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NOTES

Mutations in the C Terminus of Herpes Simplex Virus Type 1 DNA Polymerase Can Affect Binding and Stimulation by Its Accessory Protein UL42 without Affecting Basal Polymerase Activity

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We have analyzed the effects of mutations in the herpes simplex virus type 1 DNA polymerase (Pol) C-terminal UL42 binding domain on the activity of Pol and its ability to form complexes with and be stimulated by UL42 in vitro. Wild-type Pol expressed in *Saccharomyces cerevisiae* was both bound and stimulated by UL42 in vitro. C-terminal truncations of 19 and 40 amino acids (aa) did not affect the ability of Pol to be stimulated by UL42 in vitro. This stimulation as well as basal Pol activity in the presence of UL42 was inhibited by polyclonal anti-UL42 antiserum, thus indicating a physical interaction between Pol and UL42. Removal of the C-terminal 59 aa of Pol and internal deletions of 72 aa within the Pol C terminus eliminated stimulation by UL42. None of the truncations or deletions within Pol affected basal polymerase activity. In contrast with their ability to be stimulated by UL42, only wild-type Pol and Pol lacking the C-terminal 19 aa bound UL42 in a coimmunoprecipitation assay. These results demonstrate that a functional UL42 binding domain of Pol is separable from sequences necessary for basal polymerase activity and that the C-terminal 40 aa of Pol appear to contain a region which modulates the stability of the Pol-UL42 interaction.

The herpes simplex virus type 1 (HSV-1) DNA polymerase (Pol) (1,235 amino acids [aa], 135 kDa) copurifies from infected cells in a heterodimeric complex with its accessory protein, UL42 (1, 7). The DNA polymerases of some other herpesviruses, namely, Epstein-Barr virus (17), human cytomegalovirus (5, 15), and herpes simplex virus type 2 (25), have also been demonstrated to exist in a complex with their respective accessory proteins, which share homology with UL42. The HSV-1 UL42 protein (488 aa) is a nonspecific double-stranded DNA-binding protein (19). It is one of seven HSV-1 proteins required for HSV origin-dependent DNA replication in transfected cells (27) and is necessary for HSV-1 DNA synthesis during infection of cultured cells (16, 18). The activity of HSV Pol is stimulated by UL42 in vitro (4, 6), an effect resulting from an increase in the processivity of the HSV Pol enzyme (10, 13, 14) and in the affinity of the complex for its primer-template (9).

We have previously described the expression of Pol and mutant Pol proteins in the yeast *Saccharomyces cerevisiae* under the galactose-inducible galactokinase gene promoter (Fig. 1) (11, 12, 20). Several of the mutants contained deletions or truncations within the C-terminal 227 aa of Pol, a region recently shown to be necessary and sufficient for binding to UL42 (3). In this work, we have examined the abilities of these Pol mutants to functionally interact with UL42. While all the mutants retain basal polymerase catalytic activity in vitro (11), we note differences in their ability to be stimulated by HSV UL42 and to form stable complexes with UL42 in vitro. These results demonstrate that a functional UL42 binding domain is dispensable for polymerase enzymatic activity in vitro and provide insight into the regions of the Pol protein that contribute to the formation of the Pol-UL42 heterodimer and to a functional interaction with UL42.

Polymerase activity and stimulation by UL42. Extracts of yeast cells expressing the Pol proteins were prepared as described previously (12) and contained approximately 5 mg of total protein per ml. HSV Pol expressed in recombinant baculovirus-infected cells (rPol) was purified as described previously (14), except that DNA cellulose was used in place of heparin agarose. Recombinant UL42 (rUL42) was purified from insect cells infected with a recombinant baculovirus encoding the UL42 gene under the control of the baculovirus polyhedrin promoter (to be described elsewhere). An extract of yeast cells expressing the wild-type (WT) HSV Pol (RC205) was assayed for polymerase activity under high-salt reaction conditions specific for HSV Pol (12) and contained polymerase activity which could be stimulated by the addition of rUL42 (Fig. 2). The response of yeast-expressed Pol to stimulation by rUL42 was dose dependent and saturable and was comparable to that of rPol (Fig. 2).

