The herpes simplex virus type 1 DNA polymerase accessory protein, UL42, contains a functional protease-resistant domain

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Herpes simplex virus type 1 encodes its own DNA polymerase (Pol), the product of the UL30 gene, and a polymerase accessory subunit, the product of the UL42 gene, both of which are required for viral DNA replication. Pol and the UL42 protein associate to form a heterodimeric complex (Pol/UL42) which is more active and has a higher processivity than the Pol catalytic subunit alone. The Pol/UL42 complex has been reconstituted by mixing together highly purified Pol and UL42 subunits obtained from recombinant baculovirus-infected cells. We have used polymerase activity on poly(dA):oligo(dT20), a template that the Pol subunit utilizes with low efficiency, to measure the formation of the Pol/UL42 complex. Our data indicate that the association constant for the Pol/UL42 complex is $1 \times 10^7$ M$^{-1}$. Proteolytic digestions of UL42 were performed to determine whether structural domains of UL42 could be disclosed by differential amino acid accessibilities. The ability of these protease-resistant domains to form a functional complex with Pol was determined by measuring their ability to stimulate Pol activity on poly(dA):oligo(dT20). We have found that trypsin digestion of UL42 in the presence of DNA generates protease-resistant fragments of 28K and 8K which co-elute from a MonoQ column and are able to stimulate Pol activity on poly(dA):oligo(dT20). Complex formation of the 28K and 8K tryptic fragments with Pol was also shown by their co-immunoprecipitation with antibody to Pol. It was determined that the 28K fragment of UL42 comprised amino acids 1 to 245 or 1 to 254 of UL42, whereas the 8K fragment started at amino acid 255. Thus, controlled proteolysis of UL42 revealed two closely contiguous structural domains that retained the ability to complex with Pol and stimulate Pol activity.

Introduction

Herpes simplex virus type 1 (HSV-1) encodes seven genes required for viral DNA replication and for replication of origin-containing plasmid DNA (Schaffer et al., 1973; Weller et al., 1983; Marchetti et al., 1988; Wu et al., 1988). The products of two of these genes, the UL30 gene encoding the 136K viral DNA polymerase (Pol) (Purifoy et al., 1977; Chartrand et al., 1980) and the UL42 gene encoding a 65K double-stranded DNA-binding protein (UL42) (Parris et al., 1988), interact within the infected cell to form a heterodimeric complex (Pol/UL42) (Gallo et al., 1988). The use of recombinant baculoviruses to over-express Pol and UL42 as individual subunits has allowed a detailed analysis of the effect of UL42 on Pol activity (Hernandez & Lehman, 1989; Gottlieb et al., 1990; Gallo et al., 1989). Stimulation occurs because the UL42 protein increases the processivity of Pol (Gottlieb et al., 1990; Hernandez & Lehman, 1990). Recent studies also indicate that Pol/UL42 is more resistant than Pol to the salt-dependent increase in the $K_m$ values for activated calf thymus DNA and that the $V_{max}$ of Pol/UL42 increases with salt concentration (Hart & Boehme, 1992).

Although it is clear that Pol and UL42 associate with each other and that the resulting Pol/UL42 complex is more competent than Pol to replicate the 153 kbp viral genome, the manner in which complex formation occurs is less well understood. The C-terminal region of Pol, from amino acids 1008 to 1235, has been shown in co-immunoprecipitation studies to be the UL42-binding domain of the protein (Digard & Coen, 1990; Digard et al., 1993a; Tenney et al., 1993a). In similar studies we have used co-immunoprecipitation to map the Pol-binding domain of UL42 to amino acids 1 to 315, suggesting that the C-terminal region of UL42 is not essential for this function (Tenney et al., 1993b). In this communication, we used proteolytic digestions of UL42...
to generate protease-resistant domains of UL42 and analysed these domains for the biochemical functions of UL42. We found that a region of UL42 becomes resistant to trypsin digestion when UL42 is first allowed to bind to DNA. This domain was mapped to two contiguous tryptic fragments, an N-terminal 28K fragment from amino acids 1 to 245 or 1 to 254, and an 8K fragment starting at amino acid 255 of UL42. Together, these two fragments possess the ability to complex with Pol as shown by their ability to stimulate Pol activity and by their co-immunoprecipitation with Pol. The retention of these activities in these protease-resistant polypeptides suggests that the functional domain of UL42 is a well ordered structure that need not be covalently linked.

