication machinery, as it shows significant homology to HCMV ICP36 (Chee et al., 1990), which was recently shown to be a DNA polymerase-associated stimulatory factor (Ertl & Powell, 1992).

**Methods**

**Virus and cell lines.** The HSB-2 human T cell line was grown in RPMI-1640 (Gibco) supplemented with 10% fetal calf serum (FCS) and antibiotics, and infected with the GS strain of HHV-6 (Ablashi et al., 1988b). Infected cultures were routinely maintained by addition of infected cells, displaying c.p.e., to uninfected cells. COS-1 cells were grown in DMEM (Gibco) supplemented with 8% FCS plus antibiotics.

cDNA library construction and screening. HHV-6-infected HSB-2 cells were harvested when greater than 80% of the cells displayed c.p.e. and plated, and expression of fl-galactosidase fusion proteins were visualized using Stratagene's picoBlue Immunoscreening kit. Briefly, *Escherichia coli* (XL-1 Blue) were infected with recombinant phage and plated, and expression of β-galactosidase fusion proteins was induced by overlaying the plaques with IPTG-soaked filters. Filters were incubated with C5 MAb followed by incubation with an anti-mouse IgG-alkaline phosphatase-conjugated antibody. Binding of C5 MAb was visualized using colorimetric reagents, 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium as described above.

**RNA isolation and cDNA synthesis.** Total cytoplasmic RNA was extracted (Ricciardi et al., 1979) and 5 μg poly(A)+ RNA, isolated by oligo(dT)-cellulose chromatography (Stratagene), was used to construct a Lambda-Zap II cDNA expression library (Clontech). cDNAs generated with random hexamer and oligo(dT) primers were cloned into the EcoRI site of the Lambda-Zap II vector, yielding 98% recombinant phage. Plaques (105) were screened with the C5 MAb (Iyengar et al., 1991), and immunopositive plaques were visualized using Stratagene's picoBlue Immunoscreening kit. Total cytoplasmic RNA was extracted (Ricciardi et al., 1979) and 5 μg poly(A)+ RNA, isolated by oligo(dT)-cellulose chromatography (Stratagene), was used to construct a Lambda-Zap II cDNA expression library (Clontech). cDNAs generated with random hexamer and oligo(dT) primers were cloned into the EcoRI site of the Lambda-Zap II vector, yielding 98% recombinant phage. Plaques (105) were screened with the C5 MAb (Iyengar et al., 1991), and immunopositive plaques were visualized using Stratagene's picoBlue Immunoscreening kit. Briefly, *Escherichia coli* (XL-1 Blue) were infected with recombinant phage and plated, and expression of β-galactosidase fusion proteins was induced by overlaying the plaques with IPTG-soaked filters. Filters were incubated with C5 MAb followed by incubation with an anti-mouse IgG-alkaline phosphatase-conjugated antibody. Binding of C5 MAb was visualized using colorimetric reagents, 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium. Positive phage were purified by rounds of screening and isolation. In vivo excision (Stratagene) of λ clones yielded pBluescript plasmid vectors containing the cloned inserts.

**DNA sequence analysis.** Sequencing of double-stranded plasmid DNA was performed by the dideoxynucleotide chain termination method (Sanger et al., 1977) using synthetic oligonucleotide primers. Both strands of the pC5-1 insert, which contains the entire p41 open reading frame (ORF), were sequenced. Sequence data were analyzed using the University of Wisconsin GCG Sequence Analysis package (Devereux et al., 1984) and the GenEMBL and PIR databases on a Microvax 3300 (The Wistar Institute).

**Southern blot analysis.** Total cellular DNA was isolated from uninfected and HHV-6-infected HSB-2 cells (Ausubel et al., 1987). Restriction enzyme-digested DNAs were electrophoresed on a 1% agarose gel, transferred to a nylon membrane (Stratagene) by the method of Southern (1975), and probed with 32P-labelled nick-translated DNA (Bethesda Research Laboratories). Blots were exposed to Kodak XAR film at -80 °C with intensifying screens (DuPont).

**Northern blot analysis.** HSB-2 cells were synchronously infected using cell-free supernatant from HHV-6-infected cell cultures. After 1 h adsorption at 37 °C, cells were washed with PBS and incubated at 37 °C for 18 h until c.p.e. was evident. Total cytoplasmic RNA was isolated (Ricciardi et al., 1979) from both infected and uninfected HSB-2 cells, electrophoresed on 1% agarose gels containing 3% formaldehyde, and transferred by blotting to a nylon membrane (Stratagene). The DNA probe, labelled with 32PjDTCP by nick translation (Bethesda Research Laboratories), was hybridized to blotted membranes as described (Ausubel et al., 1987). Blots were exposed to Kodak XAR film at -80 °C with intensifying screens (DuPont).

