Mapping of Epitopes on the 65K DNA-binding Protein of Herpes Simplex Virus Type 1

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SUMMARY

Previously we have described the isolation of seven monoclonal antibodies (MAbs) and two polyclonal rabbit sera directed against the product of herpes simplex virus type 1 (HSV-1) gene UL42, a 65K DNA-binding protein (65KDBP) which is essential for HSV DNA replication and virus growth. We now report the synthesis of all 483 overlapping hexapeptides of this 488 amino acid protein and describe their use for the identification of epitopes recognized by these MAbs and polyclonal sera. MAb 6898, derived from one fusion, recognizes the peptides EDLDGA and DLDGAA which correspond to amino acids 363 to 369 of 65KDBP. MAbs Z4D4, Z6F3, Z1AS, Z10C1, Z3H12 and Z1F11, derived from a second fusion, all recognize the peptides GDPEDL and DPEDLD which correspond to amino acids 360 to 366. As expected both polyclonal sera recognize several different epitopes.

INTRODUCTION

A 65K DNA-binding protein (65KDBP) is induced in cells infected with herpes simplex virus type 1 (HSV-1) (Bayliss et al., 1975; Marsden et al., 1987), and is encoded by gene UL42 (Parris et al., 1988). UL42 is one of seven HSV genes essential for virus DNA replication (Challberg, 1986; Wu et al., 1988) and its DNA sequence has been determined (McGeoch et al., 1988a). Mutants have been identified with alterations in this gene that are defective in replication of virus DNA and virus growth (Marchetti et al., 1988). 65KDBP is consequently of considerable interest both for its role in virus DNA replication and for its potential as an antiviral target.

The exact role that 65KDBP plays in virus replication has not been determined; however, some of its properties have been described. The protein binds to dsDNA in the absence of other proteins and interacts, directly or via some other protein, with HSV DNA polymerase (Gallo et al., 1988). It is synthesized early in infection and is localized in the nucleus (Goodrich et al., 1989; Schenk et al., 1988; Schenk & Ludwig, 1988) initially showing a punctate distribution associated with margined heterochromatin which later becomes more disperse (Goodrich et al., 1989). The finding (Gallo et al., 1988) that 65KDBP is the serotype equivalent to the HSV-2 protein ICSP 34/35 is consistent with the earlier observations of Vaughan et al. (1984, 1985) who reported the nuclear distribution of ICSP 34/35 and its association with HSV-2 DNA polymerase.

Seven monoclonal antibodies (MAbs) and two polyclonal rabbit sera have been described recently that are reactive against 65KDBP (Marsden et al., 1987; Parris et al., 1988; Schenk et al., 1988). Because defined antibodies have proved an invaluable tool in the study of protein function we decided to characterize these MAbs and polyclonal antibodies in more detail by identifying the epitopes on 65KDBP with which the antibodies interact. The work was also undertaken to identify a MAb that might be used in immunoaffinity chromatography to provide a simple and rapid method for the purification of 65KDBP.
METHODS

Cells and virus. BHK clone 13 cells (Macpherson & Stoker, 1962) and HSV-1 strain 17 syn+ (Brown et al., 1973) were used throughout.

Radioactive labelling. Confluent monolayers in 50 mm diameter dishes or roller bottles were infected with 5 to 20 p.f.u. of HSV-1 per cell. After 1 h, unadsorbed virus was removed and the infected cell monolayer was radiolabelled with [35S]methionine (specific activity > 1000 Ci/mmol; Amersham) at a concentration of 20 μCi/ml. Labelling was performed in Eagle's medium containing 20% of the normal concentration of methionine and 2% calf serum. The label was added 5 h after the end of the adsorption period, and the cultures were harvested after approximately 18 h.

Purification of 65KoBP. 65KoBP was partially purified from a high-salt extract of BHK cells infected with 17 syn+ by DNA-cellulose chromatography as previously described (Marsden et al., 1987).