**Methods**

**Nucleic acids.** Calf thymus DNA (Sigma) was activated by DNase I treatment as described (Chang, 1973). Poly(dA):oligo(dT) was prepared by heating a solution containing 1 mg/ml poly(dA) (Pharmacia) and 0.1 mg/ml oligo(dt30) or oligo(dt40) (Pharmacia) in 10 mM-Tris–HCl pH 8, 1 mM-EDTA and 100 mM-NaCl for 3 min at 90 °C and cooling slowly to room temperature. The length of the oligo(dt) did not affect polymerase activity of either Pol or Pol/UL42. The other synthetic templates were prepared similarly.

**Antiserum.** A decapeptide corresponding to the N terminus of UL42 was synthesized and conjugated to BSA with glutaraldehyde. The peptide conjugate was administered intradermally to female New Zealand rabbits. The rabbits were given boosters at 4 week intervals and test bleeds were obtained 10 days after the boosts.

**Plasmids and recombinant baculoviruses.** The UL30 recombinant baculovirus was obtained from the laboratory of I. R. Lehman. Plasmid pNN4 (Wu et al., 1988) containing the HSV-1 UL42 gene was obtained from the laboratory of M. Chalberg and was used to make the UL42 recombinant baculovirus by co-transfecting with wild-type Autographa californica nuclear polyhedrosis virus DNA as described (Summers & Smith, 1987). Plasmid pAS510 was constructed by inserting the recognition site immediately upstream of the ATG start by the use of oligonucleotide mutagenesis. The UL42 gene was excised from the plasmid by inserting the BamHI site to an NsiI site downstream of the terminase codon and cloned into the BamHI and NsiI sites of the baculovirus expression vector pVL1393 (Webb & Summers, 1990) to construct plasmid pH107. This plasmid was used to generate the UL42 recombinant baculovirus by co-transfecting with wild-type Autographa californica nuclear polyhedrosis virus DNA as described (Summers & Smith, 1987). Plasmid pAS510 was constructed by inserting the BamHI–BglII fragment from pH107 into the BamHI site of PET16c.

**Enzyme assays.** Standard DNA polymerase assays (50 μl) were performed in 50 mM-Tris–HCl pH 8, 10% glycerol, 5 mM-MgCl2, 1 mM-DTT, 40 μg/ml activated calf thymus DNA, 5 μM each dATP, dCTP and dGTP, 5 μM-3′pHiHTP (300 to 500 c.p.m./pmol), 100 mM-NaH2SO4 and 200 μg/ml BSA. The reaction mixture was added to the polymerase samples in the wells of a vinyl microplate (Falcon 3911) kept on ice. The reaction was initiated by transfer to a 37 °C water bath and was quenched after 30 min by the addition of 50 μl 20% TCA. Incorporation of labelled nucleotide into acid-insoluble material was measured by filtration of the quenched reactions onto glass fibre filter mats using a Skatron 12-well cell harvester programmed for washing with 1 M-HCl and with 95% ethanol. The dried filter mats were either punched out into scintillation vials or were counted with the LKB B eta plate scintillation counter.

The assay to detect UL42 stimulation of Pol activity was performed similarly to the standard polymerase assay with the following modifications. The reaction mixture did not contain dATP, dCTP or dGTP, and 10 μg/ml poly(dA):oligo(dt30) was used instead of activated calf thymus DNA. Pol (30 ng) and the UL42 sample were preincubated for 15 min prior to the addition of the reaction mixture.

**Enzymes.** High five insect cells (Invitrogen) were grown in Ex-Cell 400 medium (JRH Biosciences) to 75% confluence at 27 °C and infected with the appropriate recombinant baculovirus at a m.o.i. of 5. After 60 to 80 hours at 27 °C, the infected cells were shaken off the flask and collected by centrifugation. Nuclei were isolated as described (Hernandez & Lehman, 1990) and stored at −80 °C.