**Expression of p41 in COS-1 cells.** The p41 ORF from pC5-1 was amplified by PCR and cloned into the eukaryotic expression vector pSPORT (Bethesda Research Laboratories). The synthetic primers, made complementary to the 5' and 3' ends of the p41 ORF, contained additional flanking sequences to create artificial EcoRI and XbaI sites, respectively. The PCR-amplified product was cloned into the EcoRI and XbaI sites of pSPORT. This construct, pSP41, was transfected into COS-1 cells by the calcium phosphate precipitation method (Ausubel et al., 1987). Transfected cells were harvested after 3 days, and a crude nuclear extract prepared as described by Gibson et al. (1981), with slight modifications. Briefly, cells were lysed in 20 mM-Tris–HCl pH 7.4, 100 mM-NaCl, 0.5% NP40, and nuclei were recovered by centrifugation, resuspended in 20 mM-Tris–HCl pH 7.4, 50 mM-NaCl, 1 mM-2-mercaptoethanol, and sonicated.

**Western blot analysis.** Western blotting was performed essentially as described (Iyengar et al., 1991). Infected and uninfected HSB-2 cells were washed in PBS and resuspended in SDS–PAGE sample buffer (106 cells/ml) and sonicated briefly. Extracts from transfected and non-transfected COS-1 cells were normalized for protein content (Bio-Rad) before addition of SDS–PAGE sample buffer. Proteins were separated on a 12% SDS–polyacrylamide gel and electrophoretically transferred to an Immobilon-P membrane (Millipore). Membranes were blocked for 1 h in Western blotting buffer [20 mM-Tris–HCl pH 7.4, 150 mM-NaCl, 0.5% Tween-20 and 5% dry milk (Carnation)], incubated for 1 h with C5 MAB (diluted 1:5000 in Western blotting buffer), and incubated for 1 h with alkaline phosphatase-conjugated anti-mouse IgG; all steps were performed at room temperature. Bound antibody was colorimetrically visualized using 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium as described above.

**Immunofluorescence.** The p41 expression vector (pSP41) was transfected into COS-1 cells and, after 3 days, cells were fixed in methanol and incubated for 1 h at room temperature with C5 MAB (diluted 1:500), and then for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Cappel Research Products). Staining was examined using an Olympus fluorescence microscope.

In vitro transcription/translation. The p41 ORF from pC5-1 was PCR-amplified as above and subcloned into the EcoRI and XbaI sites of pBluescript. The construct was linearized with NotI, which cuts immediately downstream of the ORF, and transcribed in vitro (Stratagene) using T7 RNA polymerase. The transcripts were then translated in a rabbit reticulocyte lysate system (New England Nuclear) in the presence of [35S]methionine (Amersham). The labelled products were electrophoresed on a 12% SDS–polyacrylamide gel, which was fluorographed with Entensify (DuPont), dried, and exposed to Kodak XAR film at ~80 °C with intensifying screens (DuPont).

**Immunoprecipitation.** Translation reactions (25 μl) were brought to 250 μl with immunoprecipitation buffer (IP buffer) (50 mM-HEPES pH 7.0, 250 mM-NaCl, 0.1% NP40) (Dyson et al., 1990) and incubated with 2 μl of C5 MAB for 1 h at 0 °C. One-hundred μl of Protein A-Sepharose beads (Pharmacia) (10%) in IP buffer was added and reacted for 1 h at 0 °C. The beads were washed three times in IP buffer and SDS–PAGE sample buffer was added to the pelleted beads. Samples were separated by SDS–PAGE and analysed by fluorography as above.

**DNA-cellulose chromatography.** Translation reactions were diluted in buffer C (20 mM-Tris–HCl pH 7.4, 50 mM-NaCl, 1 mM-EDTA, 1 mM-2-mercaptoethanol) and applied directly to a 0.2 ml calf thymus
ssDNA-cellulose (Sigma) column, or to a cellulose (Sigma) control column that had been equilibrated in buffer C. Chromatography was carried out essentially as described (Gibson et al., 1981). Columns were washed with buffer C, followed by step-gradient elution using 0.5 ml aliquots of increasing NaCl concentration. Aliquots of the column fractions were separated by SDS-PAGE and analysed by fluorography as described above.