Antisera. The anti-65KoBP rabbit polyclonal antibodies and mouse MAbs used in this study are listed in Table 1 together with their immunoglobulin subtypes. Sera 13809 and 13810 and MAb 6898 were produced by immunizing rabbits or BALB/c mice with 65KoBP partially purified from BHK cells infected with HSV-1 strain 17+ (Marsden et al., 1987; Parris et al., 1988). MAbs Z4D4, Z6F3, Z1A8, Z10CI, Z3H12 and Z1F11 were produced by immunizing BALB/c mice with a crude extract of A31 cells infected with HSV-1 strain F (Schenk et al., 1988).

Immunoprecipitation. One-hundred μl of peptide was preincubated for 1 h at room temperature with MAb Z1F11 (cell culture supernatant) in extraction buffer (Zweig et al., 1980). The concentration of peptide and antibody are discussed in the results section. To this mixture 100 μl of partially purified 65KoBP was then added and incubated for 18 h at 4 °C. Antibody, together with any bound protein, was precipitated by incubation with Protein A-Sepharose (1 h at 4 °C) and the immunoprecipitate was washed several times with 0.6 M-LiC1 containing 0.1 M-Tris–HCl pH 8.0 and 1% 2-mercaptoethanol. Bound protein was eluted with 0.125 M-Tris–HCl pH 6.8 containing 2% SDS, 20% glycerol, 5% 2-mercaptoethanol and bromophenol blue (for staining).

Gel electrophoresis. SDS–PAGE was carried out by using the buffer system of Laemmli (1970) with 5% to 12.5% gradient gels cross-linked with 5% (w/w) NN'-methylenebisacrylamide.

Autoradiography. Slab gels were fixed and treated with En3Hance (New England Nuclear), dried, and exposed to Kodak X-Omat XSI film at -70 °C.

Purification of antibodies for epitope mapping

Trisacryl anion-exchange chromatography. Rabbit serum was extensively dialysed using 25 mM Tris–HCl, 35 mM-NaCl pH 8.8. It was then applied to an anion-exchange column of DEAE–Trisacryl M (IBF). The serum was adsorbed for 2 h at room temperature and immunoglobulin was then eluted from the column using the same buffer; all other serum proteins were bound by the Trisacryl.

Mono Q anion-exchange chromatography. One ml of ascites fluid was diluted with 9 ml of 20 mM-Tris–HCl pH 8-6 (buffer A). It was then fractionated on an 8 ml Mono Q column (Pharmacia) which had previously been equilibrated with buffer A. Proteins were eluted in 1 ml fractions from the column with a linear gradient of NaCl (0 to 2.0 M) in buffer A.

Protein A-Sepharose affinity chromatography. Ascites fluid (0.5 ml) was mixed with an equivalent volume of 1.5 M-glycine, 3 M-NaCl pH 8.9 (binding buffer) then loaded onto a 1.5 ml Protein A–Sepharose CL-4B (Sigma) column pre-equilibrated with binding buffer. The antibody was adsorbed onto the column for 2 h at room temperature, washed with binding buffer and eluted with 100 mM-citric acid pH 4.

Synthesis of pin-bound peptides and epitope mapping. Overlapping hexapeptides were synthesized by the method of Gysen (1984), modified for Fmoc chemistry, using kits from Cambridge Research Biochemicals according to the manufacturer's instructions. The peptides are synthesized in covalent linkage to polypropylene rods having the format and spacing of the wells in a microtitre plate. This allows subsequent ELISA to be rapidly performed.

ELISAs were performed, as suggested by the manufacturer, as follows. Following purification, immunoglobulin was diluted in a buffer containing 1% ovalbumin, 1% bovine serum albumin and 0.1% Tween-20 in phosphate-buffered saline (PBS) then 175 μl was pipetted into each of the wells of a microtitre plate. After incubation with immunoglobulin at 4 °C for about 16 h the pins were washed four times in PBS containing 0.05% Tween-20 and then incubated with a second antibody conjugated to hors eradish peroxidase (HRP): either HRP-conjugated Protein A (Sigma) or HRP-conjugated goat anti-mouse IgG (Bio-Rad) as appropriate. Bound HRP was visualized by incubation with the substrate, 2,2' azino-bis(3-ethylbenzthiazolone 6-sulphonic acid) (Sigma). The absorbance in each well was recorded using a Titertek Multiskan plate reader (Flow Laboratories) linked to an IBM personal computer running under the control of the epitope mapping program from Cambridge Research Biochemicals. The output gave both numerical values for the absorbance in each well and a graphical representation of the form shown in Fig. 1.
Epitopes of HSV-1 DNA-binding protein

