Adenovirus DNA polymerase: domain organisation and interaction with preterminal protein

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ABSTRACT

Adenovirus DNA polymerase is one of three viral proteins and two cellular proteins required for replication of the adenovirus genome. During initiation of viral DNA synthesis the viral DNA polymerase transfers dCMP onto the adenovirus preterminal protein, to which it is tightly bound. The domain structure of the 140 kDa DNA polymerase has been probed by partial proteolysis and the sites of proteolytic cleavage determined by N-terminal sequencing. At least four domains can be recognised within the DNA polymerase. Adenovirus preterminal protein interacts with three of the four proteolytically derived domains. This was confirmed by cloning and expression of each of the individual domains. These data indicate that, like other members of the pol α family of DNA polymerases, the adenovirus DNA polymerase has a multidomain structure and that interaction with preterminal protein takes place with non-contiguous regions of the polypeptide chain over a large surface area of the viral DNA polymerase.

INTRODUCTION

Replication of the adenovirus type 2 genome requires three virus coded proteins, preterminal protein (pTP), DNA binding protein (DBP) and the subject of this study, the adenovirus DNA polymerase (Adpol). Two cellular transcription factors and topoisomerase I are also required for efficient replication of the entire viral genome (reviewed in 1,2). After entry of the virus into the cell the 36 kb linear double stranded genome is released into the nucleus and replication is initiated at either end of the DNA by a protein priming mechanism. Prior to initiation of DNA synthesis a large nucleoprotein complex is formed at the viral origin of DNA replication. The preinitiation complex contains the three viral proteins pTP, Adpol and DBP and the two cellular transcription factors NFI (3) and NFIII (4) which are held in a precise stereospecific arrangement on the viral origin of DNA replication by a combination of DNA–protein and protein–protein interactions. Although the three-dimensional structure of the DNA binding domains of NFIII and DBP have been determined (5,6), precise details of the interactions required for assembly of the preinitiation complex have yet to be described. Like many other viral origins of DNA replication, that of adenovirus type 2 contains an essential core domain and auxiliary regions that increase the efficiency of DNA replication. The heterodimer of Adpol and pTP recognises bp 9–18 in the core of the replication origin (7) and in the case of Ad2 and Ad5 this binding is stabilised by specific interactions with NFI and NFIII which bind to adjacent DNA sequences in the auxiliary region of the replication origin (8–12). Sequence specific interactions of NFI with the origin of DNA replication is facilitated by DBP, which appears to alter the structure of origin DNA such that NFI binds more tightly to its recognition site (13,14). Immediately before initiation takes place NFI dissociates (15) and single-stranded origin DNA is exposed in an ATP independent mechanism that is thought to harness the helix destabilising properties of DBP (16,17). In the initiation reaction Adpol catalyses the formation of a phosphodiester bond between the α-phosphoryl group of the incoming dCTP residue and the β-OH group of serine 580 in pTP. DBP increases the efficiency of this reaction by decreasing the $K_m$ of Adpol for dCTP (18). The dCMP residue, covalently bound to pTP, serves as the primer for elongation of the nascent DNA strand by Adpol, but a replication intermediate of pTP-CAT accumulates as a result of priming on the complementary sequence GTA located at nucleotides 4–6 from the terminus. Replication proceeds when this intermediate ‘jumps back’ to the terminus of the genome (19), in a manner that is analogous to the sliding back model described for the linear DNA of bacteriophages Φ29 and PRD1 (20), and pTP-CAT is extended by Adpol. During the subsequent Adpol catalysed strand displacement synthesis, DBP increases the processivity of Adpol (21) and, by the co-operative formation of a protein chain on the DNA, unwinds the double helix ahead of the advancing Adpol (22). Both the dCMP transfer activity required for initiation and the DNA polymerase catalytic activity involved in elongation appear to be carried out at the same site in Adpol (23). Displaced single strands can be converted to double stranded DNA products either by serving as templates for a further round of DNA replication (24–26) or by reannealing to complementary single-stranded DNA (27). During the late phase of infection genome linked pTP is processed, via a 62 kDa intermediate, to mature terminal protein (TP) by the adenovirus coded protease (28,29). Genome linked TP appears to have diverse roles during infection and is known to determine the subnuclear location of viral DNA templates for transcription and replication (30–32). TP directly influences DNA replication by altering the structure of linked origin DNA and stabilising binding of the pTP-Adpol heterodimer to its binding site in the viral origin of DNA replication (33,34).
Recoverable in Adpol are five of the six regions conserved among eukaryotic DNA polymerases (35). Conserved regions I, II and III are thought to form the nucleotide binding domain while the 3′→5′ exonuclease has a proofreading activity (36–38). Also present in Adpol is a sequence common to DNA polymerases which employ protein priming to initiate DNA replication (39). To date mutational analysis has had limited success in identifying regions of Adpol associated with particular activities and sites required for interaction with other proteins have not been determined (40–44). However mutations in the putative zinc finger domains affect DNA binding while binding of pTP and NFI is unaffected. Here we describe the domain structure of Adpol and map the sites of interaction with pTP.