Results

Isolation and characterization of an HHV-6 cDNA encoding the C5 antigen, p41

To isolate the gene encoding the HHV-6 p41 protein, a cDNA expression library was constructed from HHV-6-infected HSB-2 T cells and screened with the C5 MAb. Five immunopositive clones were partially sequenced and found to overlap. The largest insert, from clone pC5-1, was sequenced completely and shown to contain a single large ORF of 368 codons corresponding to a protein with a calculated M r 41912 (Fig. 1). An ATG (Fig. 1, position 1) in the context of a strong Kozak consensus sequence (Kozak, 1991), is the predicted translation initiation site. The amino acid sequence indicates that the protein is basic (pI 8.54) with a hydrophilic amino terminus composed largely of His-Arg-Asp and His-Arg-Glu tripeptide repeats.

To ascertain that the pC5-1 ORF is encoded within the HHV-6 genome and does not originate from a cellular gene, which may have been induced during infection, we analysed DNA from both uninfected and HHV-6-infected T cells. These DNAs were digested with EcoRI, Southern-blotted, and probed with the DNA fragment corresponding to the ORF of pC5-1. The size of the hybridizing fragment is indicated (kb).

The p41 ORF DNA hybridizes to three HHV-6 transcripts. Ten μg of uninfected (lane 1) or infected (lane 2) HSB-2 cell RNA was fractionated on a agarose-formaldehyde gel, Northern-blotted, and probed with p41 ORF DNA. The lengths of transcripts are indicated (kb).

consensus sequence (Kozak, 1991), is the predicted translation initiation site. The amino acid sequence indicates that the protein is basic (pI 8.54) with a hydrophilic amino terminus composed largely of His-Arg-Asp and His-Arg-Glu tripeptide repeats.
Northern blot analysis was used to characterize p41 transcripts. Using a p41 ORF DNA probe, two major RNAs of approximately 2.6 and 2.0 kb, and one minor RNA of 1.7 kb, were detected in infected cells but no transcripts were detected in uninfected cells (Fig. 3). All three RNAs are of sufficient length to encode the 1.1 kb ORF. It remains to be determined whether they represent differentially spliced forms of p41 mRNA.

The pC5-1 ORF was expressed in vivo and compared to the p41 produced in HHV-6-infected cells. The entire ORF was introduced downstream of the simian virus 40 early promoter in the eukaryotic expression vector, pSPORT, and this construct (pSP41) was transfected into COS-1 cells. Extracts from transfected cells and from HHV-6-infected cells were compared by Western blot analysis, using the C5 MAb. A single protein with an apparent Mr of 41K was seen in both transfected and virus-infected cells, but not in non-transfected or uninfected cells (Fig. 4). Since the protein products produced by the transfected and the infected cells comigrated and were recognized by the C5 MAb, we conclude that the cloned ORF encodes the p41 protein of HHV-6.

To assess the intracellular localization of cloned p41 protein in mammalian cells, the p41 expression vector pSP41 was transfected into COS-1 cells, which were then examined by indirect immunofluorescence using the C5 MAb (Fig. 5). A distinct nuclear fluorescence was observed. Nucleoli were stained less intensely, and there was negligible cytoplasmic staining. HHV-6 p41 was also observed in the nuclei of infected cells (Iyengar et al., 1991). This result indicates that nuclear localization and retention of p41 occurs independently of other viral proteins.

**HHV-6 p41 is homologous to HCMV ICP36**

A search of the GenBank and PIR databases revealed significant homology between HHV-6 p41 and the HCMV DNA-binding protein, ICP36 (44% amino acid identity, 64% amino acid conservation) (Fig. 6). ICP36
HHV-6 p41 binding to ssDNA

We tested the affinity of HHV-6 p41 for DNA. In vitro transcription/translation of p41 produced a single predominant polypeptide of 41K (Fig. 7a). This 41K protein comigrated with the p41 protein synthesized in HHV-6-infected cells (data not shown). The p41 translated in vitro was immunoprecipitated by C5 MAb but not by a control antibody (Fig. 7a).

The p41 translation product was applied to a ssDNA–cellulose column, and eluted stepwise with increasing concentrations of salt (Fig. 7b, bottom). The p41 protein was retained by the DNA column in 50 mM-NaCl, and was efficiently eluted at 300 mM-NaCl. p41 failed to bind to the cellulose control column showing that binding is specific for DNA (Fig. 7b, top). Identical results were obtained when p41, expressed in COS cells, was subjected to DNA affinity chromatography (data not shown). The ability to bind DNA is further evidence that HHV-6 p41 is functionally similar to HCMV ICP36.

Discussion

MAb C5 recognizes an early nuclear antigen produced in HHV-6-infected cells. Because C5 stains nuclei of cells infected by all HHV-6 serotypes examined so far (Iyengar et al., 1991), the p41 antigen serves as an important diagnostic marker. In this study we have cloned a cDNA that encodes the p41 protein of HHV-6. The cloned protein localizes to the nucleus and has the same apparent M, as p41 produced in HHV-6-infected T cells.