Synthesis of free peptides. Peptides were synthesized by continuous flow Fmoc chemistry (see reviews by Atherton et al., 1979; Sheppard, 1983) using an LKB Biolyx peptide synthesizer. All chemicals were purchased from LKB with the exception of dimethylformamide (Rathburn Chemicals), trifluoroacetic acid (Aldrich) and resin to which the first amino acid had been coupled via an acid-labile linkage agent (Peptide and Protein Research). Following synthesis, peptides were cleaved from the resin and side chain protecting groups were removed with 95% trifluoroacetic acid in H₂O (plus 4% w/v phenol for the arginine-containing peptide) using standard protocols.

Peptide purity was determined by reverse phase HPLC and varied between 68% and 91%. The M_r values of the peptides were determined by mass spectrometry (M-Scan Ltd.) which gave values identical to those expected.

RESULTS

The antibodies to be used for mapping were selected for their ability to react with 65K DBP on Western blots. We reasoned that antibodies reactive with denatured proteins were likely to recognize sequential epitopes. The two polyclonal antibodies and seven MAbs used in this study are shown in Table 1. Immunoglobulin was purified by the method recorded in Table 1.

In separate experiments the concentration of purified immunoglobulin necessary to produce a good signal on Western blots was determined (data not shown) and this concentration was used for epitope mapping. Sufficient immunoglobulin was obtained from 0.5 to 1.0 ml of ascites fluid, rabbit sera or ammonium sulphate-concentrated (33-fold) hybridoma cell culture supernatant. To identify these epitopes and to assess the reproducibility of the technique, two independent complete sets of the 483 overlapping hexapeptides of the 488 amino acid protein (McGeoch et al., 1988a) were synthesized. MAbs Z1A8 and Z1F11 were located on the two sets of hexapeptides (Fig. 1). The reactivity of each hexapeptide was determined by ELISA and is represented in the figure as a vertical line of height corresponding to the A_405 produced. The data show that hexapeptides 360 (GDPEDL) and 361 (DPEDLD) from each synthesis were recognized by both MAbs Z1A8 and Z1F11. Thus the two antibodies recognize the same epitope and the method is reproducible. Repeated experiments on each of the sets of synthesized hexapeptides have confirmed these results.

Next, MAbs Z4D4, Z6F3, Z10Cl, Z3H12 and 6898 were mapped (Fig. 1). MAbs Z4D4, Z10Cl, Z3H12 and possibly Z6F3 recognized hexapeptides 360 and 361 whereas MAb 6898 consistently recognized hexapeptides 363 (EDLDGA) and 364 (DLDGAA). Two features merit comment. First, the signals from hexapeptides 360 and 361 with Z6F3 were very weak (although both were 1.5-fold higher than the signal from the next highest hexapeptide). This is probably because MAb Z6F3 is IgM which is not readily recognized by the detecting antibody. Secondly, the background for MAb 6898 is higher than for any other antibody. The reason for this is not yet known.

The mapping of epitopes using rabbit polyclonal antibodies 13809 and 13810 is also shown in Fig. 1, together with that of the respective preimmune (pi) sera (13809pi and 13810pi). Strong reactivities of immune sera but not preimmune sera were found reproducibly with only a few