Extracts of yeast cells expressing mutant Pol proteins (Fig. 1) were assayed for Pol activity in the absence and presence of 30 ng of rUL42 (Fig. 3). All of the Pol mutants exhibited polymerase activity that was indistinguishable

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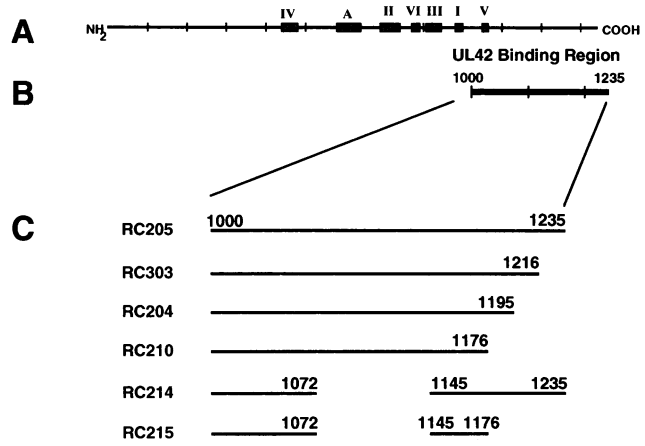

FIG. 1. HSV-1 polymerase and polymerase mutants expressed in *S. cerevisiae*. (A) An illustration of the 1,235-aa HSV Pol protein showing boxed regions of sequence homology with other DNA polymerases (8, 26). (B) The 235-aa C-terminal region of HSV Pol shown to be responsible for binding to UL42 (3). (C) The mutations of the HSV-1 (KOS) Pol that lie within the C-terminal 235 aa of the protein. The construction of these mutants and their expression in yeast cells have been described previously (11). Deleted portions of the protein are indicated by the amino acid number at the terminus of the protein (C-terminal truncations) or the residues bounding internal deletions. Not shown is the JS1 mutant, which has an N-terminal truncation, expressing Pol aa 228 to 1235 (11). All mutants express only the Pol sequences indicated, except for RC303, which expresses 4 additional aa (SLVS) after residue 1216 because of the introduction of a frameshift.

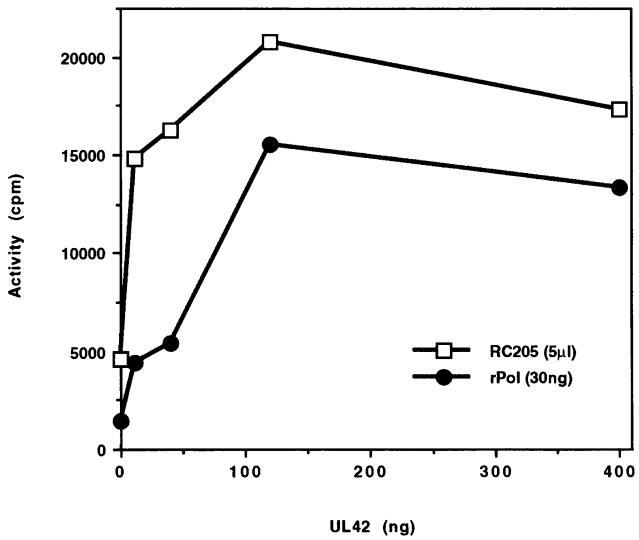

FIG. 2. Stimulation of HSV-1 Pol expressed in yeast cells by UL42. WT HSV-1 Pol yeast extract (RC205, 5 μl) or 30 ng of rPol was coincubated with various amounts of rUL42 and assayed for polymerase activity on activated calf thymus DNA (12). Five microliters of yeast extract and rUL42 was incubated 15 min on ice in 15 μl (total volume) of 50 mM NaCl–500 μg of bovine serum albumin (BSA) per ml–50 mM Tris-HCl (pH 8.0)–10% glycerol–1 mM dithiothreitol, after which was added 35 μl of reaction buffer {30 μg of activated calf thymus DNA per ml; 100 mM $(NH_4)_2SO_4$; 5 μM (each) dATP, dCTP, dGTP, and [^{3}H]dTTP (540 cpm/pmol); 5 mM $MgCl_2$; 1 mM dithiothreitol; 100 μg of BSA per ml; 50 mM Tris-HCl (pH 8.0)}. After 20 min at 37°C, reaction mixtures were quenched with 50 μl of 10% cold trichloroacetic acid, incubated on ice for 10 min, filtered onto glass fiber filters, washed twice with 1 N HCl and once with ethanol, and counted.