HSV-1 Pol was isolated from UL30 recombinant baculovirus-infected cells as described (Hernandez & Lehman, 1990) except that single-stranded DNA-cellulose was used instead of heparin–agarose. The Pol/UL42 complex was isolated similarly from cells co-infected with the UL30 and UL42 recombinant baculoviruses except that hydrophobic interaction chromatography was performed before gel filtration by the following procedure. The pooled fractions from the single-stranded DNA-cellulose column were adjusted to 670 mM-ammonium sulphate by the addition of 2 mM-ammonium sulphate solution in Buffer H (20 mM-HEPES pH 8, 1 mM-EDTA, 10% glycerol, 10 mM-2-mercaptoethanol and 1 mM-PMSF). The sample was applied to a 0.46 x 5 cm POROS PH column and eluted with a 30 ml linear gradient from 670 to 0 mM-ammonium sulphate in Buffer H. Fractions containing Pol/UL42 were concentrated by centrifugation in a Centricon 30 microconcentrator and applied to a Superose 12 gel filtration column.

UL42 protein was isolated from UL42 recombinant baculovirus-infected cells by the following procedure. Nuclei were extracted with one half volume of 5 M-NaCl at 0 °C for 30 min. The nuclear extract was clarified by centrifugation at 100 000 g for 60 min. The supernatant was dialysed against 20 mM-Hepes pH 8, 10% glycerol, 10 mM-2-mercaptoethanol and 1 mM-PMSF) until its conductivity was equivalent to Buffer T containing 50 mM-NaCl. The dialysate was clarified by centrifugation and applied to a Q-Sepharose column equilibrated in Buffer T with 50 mM-NaCl. After washing extensively with Buffer T with 50 mM-NaCl, the Q-Sepharose column was eluted with Buffer T containing 0.4 M-NaCl. The 0.4 M-NaCl eluate was dialysed for 3 h against Buffer T and then applied to a dsDNA–cellulose column equilibrated with Buffer T containing 0.1 M-NaCl. The column was washed with 0.1 M- and 0.3 M-NaCl in Buffer T and was then eluted with Buffer T containing 0.7 M-NaCl. The 0.7 M eluate was dialysed against Buffer T and then applied to a Blue-Sepharose column. The Blue-Sepharose column was washed with Buffer T with 0.2 M-NaCl and then eluted with Buffer T containing 0.5 M-NaCl. Solid ammonium sulphate was added to the 0.5 M eluate to 40% saturation and the precipitate was collected by centrifugation. The precipitate was dissolved in a small volume of Buffer T and was subjected to gel filtration chromatography on a Superose 12 HR 10/30 column. Quantification of the purified proteins was performed by absorbance measurements at 280 nm (Gill & von Hippel, 1989).
Sequencing grade trypsin, endoproteinase Lys-C and endoproteinase Asp-N were from Boehringer Mannheim. SDS-PAGE and Western blots. Samples for SDS-PAGE in 2% SDS, 100 mM-DTT, 10% glycerol and 0.001% bromophenol blue were heated for 5 min at 98 °C prior to electrophoresis. Electrophoresis was performed in pre-cast 10 to 20% gradient gels (NOVEX) using 0.1% SDS, 100 mM-Tris-HCl and 100 mM-tricine as the running buffer. Proteins were visualized either by Coomassie blue or by silver staining. For Western blots, proteins were electrophoretically transferred to Immobion-P membranes (Millipore) in 25 mM-Tris-HCl and 192 mM-glycine for at least 1 Ah. Alkaline phosphatase conjugated to goat anti-rabbit IgG (Promega) was used to detect antibody–antigen complexes.

Co-immunoprecipitation of UL42 trypsin digestion products with Pol. In vitro transcription/translation and co-immunoprecipitation were performed as described (Tenney et al., 1993b). Briefly, 35S-labelled UL42 protein was produced by transcription of a plasmid (pAS510) containing the UL42 gene and a 15 amino acid leader sequence driven by a T7 RNA polymerase promoter and its translation using a reticulocyte lysate system. The translation mixture was digested with trypsin, incubated with purified Pol, and immunoprecipitated with antibody to Pol and Protein A-Sepharose. The immunoprecipitated material was analysed by SDS–PAGE and fluorography.