Table 1. Antisera and immunoglobulin purifications

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antibody type</th>
<th>Immunoglobulin subtype</th>
<th>Method of immunoglobulin purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>13809</td>
<td>Rabbit polyclonal</td>
<td>ND*</td>
<td>Anion-exchange (Trisacryl)</td>
</tr>
<tr>
<td>13810</td>
<td>Rabbit polyclonal</td>
<td>ND</td>
<td>Anion-exchange (Trisacryl)</td>
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<td>Protein A-Sepharose</td>
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<td>Mouse monoclonal</td>
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<td>Anion-exchange (Mono Q)</td>
</tr>
<tr>
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<td>Mouse monoclonal</td>
<td>G2a</td>
<td>Anion-exchange (Mono Q)</td>
</tr>
<tr>
<td>Z10Cl</td>
<td>Mouse monoclonal</td>
<td>G1</td>
<td>Anion-exchange (Mono Q)</td>
</tr>
<tr>
<td>Z3H12</td>
<td>Mouse monoclonal</td>
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<td>Anion-exchange (Mono Q)</td>
</tr>
<tr>
<td>Z1F11</td>
<td>Mouse monoclonal</td>
<td>G1</td>
<td>Anion-exchange (Mono Q)</td>
</tr>
</tbody>
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* ND, Not determined.
Fig. 1. Identification of the sequential epitopes on 65K<sub>DPB</sub> recognized by MAbs and polyclonal antibodies. Two sets of the 483 overlapping hexapeptides of 65K<sub>DPB</sub> were independently synthesized and reacted against purified immunoglobulin. The reactivity of each hexapeptide was measured by $A_{405}$ from the ELISA and is represented in the figure as a vertical line. The position in the protein of the first amino acid of each hexapeptide is shown on the abscissa. The data are arranged in groups of ten and every fiftieth one is numbered (1, 51, 101 etc.).
Epitopes of HSV-1 DNA-binding protein

Fig. 2. Immunoprecipitation of 65K_{DBP} by MAb Z1F11 is specifically blocked by an oligopeptide from 65K_{DBP} encompassing the epitope recognized by MAb Z1F11. This MAb was used to immunoprecipitate [35S]methionine-labelled 65K_{DBP} from a partially purified extract of HSV-1-infected cells (EXT) in the presence of various concentrations of the heptadecapeptide VSHGDPELDGAARAGE, which corresponds to amino acids 357 to 373 of 65K_{DBP} and contains the hexapeptide previously identified as the epitope recognized by MAb Z1F11 (Fig. 1). The unrelated pentadecapeptide YEENLRAGLDPSTEL was used as a control. Immunoprecipitated proteins were analysed by SDS-PAGE. The immunoprecipitations were performed in triplicate and all are shown. The extract was run on a separate gel and is shown in the first lane of the figure. Numbers above the lanes represent the concentrations of oligopeptides (µg/ml) present in the immunoprecipitation reactions.

hexapeptides. For both 13809 and 13810 these were hexapeptides 2 (TDSPGG) and 347 (TDSQDS) whereas 13810 also recognized consistently hexapeptides 412 (PKTGSP), 423 (ADPVPL), 435 (DAADGT), 477 (GGPQTP) and 478 (GPQTPY).

To obtain independent evidence that the epitopes identified by this technique (Geysen et al., 1984) do correspond to the peptides with which the antibodies interact, we tested to see whether immunoprecipitation of 65K_{DBP} by MAb 6898 or Z1F11 could be blocked by a peptide encompassing the predicted epitope. The concentration of MAb necessary to immunoprecipitate 65K_{DBP} was first determined (data not shown). Then using this concentration of antibody, immunoprecipitations were performed in the presence of various amounts of both the heptadecapeptide VSHGDPELDGAARAGE which corresponds to amino acids 357 to 373 of 65K_{DBP} and contains the hexapeptides recognized by the MAb and the pentadecapeptide YEENLRAGLDPSTEL (corresponding to a tyrosine plus the 14 carboxy-terminal amino acids of a putative adenovirus type 40 Elb 19K protein; van Loon et al., 1987). This pentadecapeptide was chosen as a control because of its similar overall size and charge.

Immunoprecipitated proteins were analysed by SDS-PAGE. The autoradiograph (Fig. 2) shows that the MAb Z1F11–65K_{DBP} interaction was specifically blocked by 1 µg/ml of the
It was found that the control peptide did not block the interaction when used at concentrations as high as 1000 μg. Similar results (not shown) were obtained with another control peptide FHVNPVVVFDFASY (corresponding to amino acids 708 to 722 of HSV-1 DNA polymerase; Quinn & McGeoch, 1985; Gibbs et al., 1985) and with MAb 6898.