MATERIALS AND METHODS

Purification of Adpol and pTP

Adpol and pTP were expressed in recombinant baculovirus infected Spodoptera frugiperda sf9 cells as described before (8,45). pTP was purified as described (46). One litre of a suspension of sf9 cells infected with baculovirus containing the Adpol gene was collected by centrifugation at 2000 r.p.m., and washed with phosphate buffered saline. Cells were resuspended on ice in 6 ml ice cold buffer I (25 mM Tris pH 8.0, 5 mM KCl, 0.5 mM MgCl2 with protease inhibitors PMSF, TPCK, TLCK, leupeptin, pepstatin and E64). After incubation on ice for 10 min cells were subjected to 20 strokes with pestle B in a Dounce homogeniser and nuclei collected by centrifugation for 3 min at 4°C. Nuclei were extracted by resuspension in 6 ml cold buffer II (25 mM bicine pH 7.5, 0.2 M NaCl, 0.5 mM MgCl2, 10 mM DTT and protease inhibitors as above) and left on ice for 30 min before debris was removed by centrifugation at 60 000 r.p.m. for 20 min at 4°C in a Beckman TL100 ultracentrifuge. The supernatant was diluted 1 to 5 in buffer III (25 mM bicine pH 7.5, 0.2 M NaCl, 1 mM EDTA, 10% glycerol) and loaded onto a 5 ml single stranded DNA cellulose column equilibrated with buffer III containing 0.6 M NaCl and 2 mM DTT. Fractions containing Adpol were pooled, divided into small fractions and snap frozen in liquid nitrogen prior to storage at −70°C. In some instances Adpol was further purified by virtue of its affinity for pTP. Fractions containing Adpol were pooled, divided into small fractions and snap frozen in liquid nitrogen prior to storage at −70°C. In some instances Adpol was further purified by virtue of its affinity for pTP. Peak fractions from the single stranded DNA cellulose column were pooled and loaded with recirculation onto a Hi-trap column containing covalently bound pTP. The supernatant was diluted 1 to 5 in buffer III (25 mM bicine pH 7.5, 0.2 M NaCl, 1 mM EDTA, 10% glycerol) and loaded onto a 5 ml single stranded DNA cellulose (Sigma) column equilibrated with buffer III containing 2 mM DTT. After washing with at least five times column volume of equilibration buffer Adpol was eluted in 0.5 ml fractions in buffer III containing 0.6 M NaCl and 2 mM DTT. Fractions containing Adpol were pooled, divided into small fractions and snap frozen in liquid nitrogen prior to storage at −70°C. In some instances Adpol was further purified by virtue of its affinity for pTP. Peak fractions from the single stranded DNA cellulose column were pooled and loaded with recirculation onto a Hi-trap column containing covalently bound pTP. The supernatant was diluted 1 to 5 in buffer III (25 mM bicine pH 7.5, 0.2 M NaCl, 1 mM EDTA, 10% glycerol) and loaded onto a 5 ml single stranded DNA cellulose column (Sigma) equilibrated with buffer III containing 2 mM DTT. Fractions containing Adpol were pooled, divided into small fractions and snap frozen in liquid nitrogen prior to storage at −70°C.