The p41 protein sequence is highly homologous to ICP36, the major late DNA-binding protein of HCMV, and from a functional perspective HHV-6 p41 binds ssDNA with the same apparent affinity as demonstrated for HCMV ICP36 (Gibson et al., 1981). ICP36 was recently shown to be an HCMV DNA polymerase–associated stimulatory factor (Ertl & Powell, 1992). The DNA polymerase interacts directly with the ICP36 protein in vivo, and the complex displays increased incorporation of deoxynucleotides compared to polymerase alone in in vitro assays utilizing primed ssDNA templates. Owing to the sequence and DNA-binding similarities to ICP36, it is likely that p41 serves a similar function.

Like HCMV ICP36, the herpes simplex virus type 1 (HSV-1) UL42 and Epstein–Barr virus (EBV) BMRF1 products have been reported to enhance the activity of their respective viral polymerases (Kiehl & Dorsky, 1991; Gottlieb et al., 1990). Furthermore, the varicella-zoster virus (VZV) gene 16 has been suggested to encode an HSV-1 UL42 homologue (McGeoch, 1989). Thus, it is likely that all the human herpesviruses encode polymerase stimulatory factors. To examine the relatedness of these proteins, we compared their amino acid sequences in pairwise combinations (Table 1). The alignments show limited amino acid sequence similarity (15 to 25% identity, 36 to 47% conservation), except when p41 was compared to ICP36 (44% identity, 64% conservation). Interestingly, in any aligned pair, conserved amino acids are evenly distributed throughout the length of the two proteins; however, no consensus sequence can be found for all five herpesvirus proteins.

While this study was in progress, Chang & Balachandran (1991) reported the isolation of an HHV-6-encoded cDNA from an expression library using MAb 9A5D12. MAb 9A5D12 stains the nuclei of HHV-6-infected cells, and immunoprecipitates a 41K phosphoprotein and a minor 110K polypeptide. The 9A5D12 cDNA contains a single ORF of 623 codons, which is open at the 5' end. The coding region for the p41 DNA-binding protein, which we have cloned, corresponds to codons 21 to 389 in the 623 codon ORF. Chang & Balachandran (1991) originally predicted that the 41K protein recognized by 9A5D12 is encoded downstream of the open reading frame.
Fig. 7. HHV-6 p41 binds ssDNA. (a) In vitro transcription/translation and immunoprecipitation of p41. Run-off transcripts of the p41 ORF were synthesized and capped, followed by translation in rabbit reticulocyte lysate. Lanes 1 and 2, in vitro translation with no RNA added (lane 1) or with 1 μg p41 RNA (lane 2). Lanes 3 and 4, in vitro translated p41 was immunoprecipitated with a control antibody directed against the HSV-1 DNA polymerase (lane 3), or with the C5 MAb (lane 4). (b) DNA affinity chromatography. In vitro translated p41 was applied to a ssDNA-cellulose column (bottom panel), or a cellulose control column (top panel). Lane 1, p41 translation reaction; lane 2, column flowthrough. Columns were extensively washed and then eluted with the following concentrations of NaCl: 0.05, 0.15, 0.30, 0.50, 0.75, 1.00 and 2.00 M, lanes 3 to 9, respectively.

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<td>22 (40)</td>
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* Protein sequence comparisons were accomplished as in Fig. 6. Percent amino acid identity values are shown with percent amino acid conservation values in parentheses.

of codon 227. Thus, the relationship between these two 41K proteins was unclear. Following an exchange of information, a sequence error, which places a stop codon at amino acid 381, was acknowledged by Chang & Balachandran (1991, correction). They now predict that the 41K protein recognized by 9A5D12 is specified by codons 21 to 389. This is in complete agreement with our results. To confirm that MAbs C5 and 9A5D12 recognize the same 41K protein, we used 9A5D12 in an immunofluorescence assay on cells transfected with our p41 expression vector. The nuclear staining observed was identical to that seen with the C5 MAb (data not shown).

Although the complete medical relevance of HHV-6 is unclear at present, the virus is recognized as the causative agent of roseola infantum, may be involved in some cases of infectious mononucleosis, and may act as a cofactor in the progression of AIDS. If p41 proves to be a stimulatory factor that binds directly to the HHV-6 DNA polymerase, then disruption of this interaction may prevent viral replication. Indeed, the UL42 gene product of HSV-1 has been shown to be essential for viral replication (Marchetti et al., 1988). The p41 protein thus has the potential of serving as an ideal target for antiviral therapy. Recombinant HHV-6 DNA polymerase and antibodies to this protein will be produced for use in future experiments aimed at confirming the ability of p41 to associate with and to stimulate the activity of the viral polymerase.

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References