Results

UL42 stimulation of HSV-1 DNA polymerase

The HSV-1 UL42 protein forms a complex with HSV-1 Pol and stimulates polymerase activity (Gallo et al., 1988, 1989; Hernandez & Lehman, 1990; Gottlieb et al., 1990). In order to establish a sensitive assay for the presence of the Pol/UL42 complex, we evaluated a number of DNA templates for their ability to serve as substrates for the Pol/UL42 complex and for the Pol catalytic subunit. The results, shown in Table 1, confirm previous observations that the Pol/UL42 complex is more active on activated calf thymus DNA (Gallo et al., 1989) than the Pol catalytic subunit. However, the Pol catalytic subunit still retains significant activity on this template as well as on poly(dC): oligo(dG10). In contrast,

Table 1. Template:primer utilization by Pol and Pol/UL42 complex

<table>
<thead>
<tr>
<th>Template:primer</th>
<th>Pol (pmol)</th>
<th>Pol/UL42 complex (pmol)</th>
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<tbody>
<tr>
<td>Activated calf thymus DNA</td>
<td>26±8</td>
<td>50±4</td>
</tr>
<tr>
<td>Poly(dA): oligo(dT18)</td>
<td>3.0</td>
<td>57.2</td>
</tr>
<tr>
<td>Poly(dA): oligo(dT20)</td>
<td>3.1</td>
<td>383</td>
</tr>
<tr>
<td>Poly(rA): oligo(dT10)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Poly(dT): oligo(dA18-18)</td>
<td>2.1</td>
<td>1.9</td>
</tr>
<tr>
<td>Poly(dT): oligo(dA18-18)</td>
<td>9.9</td>
<td>13.1</td>
</tr>
<tr>
<td>Poly(dC): oligo(dG10)</td>
<td>69±8</td>
<td>207</td>
</tr>
<tr>
<td>Poly(dC): oligo(dG10)</td>
<td>0</td>
<td>1.5</td>
</tr>
</tbody>
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* Nucleotide incorporation measurements were performed as in Methods using 10 μg/ml DNA for 15 min at 37 °C with 30 ng of Pol and an amount of Pol/UL42 complex containing 39 ng of the polymerase subunit as determined by Western blot analysis of Pol and Pol/UL42.

Fig. 1. Stimulation of Pol by UL42. (a) The indicated amounts of UL42 protein were added to 33 ng of Pol prior to assaying for polymerase activity on poly(dA): oligo(dTm) as described in Methods. (b) The indicated amounts of Pol/UL42 purified as a complex from insect cells co-infected with recombinant Pol and UL42 baculoviruses were assayed for polymerase activity on poly(dA): oligo(dTm). (c) UL42 stimulation of Pol was performed as for (a) except that either 10 μg/ml (■) or 20 μg/ml (□) poly(dA): oligo(dTm) was used.

the Pol catalytic subunit is severely limited in its ability to utilize poly(dA): oligo(dT) as a template:primer whereas the Pol/UL42 complex is highly active on this template:primer. Thus, polymerase activity on poly(dA): oligo(dT) is a sensitive indicator for the Pol/UL42 complex.
We reconstituted the Pol/UL42 complex by mixing together purified Pol catalytic subunit and UL42 protein and used polymerase activity on poly(dA): oligo(dT20) to measure the amount of functional Pol/UL42 complex formed. Shown in Fig. 1(a) is the stimulation of Pol activity by UL42 on poly(dA): oligo(dT20). Fig. 1(b) shows a titration of Pol/UL42 complex isolated from cells co-infected with Pol and UL42 recombinant baculoviruses. This complex, like the complex isolated from HSV-1-infected cells, is a heterodimer of Pol and UL42 in a 1:1 molar ratio. Since polymerase activity on poly(dA): oligo(dT20) is proportional to the concentration of Pol/UL42 present in the assay (Fig. 1b), we were able to determine the amount of Pol/UL42 complex formed for the various amounts of UL42 added to Pol. The molar amounts of Pol/UL42 formed were subtracted from the initial amounts of Pol and UL42 to determine the molar amounts of uncomplexed Pol and UL42. The association constant for complex formation was calculated from each data point in Fig. 1(a) and averaged to give 1.2 ± 0.2 × 10^8 M⁻¹. Although UL42 stimulation of Pol activity on poly(dA): oligo(dT20) could also be due to an interaction of UL42 with the DNA rather than with Pol, it is unlikely because the amounts of UL42 required for stimulation are much lower than the amount of DNA present in the assay (nM versus µM nucleotides) and because increasing the amount of DNA does not increase the amount of UL42 required for Pol stimulation (Fig. 1c). As expected from the role of UL42 as a processivity factor (Hernandez & Lehman, 1990; Gottlieb et al., 1990), the processivity of Pol on the poly(dA): oligo(dT) template increases from one or two nucleotides to greater than 200 nucleotides in the presence of UL42 (Fig. 2).