Endoproteinase lys C digestion of purified Adpol

Purified Adpol was incubated with the indicated ratios (w/w) of endoproteinase lys C for 1 h at 37°C. Reactions were stopped by the addition of SDS and mercaptoethanol, heated to 100°C for 3 min prior to electrophoresis in a polyacrylamide gel containing SDS. In protection experiments Adpol was preincubated at 37°C with purified pTP at the molar ratios indicated before endolys C digestion under standard conditions.

Electrophoresis and immunoblotting

Protein samples were denatured and reduced, by heating to 100°C for 3 min in the presence of 1.25% SDS and 0.35 M β-2-mercaptoethanol. Polypeptides were separated in 10% polyacrylamide minigels containing SDS (SDS–PAGE) prior to staining with Coomassie brilliant blue or silver as appropriate. Proteins to be analysed by immunoblotting were resolved by SDS–PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes (Sigma). Membranes were incubated in blocking buffer (phosphate buffered saline containing 10% non-fat milk and 0.05% Tween 20) and then with the individual primary antibody diluted in blocking buffer. After washing five times with blocking buffer, antibody–antigen complexes were detected by incubation with horseradish peroxidase conjugated anti rabbit or anti mouse antibodies and an enhanced chemiluminescence system (Amersham). Primary antibodies were mouse monoclonal antibodies to pTP (46) and a rabbit antiserum (pol D) raised against Adpol purified from recombinant baculovirus infected insect cells (47).

Solid phase interaction assays

Proteins were resolved by SDS–PAGE, electrophoretically transferred on to PVDF and membranes incubated in blocking buffer as described above. Membranes were then washed in pTP binding buffer (PKT, 50 mM potassium phosphate buffer pH 7.0, 400 mM KCl, 0.05% Tween 20) with 10% non-fat dry milk before incubation with purified pTP or extract of sf9 cells infected with recombinant baculoviruses carrying the pTP gene (TL100 extract described above) or BSA as a control protein. Bound pTP was detected with the 10B1 and 6E2 monoclonal antibodies (46), as described above for immunoblotting. 10B1 and 6E2 are conformation specific monoclonal antibodies and thus detect only native pTP immobilised on the filter by virtue of its interaction with Adpol or its digestion products.

N-terminal peptide sequencing

Peptide products of endolys C digestion of Adpol were separated by SDS–PAGE as described above using freshly made acrylamide–piperazine diacrylamide solution (37.5:1) with the addition of sodium thioglycollate to 0.1 mM to the upper electrophoresis buffer. Fragments were electrophoretically transferred to PVDF membranes (Amersham). Membranes were stained with Coomassie Brilliant Blue for a few seconds before bands were excised. Excised membrane fragments were washed extensively in distilled water. Sequence was determined by Paul Talbot and Graham Kemp using a Ponce microsequencer (Applied Biosystems) with on line phenylthiohydantoin analysis.

In vitro transcription/translation of Adpol fragments

Adpol fragments 1–4 were amplified by PCR from Ad2 genomic DNA using primers introducing N-terminal BamHI and C-terminal EcoRI sites. Oligonucleotides used were as follows: fragment pol 1, 5′-GGG GGG ATG ATC ACAC TGT GCT CCC TTC TTA A and 5′-GGA GGA ATT CCC TTT GTA AAA AAT GGC GCA; fragment pol 2, 5′-GGG GGG ATG ATC CAT GAG CCC ATC TGT CTA GGT TCA A and 5′-GCA GGA ATT CCC TTT GCA ACG GTA