from WT Pol expressed in yeast cells (RC205) or recombinant baculovirus-infected cells (rPol), and no activity was detected in extracts of yeast cells containing the expression vector alone (yEP352), as previously reported (11). However, in contrast with basal polymerase activity, the mutants differed in the ability to be stimulated by UL42. Only Pol mutants containing C-terminal deletions of 19 (RC303) and 40 (RC204) aa or an N-terminal deletion of 227 aa (JS1) were stimulated to levels equivalent to those of the full-length Pol (RC205) and rPol (Fig. 3) by the addition of UL42. On the other hand, mutants with a C-terminal deletion of 59 aa (RC210) or a 72-aa deletion within the UL42 binding domain (RC214 and RC215) failed to be stimulated by UL42. These results indicate that C-terminal truncations of 19 or 40 aa do not affect the ability of HSV Pol to form a functional interaction with UL42 and also demonstrate that the ability of Pol to functionally interact with UL42 is not required for polymerase catalytic activity in vitro.

Coimmunoprecipitation of yeast-expressed Pol and rUL42. To determine whether stimulation by UL42 was correlated with the ability of the Pol mutants to form stable complexes with UL42 in vitro, coimmunoprecipitation assays were performed. These experiments employed UL42 radiolabeled with [^{35}S]methionine that was produced by in vitro runoff transcription followed by translation in programmed rabbit reticulocyte lysates (Promega). Runoff transcripts were produced from a linearized plasmid that contained the complete HSV-1 (KOS) UL42 open reading frame (nucleotides 93113 to 94579 [21]; GenBank-EMBL accession no. X14112) cloned into pTZ18U (United States Biochemical). The yeast extracts containing Pol were incubated with the radiolabeled UL42 to allow complexes to form and were immunoprecipitated by using polyclonal rabbit antiserum raised against rPol. This antiserum was specific for HSV Pol and did not immunoprecipitate UL42 (data not shown). Radiolabeled UL42 was coimmunoprecipitated by Pol antiserum after incubation with WT Pol (RC205), the N-terminal deletion mutant (JS1), and the Pol mutant lacking the C-terminal 19 aa (RC303) (Fig. 4). No complex formation was evident after incubation with the control yeast extract (yEP352) or other mutant Pol extracts. Interestingly, the mutant Pol lacking 40 aa at its C terminus (RC204) failed to form a stable complex with UL42, although its activity was effectively stimulated by UL42 (Fig. 3). For a control, we ensured that the polyclonal anti-Pol antiserum used in the assays could immunoprecipitate the Pol mutants (Fig. 5).

It is noteworthy that the Pol mutant with a deletion of its C-terminal 40 aa (RC204) appears to functionally interact with UL42, resulting in a stimulation of polymerase activity; however, complexes with UL42 were undetectable in the coimmunoprecipitation assay. The inability of this Pol mutant to mediate coimmunoprecipitation of UL42 in vitro may reflect a lower stability of complex formation with UL42. In order to explore this possibility, coimmunoprecipitation assays using washes containing less detergent {0.1% Nonidet P-40 (rather than 1% Triton, 0.2% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS])} were used in an attempt to detect less stable interactions. Despite these measures, however, radiolabeled UL42 was coimmunoprecipitated only by the full-length Pol (RC205) and Pol containing a C-terminal deletion of only 19 aa (RC303; data not shown).

Neutralization of Pol-UL42 complexes by anti-UL42 anti-

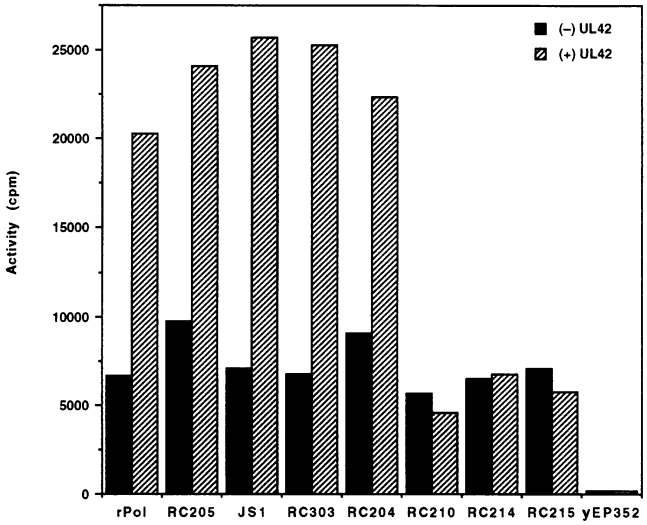

FIG. 3. Stimulation of HSV Pol mutants by UL42. Five microliters of yeast extracts containing HSV-1 Pol mutants (Fig. 1) was assayed for stimulation by UL42, as described in the legend to Fig. 2. Thirty nanograms of rPol and an extract of yeast cells containing only the yeast expression vector lacking HSV-1 sequences (yEP352) were analyzed in parallel.

serum. To further investigate the ability of Pol mutants to form a functional, physical interaction with UL42 in vitro, we have used polyclonal rabbit antiserum raised against purified rUL42. The activity of the Pol-UL42 complex is neutralized by the antiserum in vitro, but the activity of the catalytic subunit of Pol alone is unaffected (RC205, Fig. 6).