**Protease-resistant domain of UL42**

The above result (Fig. 1), as well as our previous results and the work of others (Hernandez & Lehman, 1990; Gottlieb et al., 1990; Digard & Coen, 1990; Tenney et al., 1993a), has shown that HSV-1 Pol and UL42 readily form the Pol/UL42 complex upon mixing of the subunits. In order to deduce the region of UL42 responsible for complex formation with Pol, UL42 was digested with proteases and functional activities were...
correlated with specific proteolytic fragments. The initial experiments surveyed three endoproteinases for their effects on the UL42 polypeptide. Purified, recombinant UL42 was digested with various amounts of trypsin, endoproteinase Lys-C and endoproteinase Asp-N and the digestion products were analysed by SDS–PAGE (Fig. 3). UL42, containing 30 arginines and 13 lysines, was digested very efficiently by trypsin. However, large fragments of UL42 could be generated at the lowest trypsin concentration (lane 2). UL42 was more resistant to endoproteinase Lys-C digestion and a stable protease-resistant fragment of 24K could be observed (lanes 6 and 7). Asp-N digestion of UL42 also produced large fragments (lanes 9 and 10) even though it contains 29 aspartic acid residues. These results indicate that structural domains of UL42 may be disclosed by the differential amino acid accessibilities to endoproteinase digestion.

UL42 is a dsDNA-binding protein, so the trypsin digestion of UL42 was performed in the presence of DNA to determine whether binding to DNA afforded some protection against proteolysis. As shown in Fig. 4, trypsin digestion of UL42 in the presence of DNA resulted in major products of Mr 36K and 28K and 8K and minor products of Mr 36K and 21K (lane 1). None of these polypeptides are present in the UL42 preparation, the DNA preparation, or trypsin (lanes 3 to 5, respectively). No stable partial proteolysis products accumulated to significant levels following trypsin digestion under identical conditions in the absence of DNA (lane 2). The trypsin digestion pattern of BSA, which is not a DNA-binding protein, is not altered by the addition of DNA (data not shown) suggesting that the UL42 protease-resistant domains result from binding of UL42 to DNA rather than by inhibition of trypsin by DNA. The relative abundance of the 28K and 8K proteolytic fragments indicates that they represent a major portion of the input UL42 and are therefore highly resistant to trypsin despite the large numbers of arginines and lysines distributed throughout UL42.

Polypeptides retaining the N terminus of UL42 were identified by Western blots using antibody raised against an N-terminal decapeptide of UL42. As shown in Fig. 4, this antibody reacts with intact UL42 (lane 8) and with the 36K, 28K and 21K proteolytic fragments (lane 6) thus identifying these polypeptides as retaining the N terminus of UL42. Interestingly, an N-terminal 28K degradation product of UL42 can be detected by Western blots in the undigested UL42 sample (lane 8) suggesting that the region flanking the 28K domain is highly sensitive to proteases present within the cells or during purification.

The 8K fragment did not react with the antibody raised against the N terminus of UL42 indicating that it is an internal polypeptide. The N terminus of the 8K fragment was therefore determined by amino acid sequencing of the polypeptide. The 8K fragment was isolated by SDS–PAGE of trypsin-digested UL42 followed by electrophoretic transfer to Immobilon-P membrane. The band corresponding to the 8K fragment was excised and analysed with an Applied Biosystems 473A sequenator (data not shown). The N-terminal sequence of the 8K fragment, TVYG, corresponds unambiguously to amino acids 255 to 258 of the deduced UL42 sequence (McGeoch et al., 1988) and indicates that cleavage occurred after lysine 254 to generate the N terminus of the 8K fragment.