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RESULTS

Domain organisation of Adpol

Limited digestion by proteases with defined cleavage sites has provided a useful means of investigating the structure of large multidomain proteins (46) and has been particularly useful in the investigation of the domain structure of DNA polymerases. Such proteins are often composed of a number of independently folded regions connected by flexible linker sequences. While the highly ordered domains are resistant to proteolytic cleavage the surface exposed unstructured connecting regions are susceptible to protease attack and are also most readily available for interaction with other molecules. Sequences which are part of well defined secondary structure elements or are buried within the molecule are far less readily cleaved. To determine the domain structure of Adpol the 140 kDa protein purified from insect cells (7) was subjected to limited proteolysis with a range of proteases. Limited digestion of Adpol with endoprotease lys C (endolys C) which cleaves to the C-terminus of lysine residues gave a reproducibly clearer pattern of fragments over a wider size range than that produced by cleavage with trypsin, chymotrypsin or proteinase K (Fig. 1 and data not shown). Endolys C was therefore used in all digestion experiments reported in this study. The Adpol sequence is rich in lysines (57 potential endolys C cleavage sites) which are distributed fairly evenly throughout the molecule. Treatment with endolys C reproducibly gives rise to a number of discrete species implying a strong discrimination between potential cleavage sites. Presumably, favoured cleavage sites are located in unstructured regions of the polypeptide chain exposed on the surface of the protein. Digestion of Adpol at endolys C to pol ratios (weight to weight) of 1:50 for 1 h at 37 °C gives rise to major species of apparent MW 98 kDa, 65 kDa and a number of fragments in the 32–35 kDa size range (Fig. 1A). Although fragments smaller than ∼29 kDa are not retained on a 10% polyacrylamide gel (Fig. 1A) a stable species of 14 kDa can be detected after fractionation of the digestion products in a 15% polyacrylamide gel (data not shown). At this enzyme concentration no intact Adpol remains as visualised by Coomassie brilliant blue staining of polyacrylamide gels while reduction in enzyme concentration (1:100 and 1:1000 endolys C to pol) results in some full length material remaining undigested, a greater intensity of the 98 kDa product and a minor product of 110 kDa which is presumably an intermediate between full length Adpol and the 98 kDa product and a minor product of 110 kDa which is presumably an intermediate between full length Adpol and the full length material remaining undigested, a greater intensity of the 98 kDa product and a minor product of 110 kDa which is relatively unstable domain susceptible to further digestion even under mild conditions. Adpol endolys C digestion products can also be visualised by western blotting with a polyclonal antibody raised against intact Adpol (Fig. 1C). This antibody reacts strongly with the 32/35 kDa cluster of proteolytic fragments suggesting that they contain major antigenic epitopes of the molecule. Thus the antigenic activity and protease accessibility at the boundaries of the domains represented by the 32/35 kDa fragments suggests that they occupy an exposed location on the surface of the Adpol molecule.
Figure 1. Endolys C digestion of Adpol. Baculovirus expressed Adpol (purified as described in Materials and Methods) was subjected to partial proteolysis by endoproteinase lys C at the indicated enzyme to Adpol ratios (weight to weight). Adpol digestion products were analysed by electrophoresis through a 10% SDS polyacrylamide gel. (A) Gel stained with Coomassie brilliant blue. Molecular weight of Adpol and approximate sizes of major partial proteolysis products are shown in kDa on the right of the gel. Molecular weight of standards are indicated on the left. (B) Silver stained portion of the same gel showing the 68 kDa molecular weight marker and a 50 kDa proteolytic product not visible after Coomassie brilliant blue staining. (C) Western blot of aliquots of the same digestion reactions. Proteolytic products detected with a polyclonal antibody raised against purified Adpol. Prestained MW standards are indicated on the left of the gel and approximate sizes of main digestion products to the right.