**DISCUSSION**

The sequential epitopes of 65K_DHP recognized consistently by the antibodies used in this study are summarized in Fig. 3 below the hydropathicity plot of the protein. The result for MAb Z6F3 has been included, although the signal was extremely weak and therefore not much confidence can be placed in it. The two rabbit polyclonal antibodies recognized the amino terminus of the protein and one of them (13810), the carboxy terminus. In addition they both recognized internal domains within regions of the protein predicted to be hydrophilic. These findings support the suggestion of Hopp & Woods (1981) that hydrophilic regions are likely to be antigenic; however, they are not the most hydrophilic regions in 65K_DHP and would not have been the first choice in selecting peptides with which to raise anti-peptide antibodies. It is interesting that the hexapeptides recognized by both polyclonal antibodies contain the sequence TDS suggesting that this tripeptide is largely responsible for the specificity of these antibodies. It is worth emphasizing that Fig. 3 probably does not show the only regions of 65K_DHP that are reactive with polyvalent sera 13809 and 13810 since these sera are likely to be reactive also with discontinuous epitopes.

The results obtained from the reactions of the MAbs were more surprising. They all recognized epitopes between amino acids 360 and 369 indicating perhaps that this region of 65K_DHP is immunodominant in BALB/c mice. However, as six of the seven MAbs arose from a single fusion (Schenk et al., 1988), we cannot exclude the possibility that these six are from the same clone and subsequently differentiated, within the mouse, to produce the observed immunoglobulin subtypes (G1, G2a and M). Mapping of the epitopes recognized by other MAbs derived from BALB/c mice should clarify this point.
Previously we showed (Gallo et al., 1988) that an immunoaffinity column made with MAb 6898 specifically retained the HSV DNA polymerase (pol) and we deduced that there was a physical association between 65K DBP and pol, either directly or via some intermediate molecule(s). This finding was reminiscent of that made previously by Vaughan et al. (1985) on the association of ICSP 34/35 (the HSV-2 equivalent of 65K DBP) with the HSV-2 DNA pol. As we consider it extremely unlikely that the epitope recognized by MAb 6898 (EDLDGAA) could be simultaneously interacting with the antibody and be available for association with pol, we infer that amino acids 363 to 369 are not involved directly in the interaction of 65K DBP with pol.

An alternative explanation for the specific retention of the HSV DNA pol by the MAb 6898 immunoaffinity column could be that the pol molecule contained epitopes that reacted with the antibody. A computer search within the established amino acid sequence of HSV-1 pol from strain 17 (McGeoch et al., 1988b) revealed that homologies with the epitope EDLDGAA recognized by MAb 6898 do exist. These were the tripeptides EDL and LDG at positions 388 and 487 respectively and GAA at positions 985 and 1167 in the sequence of HSV-1 pol. Furthermore, the amino acid immediately upstream of EDL in both 65K DBP and pol is P which may contribute to the reactivity of the antibody. Such putative cross-reactivities of pol with MAb 6898 were not observed by immunoprecipitation with DNA-binding proteins from HSV-1-infected cells (which would be expected to contain pol) (Parris et al., 1988) or by immunoblotting with crude extracts from infected cells (Gallo et al., 1988). We intend to investigate further whether they might exist using purified preparations of pol; however we believe that the available evidence points to a physical association between 65K DBP and pol.

Studies on the function of 65K DBP would be facilitated by a reliable and simple procedure for purification of the protein at the milligram level. The two published procedures (Marsden et al., 1987; Gallo et al., 1988) do not meet these criteria: the first because it was only a partial purification and the second because it yielded only small amounts of protein. Our observation that the MAb Z1F11–65K DBP interaction can be blocked by a heptadecapeptide encompassing the recognized sequential epitope suggests that it might be possible to develop a purification scheme, involving immunoaffinity chromatography combined with competitive peptide elution, which meets the above criteria. This approach is currently being investigated.

Anti-oligopeptide sera have proved valuable research tools because of their predetermined specificity. However, these sera suffer from two disadvantages. First they are often of low titre and affinity. Secondly they are usually produced in rabbits and are therefore only available in limited amounts. MAbs generally do not suffer these problems. They can be regrown from the parent hybridoma cell lines and are often of high affinity. By the MAb epitope mapping as described here we combine the advantages of both types of antibodies. The pin-bound peptides appear stable over 6 months and can be reused at least 15 times so that they are available for mapping of sequential epitopes recognized by additional MAbs.

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