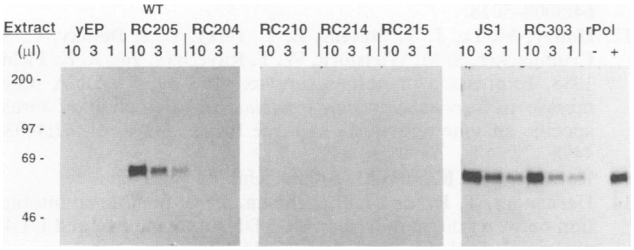

FIG. 4. Coimmunoprecipitation of HSV-1 Pol and UL42 complexes. A total of 10, 3, or 1 μl of yeast extract (or 60 ng of rPol or no Pol) was incubated with 4 μl of radiolabeled UL42 in 15 μl (total volume) of yeast extract buffer (see legend to Fig. 2) for 90 min on ice. A total of 100 μl of IP buffer (0.1% Nonidet P-40, 100 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl [pH 7.6], 0.02% Na azide) was added along with 2 μl of polyclonal rabbit anti-Pol antiserum to the buffer, and the resulting buffer was incubated for 60 min on ice. Thirty-five microliters of protein A-Sepharose (10% [wt/vol] in phosphate-buffered saline) was added, and the samples were rocked at 4°C for 15 min. The precipitates were washed once with 750 μl of IP buffer and twice with RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.2% sodium deoxycholate, 0.1% SDS, 20 mM Tris-HCl [pH 7.5]) and then resuspended in 2× protein sample buffer (6% SDS, 10% β-mercaptoethanol, 100 mM Tris-HCl [pH 6.8], 0.02% bromphenol blue, 20% glycerol). The samples were boiled 3 min, the Sepharose was pelleted, and the supernatants were analyzed by electrophoresis in precast SDS–10% polyacrylamide gels (Integrated Separation Systems, Hyde Park, Mass.). After electrophoresis, the gels were fixed in 5% methanol–7.5% acetic acid, rinsed in H_2O, soaked in 1 M salicylate, dried, and autoradiographed. Numbers at left are molecular sizes in kilodaltons.

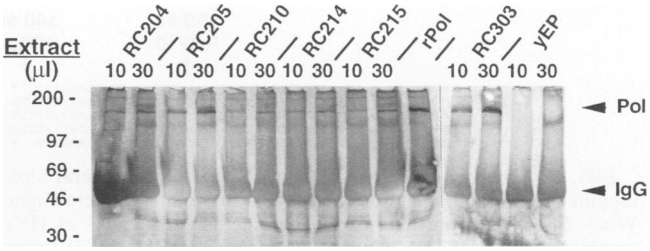

FIG. 5. Immunoprecipitation of Pol mutants by anti-Pol antiserum. A total of 10 or 30 μl of various yeast extracts or 300 ng of rPol was immunoprecipitated with anti-Pol antiserum, as described in the legend to Fig. 4. After electrophoresis, samples were detected by Western blotting (immunoblotting) using polyclonal rabbit anti-Pol antiserum and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) antiserum followed by detection with 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium (BCIP-NBT) (essentially as described previously [22]). Numbers at left are molecular sizes in kilodaltons.

Neutralization of Pol activity in the presence of UL42, therefore, is indicative of a physical interaction between the two proteins. As expected, none of the Pol mutants were affected by the anti-UL42 serum when tested in the absence of UL42; however, in polymerase assays containing purified UL42, the WT Pol and those mutants which could be stimulated by UL42 were strongly inhibited (Fig. 6). The catalytic activities of those mutants which were not stimulated by UL42 showed no UL42-dependent neutralization. Preimmunization antiserum did not inhibit polymerase activity (data not shown).