Identification and expression of Adpol domains

To identify sites in Adpol cleaved by endolys C large scale digests of Adpol (endolys C:pol ratio of 1:50) were separated by SDS–PAGE and the polypeptides transferred onto PVDF for N-terminal sequencing. Five digestion product peptides yielded sequences that could be unambiguously identified after 5–10 rounds of N terminal sequencing (Fig. 2B). Sequence could not be obtained from either the 98 or the 65 kDa digestion products which may indicate that they are derived from the N-terminus of Adpol which is blocked and refractory to Edman degradation (data not shown). The 32 kDa fragment contained two Adpol derived species that could be assigned as they were present in different molar amounts (Fig. 2B). While the N-termini of the digestion products could be unambiguously established the C-termini of the fragments were less certain and these were estimated based on the apparent molecular weights of the fragments and the locations of lysines to act as potential cleavage sites for endolys C. The location of the major endolys C cleavage sites in Adpol and the resultant peptide fragments are shown in Figure 2A along with previously recognised motifs known to be important for Adpol function. On the basis of this analysis Adpol was divided into four fragments designated pol 1–4 (Fig. 2) for further study. Pol 1 (predicted molecular weight 25 679) extends from the N-terminus of the molecule to the start of pol 2. Although this is the only fragment not corresponding directly to an endolys C digestion product it does contain the 14 kDa digestion product with N-terminus at P76. It seems probable that the sequences N terminal of those found in the 14 kDa excised band 6 are digested to fragments too small to be detected by polyacrylamide gel electrophoresis. Pol 2 (predicted molecular weight 35 193) extends from S237, mapped as the N-terminus of the 32 kDa proteolytic fragment to the predicted C-terminus of the other 32 kDa fragment which is generated by cleavage after K295. Pol 3 (predicted molecular weight 56 609) with its N-terminus at A521 overlaps at its N-terminus with pol 2 and at its C-terminus with pol 4. Pol 4 corresponds to the 35 kDa proteolytic fragment with N-terminus at S879 and extends to the

Figure 2. Sequencing of Adpol endolys C digestion products. (A) Schematic of Ad 2/5 DNA polymerase showing motifs conserved across DNA polymerases (CR1–5). Also indicated are: a conserved sequence present in DNA polymerases which use protein priming to initiate DNA replication (PP), amino acid sequences conserved in exonuclease enzymes (exo1–3), the nuclear localisation signal (NLS) and putative ‘zinc finger’ DNA binding domains (Zn1, Zn2). Sites of endolys C cleavage giving rise to sequenced digestion products are indicated by arrowheads while the regions corresponding to fragments (pol 1–4) used in the rest of this study are shown by double headed arrows, the N- and C-terminal amino acids of each shown below in brackets. (B) Adpol endolys C digestion products were subjected to 5–10 rounds of Edman degradation N-terminal sequencing. The molecular weight of fragments obtained, the sequences obtained, location of lysines predicted to be N-terminal to the endolys C cleavage site and the corresponding cloned fragments are indicated.

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C-terminus of the polymerase. To confirm this analysis and to provide material for further study, DNA corresponding to each of these fragments was cloned into expression plasmids. For eukaryotic expression each pol fragment was fused at its N-terminus to an epitope from Simian Virus 5 (PK pol series) that is recognised by a previously characterised monoclonal antibody (48) and inserted into pcDNA3, thus permitting [35S] labelled isotope to be transferred onto PVDF for solid phase interaction assays. PVDF membranes were incubated either in the presence of purified pTP (+pTP) (A), or in the absence of pTP (−pTP) (B), and bound pTP detected by interaction with conformation specific anti-pTP monoclonal antibodies 10B1 and 6E2. Arrowheads to the right of (A) indicate positions of Adpol and partial digestion fragments interacting in this assay with pTP.