Analysis of the UL42 trypsin digest

The trypsin digestion of UL42 in the presence of DNA was scaled up in order to produce enough material for further analysis. SDS–PAGE and Western blots confirmed that the digestion mixture contained the same proteolytic fragments seen in Fig. 4 (data not shown). In this experiment an additional band at 26K was also produced as a minor species. The 26K polypeptide reacted with the antibody raised against the N-terminal decapetide of UL42 and we therefore conclude that this species is produced from sequences within the major 28K fragment. No intact UL42 was observed in the digestion mixture by Coomassie blue staining or Western blots using either the antibody to the N terminus of UL42 or an antibody against recombinant UL42 protein. Since a

![Fig. 4. Trypsin digestions were performed with UL42 protein (68 μg), double-stranded plasmid DNA (6.9 μg) and trypsin (1.25 μg).](image-url)
Fig. 5. Pol stimulation by UL42 tryptic fragments. UL42 protein was digested with trypsin in the presence of DNA as described in Fig. 3 and the digestion was quenched by the addition of 1 mM-PMSF. The indicated amounts of trypsin-digested UL42 (■) or intact UL42 (□) were added to Pol and assayed for Pol stimulation as described in Methods.

minimum of 15 ng of intact UL42 could be detected with our antibodies and 375 ng of digested UL42 was loaded for the Western blot analysis, we conclude that over 95% of the UL42 was cleaved by trypsin.

In order to determine whether the tryptic fragments of UL42 retained any UL42 functions, the digestion mixture was assayed for Pol stimulation activity on poly(dA):oligo(dT20) (Fig. 5). Comparison of the Pol stimulation activity with that of intact UL42 revealed that the digested material stimulated Pol to the same extent as undigested UL42. Since the stimulation of Pol occurs through the formation of a complex capable of utilizing poly(dA):oligo(dT20), this suggests that the fragments produced by trypsin digestion of UL42 are capable of productively interacting with Pol and eliciting its enzymatic activity on this template:primer. Moreover, since the specific stimulating activities of the digested and intact UL42 are very similar, the association constant of the digested UL42 fragments must also be very similar to that of intact UL42.

The trypsin digestion mixture that was active for Pol stimulation was subjected to chromatography using the SMART system (Pharmacia) and a MonoQ PC 1.6/5 column. The samples were applied at low salt and were eluted with a linear NaCl gradient. Aliquots of the collected fractions were assayed for Pol stimulation activity (Fig. 6a) and the active fractions analysed by SDS-PAGE (Fig. 6b). The fractions containing the Pol stimulation activity were eluted from the MonoQ column by 270 mM-NaCl and corresponded to the presence of the 28K and 8K polypeptides. Although the 21K polypeptide could be detected in the active fractions, it did not co-elute with the Pol stimulation activity or the other polypeptides. Therefore, we attribute the Pol stimulation activity in the trypsin digestion mixture of UL42 to the 28K and 8K polypeptides. The 8K polypeptide also co-eluted with the 28K polypeptide during chromatography on dsDNA–cellulose and gel filtration columns (data not shown). The inability to separate these proteolytic fragments by different purification techniques suggests that they may remain associated after trypsin digestion of UL42.
Trypsin digestion of in vitro translated UL42

Although we have inferred from the Pol stimulation data that the trypsin-resistant fragments of UL42 formed a complex with Pol, we also wished to demonstrate this directly. We have previously used 35S-labelled UL42 and truncations of UL42 in co-immunoprecipitation experiments with Pol to establish the minimal region of UL42 required for complex formation (Tenney et al., 1993b). We have determined the ability of tryptic digests of 35S-labelled UL42 to co-immunoprecipitate with Pol in a similar manner. For these experiments, the UL42 produced by the programmed reticulocyte lysates contained an N-terminal 15 amino acid leader sequence from the pET1lc plasmid used for expressing UL42 as described in Methods. Trypsin digestion of the 35S-labelled UL42 was performed and generated the 28K and 8K fragments as the major species (Fig. 7, lane 3) as was also seen with the purified recombinant UL42 (Fig. 4, lane 1). The digestion mixture was incubated with or without Pol, immunoprecipitated, and analysed by SDS-PAGE and fluorography. The 28K and 8K fragments co-immunoprecipitated with Pol whereas the other minor tryptic fragments, with the exception of the 26K fragment, did not (Fig. 7, lane 1). None of the tryptic fragments were immunoprecipitable in the absence of Pol (lane 2). These results show that the 28K and 8K fragments of UL42 form a complex with Pol.