The data therefore indicate that Pol sequences C terminal to aa 1195 (RC204) or N terminal to aa 228 (JS1) are dispensable for functional interaction of Pol with UL42 in vitro. The inability of Pol with a deletion to aa 1195 to form a stable coimmunoprecipitable complex with UL42, however, suggests that a region distal to this position modulates the stability of the Pol-UL42 complex. Residues between aa

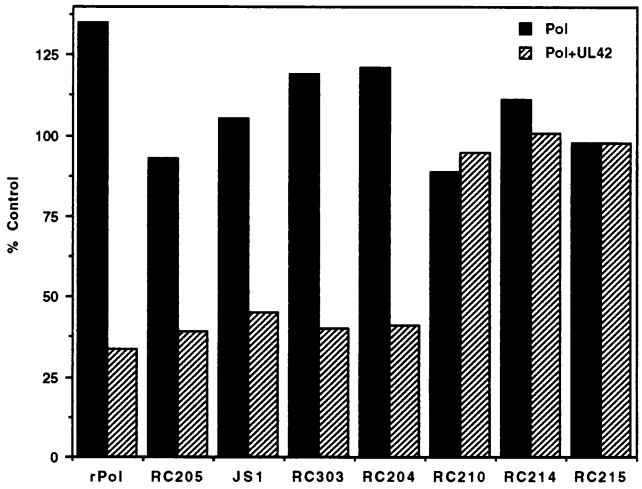

FIG. 6. Effect of anti-UL42 antiserum on Pol activity and stimulation by UL42. Five microliters of rabbit polyclonal anti-UL42 antiserum was included in the 15-min preincubation of the yeast extracts and rUL42. Afterwards, the polymerase assays were performed as described in the legend to Fig. 2. Results are expressed as the activity relative to that in the absence of antiserum.

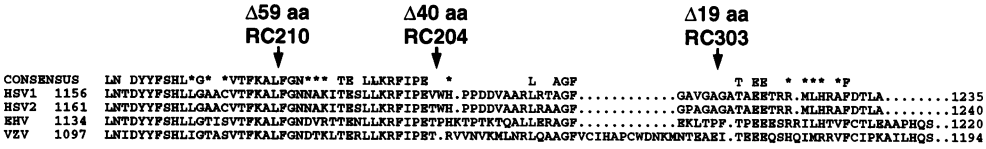

FIG. 7. Comparison of the C-terminal regions of several alphaherpesvirus Pol proteins. The amino acid sequences of the C-terminal regions of several alphaherpesvirus DNA polymerases were aligned according to the Genetics Computer Group Pileup algorithm (Madison, Wis.). Shown are portions of the polymerase sequences of HSV-1 (KOS) (8) (accession no. A00714), herpes simplex virus type 2 (24) (accession no. M16321), equine herpesvirus 1 (EHV) (23) (accession no. M86664), and varicella-zoster virus (VZV) (2) (accession no. X04270). The consensus sequences are indicated by the common amino acids shown above the alignment, and conservative residue positions are noted with asterisks. The locations of termini of the truncation mutants are indicated: RC303, 19-aa truncation; RC204, 40-aa truncation; and RC210, 59-aa truncation.

1195 (RC204) and 1216 (RC303) appear to be particularly important for the stability of the complex such that they are preserved throughout the stringent coimmunoprecipitation assay. It is intriguing to note that secondary structure analysis of the C terminus of Pol predicts a region of alpha-helix (aa 1202 through 1235 [12]). Perhaps an alpha-helical structure formed by this region contributes to the interaction of Pol with UL42. The observation that mutant RC214 (deletion of aa 1073 to 1144) failed to form a functional interaction with UL42 even though it retains the predicted alpha-helix region suggests that the predicted alpha-helix alone is not sufficient for the formation of a functional Pol-UL42 interaction.

The Pol mutant terminating at aa position 1176 (RC210) failed to be stimulated by, or form complexes with, UL42. These results clearly implicate critical residues between aa 1176 and 1195 of Pol in productive interaction with UL42. Comparison of the C-terminal amino acid sequences of several alphaherpesvirus DNA polymerases (Fig. 7) illustrates that this region is more highly conserved than distal sequences in the C terminus which are dispensable for in vitro stimulation. Alignment of the alphaherpesvirus DNA polymerases revealed 45% sequence identity within the entire protein and across the 227-aa C-terminal UL42 binding domain (data not shown), whereas the critical region between residues 1176 and 1195 is more highly conserved (68%). In contrast, only 8 residues of the dispensable 40 aa at the C terminus (20%) are identical. Overall, it appears that the more highly conserved sequences of the Pol C terminus are essential for a productive interaction with UL42 in vitro and that more variable residues at the extreme C-terminal end contribute to, but are not required for, this interaction.

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