The 35S-labelled UL42 and UL42 truncations used previously to define the minimal Pol-binding domain (Tenney et al., 1993b) were also used to map the C terminus of the 28K fragment. The UL42 truncation produced by a NotI digest of the UL42 expression plasmid (amino acids 1 to 248) has an electrophoretic mobility close to that of the 28K fragment. The potential trypsin cleavage sites around amino acid 248 are arginines at positions 224 and 263 and lysines at positions 245 and 254. Since Lys-C digestion of UL42 also produces the N-terminal 28K fragment (data not shown), the 28K fragment must be produced by cleavage at lysine 245 or 254. Cleavage at lysine 245 would make the 28K fragment three amino acids smaller than the NotI truncation whereas cleavage at lysine 254 would make it six amino acids larger. Because the in vitro synthesized UL42 used in these experiments contained a 15 amino acid leader sequence which could also be cleaved by trypsin at either the arginine at position 12 or lysine at position 15, we cannot unambiguously assign the C terminus to lysine 245 or 254. However, since the 28K fragment (Fig. 7, lane 3) migrates faster than the NotI truncation product (Fig. 7, lane 5) it is likely that the cleavage by trypsin to generate the major 28K product occurs at lysine 245.

Discussion

HSV-1 UL42 protein is a processivity factor for HSV-1 Pol. The association of DNA polymerases with subunits that increase processivity seems to be a common theme among replicative polymerases. The eukaryotic DNA Pol δ and proliferating cell nuclear antigen (Prelich et al., 1987), Escherichia coli Pol III and the β subunit (Fay et al., 1982), and bacteriophage T7 Pol and thioredoxin (Tabor et al., 1987) are some of these replicative polymerases and processivity factors. The Pol/UL42 complex can be readily reconstituted from the individual subunits as demonstrated by this work and the work of others (Hernandez & Lehman, 1990; Gottlieb et al., 1990; Digard & Coen, 1990; Tenney et al., 1993a, b). Their strong affinity for each other, as shown by an association constant of 1 x 10^8 M^-1, and the large excess of UL42 within HSV-1-infected cells (Gottlieb et al., 1990) would suggest that Pol exists solely as the Pol/UL42 complex during infection. Indeed, numerous workers have observed that their highly purified Pol preparations from HSV-1-infected cells are in fact the
Pol/UL42 complex (Gottlieb et al., 1990; Crute & Lehman, 1989; R. K. Hamatake et al., unpublished). The preponderance of Pol as the Pol/UL42 complex within infected cells suggests that the ability of UL42 to complex with Pol is of critical importance during HSV-1 replication. In support of this hypothesis are recent genetic data that correlate the ability of mutant UL42 proteins to bind Pol with the ability to support viral growth by either complementation of a UL42 null mutant (Digard et al., 1993b) or isolation of UL42 deletion mutants (Gao et al., 1993). Similarly, Pol mutants that retain catalytic activity but are unable to complex with UL42 are incapable of supporting viral replication (Digard et al., 1993a) or origin-dependent DNA synthesis (Stow, 1993).

DNA synthesis activity on poly(dA):oligo(dT9) was used to assay for the reconstitution of the Pol/UL42 complex from the individual subunits, based on the observation that Pol alone is inactive on this template whereas Pol is highly active when complexed with UL42. In the absence of UL42, Pol extends the primers by one or two nucleotides, but in the presence of UL42 the sizes of the products formed on poly(dA):oligo(dT) are increased and are indicative of highly processive synthesis suggesting that the UL42 stimulation of Pol activity on poly(dA):oligo(dT) is mechanistically similar to that on primed single-stranded bacteriophage DNA (Hernandez & Lehman, 1990; Gottlieb et al., 1990). Although processive synthesis on primed single-stranded bacteriophage DNA has been used by others to measure UL42 function (Owsianka et al., 1993), Pol activity on poly(dA):oligo(dT) is an alternative assay for UL42 function that is more rapid and sensitive.

We have used trypsin digestion of UL42 to generate two proteolytic fragments of M, 28K and 8K whose resistance to trypsin cleavage is dependent upon prior binding of UL42 to DNA. The 28K polypeptide is also generated by endoproteinase Lys-C digestion of UL42 although in this case it appears to be a relatively protease-resistant polypeptide. The resistance of the 28K polypeptide to further digestion by Lys-C and its sensitivity to trypsin in the absence of prior binding to DNA suggests that the arginine residues within this region are protected from trypsin digestion by DNA binding and that the lysine residues are relatively resistant to proteolytic digestion independent of DNA binding.

The major protease-resistant polypeptides of UL42 comprise a nearly contiguous region of the amino-terminal two-thirds of UL42. The 28K polypeptide has been mapped to amino acids 1 to 245 or 1 to 254 of UL42. The carboxy terminus of the 8K fragment has not been determined precisely but we have shown that its amino terminus is at amino acid 255 of UL42. Based on its size, the 8K fragment could be generated by carboxy-terminal cleavage at lysine 319, arginine 340 or arginine 370. This region encompasses the minimal Pol-binding domain of UL42 (amino acids 1 to 315) as shown by co-immunoprecipitation studies (Tenney et al., 1993b). This region also encompasses a region of UL42 (amino acids 1 to 348) that has recently been shown to have all the functions required for a productive viral infection in tissue culture (Gao et al., 1993). In this study, we have also used co-immunoprecipitation with Pol to show that the 28K and 8K polypeptides complex with Pol. Since the 28K polypeptide is smaller than the minimal Pol-binding domain of UL42, it is reasonable to infer that the 28K and 8K polypeptides must remain associated in order for complex formation with Pol to occur. The inability to separate the 28K and 8K polypeptides from each other using several different chromatographic conditions also suggests that they remain tightly associated after trypsin digestion of UL42. This association does not occur by disulphide linkages since the 28K and 8K polypeptides can be separated during SDS-PAGE without prior treatment with reducing agents (data not shown). These two proteins co-elute during chromatography on a Superose 12 gel filtration column after intact UL42 (data not shown) suggesting that trypsin digestion of UL42 does not result in the association of all of the tryptic fragments with the 28K and 8K polypeptides. Although we cannot eliminate the possibility that very small peptides are associated with the 28K and 8K polypeptides, the previous mapping of UL42 functional domains (Tenney et al., 1993a,b) indicates that these peptides would not contribute to the activities of the 28K and 8K polypeptides.

Several studies have investigated the UL42 interaction with Pol and indicate that the Pol-binding domain of UL42 is not a simple linear array of amino acids. A complete set of overlapping pentadecapeptides spanning the entire 488 amino acids of UL42 did not contain any sequences capable of specifically interfering with Pol activity (Owsianka et al., 1993). Carboxy-terminal truncations of UL42 that extend past amino acid 315 abolish the abilities of the UL42 truncations to coinmunoprecipitate with Pol, to stimulate Pol activity and to bind to dsDNA (Tenney et al., 1993b). One particular truncation of UL42 composed of amino acids 1 to 248 retained all of the amino acids contained in the 28K polypeptide described in this study yet could not perform any of the known UL42 activities in vitro. The association of the 8K polypeptide with the 28K polypeptide thus seems to be essential for the UL42 activities described in this study. A minor trypsin-resistant fragment of 26K with sequences contained within the 28K polypeptide, in conjunction with the 8K polypeptide may also be capable of complex formation since it is
present in fractions that stimulate Pol activity (Fig. 6b) and co-immunoprecipitate with Pol (Fig. 7). The 8K polypeptide may be a domain that is important for maintaining the structural integrity of other regions of UL42 although a more direct contribution to UL42 function by binding to DNA or Pol cannot be ruled out for the 8K polypeptide. Further dissection of UL42 domains by a combined biochemical and genetic approach may partition them into separable domains and allow a more complete understanding of how they interact with each other and with Pol in order to confer highly processive synthesis activity to Pol.

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References


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