Interaction of Adpol digestion products with pTP

In vivo Adpol forms a stable heterodimer with the viral pTP. Solid phase interaction assays were used to determine which domains of Adpol participate in this interaction. Endolys C digestion products of Adpol were resolved by SDS–PAGE and transferred onto PVDF membrane. Membranes were blocked and incubated either in the presence (Fig. 3A) or absence (Fig. 3B) of pTP in conditions favouring Adpol/pTP interaction and unbound pTP removed by extensive washing in the same buffer. Bound pTP was detected with anti-pTP monoclonal antibodies 10B1 and 6E2 which recognise native pTP. These antibodies do not detect pTP bound to PVDF after SDS denaturation (46) and thus react only with pTP immobilised by virtue of interaction with membrane bound Adpol and its proteolysis fragments. Fragments which most likely correspond to the 98 kDa endolys C digestion product (detected at endolys C:pol ratio of 1:50) and the 32–35 kDa cluster of products (detected at endolys C:pol ratios of 1:25 and 1:50) bound pTP in this assay. Untreated Adpol and undigested material present after incubation without endolys C or at an enzyme:Adpol ratio of 1:50 also bound pTP (Fig. 3A). As Adpol immobilised on the membrane has been denatured in SDS it is likely that pTP is binding to individual, linear sequences of Adpol. No reactive species are detected by anti-pTP antibodies after incubation in the absence of pTP (Fig. 3B). In addition pTP failed to bind to a range of other proteins (molecular weight markers) immobilised on PVDF (data not shown). To confirm specificity of the Adpol/pTP interaction, binding of pTP to immobilised Adpol was competed by addition of soluble Adpol. Adpol digestion products were resolved and transferred onto PVDF as described and membranes incubated with pTP in the presence (Fig. 4B) or absence (Fig. 4A) of an equimolar amount of purified Adpol. pTP/Adpol interaction was detected by subsequent incubation with conformational anti-pTP monoclonal antibodies 10B1 and 6E2. Adpol in solution competes efficiently for pTP interaction with the membrane bound 32/35 kDa Adpol fragments (Fig. 4B). In this experiment the higher molecular weight species were not sufficiently transferred onto the membrane but longer exposure of the same gel indicates that, as expected, Adpol present in the pTP incubation mix also competes effectively for pTP binding to immobilised 98 and 110 kDa digestion products and to undigested Adpol (data not shown).

Interaction of Adpol domains with pTP

To unambiguously determine which Adpol domains interact with pTP, [35S]methionine labelled, epitope tagged versions of the domains were generated by coupled in vitro transcription and translation of the corresponding cDNAs. 35S labelled proteins representing Adpol fragments 1–4 (Fig. 5C) were mixed with an extract from uninfected insect cells or insect cells infected with baculovirus expressing pTP (A) or a 1:1 molar ratio of pTP and Adpol (B). Bound pTP was detected by reaction with conformation specific anti-pTP monoclonal antibodies. Arrowheads to the right of each blot show the main interacting fragments while an arrow indicates undigested Adpol. Molecular weight standards are to the left.
Figure 5. Immunoprecipitation of Adpol fragments bound to pTP with pTP specific antibodies. [35S]methionine labelled in vitro transcribed and translated (IVTT) Adpol fragments 1–4 were incubated with extract from insect cells either infected with a recombinant baculovirus containing the pTP gene (A) or uninfected (B). Complexes between pTP and Adpol fragments were captured by a cocktail of pTP monoclonal antibodies bound to protein A–Sepharose. After extensive washing, bound proteins were analysed in a 10% polyacrylamide gel containing SDS and [35S]radioactivity in the dried gel detected by phosphorimaging. The IVTT protein present and antibody used to immunoprecipitate resulting complexes are shown for each reaction. ‘–’ represents an IVTT reaction containing empty pcDNA3 vector and ‘mix’ a mixture of equal amounts of pol 1–4 (as judged by quantitation of IVTT reactions analysed by SDS–PAGE and exposure to a phosphorimager screen). As controls an unrelated mAb (10B, specific for IκBα) bound to protein A beads alone (0) were used in immunoprecipitation reactions of a mixture of each of the IVTT pol fragments 1–4 after incubation with extract from pTP expressing (A) or uninfected (B) insect cells. (C) Phosphorimager scan of SDS–PAGE gel of input IVTT reactions. The positions of molecular weight markers are indicated on the left of the panels.

within the pol 2 fragment are bound strongly by pTP in solution. Adpol fragment 1 failed to immunoprecipitate with pTP over a wide range of conditions employed (Fig. 5A). No labelled fragments were immunoprecipitated with a control antibody (Fig. 5A) or when pTP was absent from the binding reaction (Fig. 5B). Identical results were obtained if complexes between pTP and Adpol domains were immunoprecipitated with individual anti-pTP monoclonal antibodies (7H1, 7A2, 11F11 and 10B1) rather than the cocktail of antibodies (data not shown). These data were confirmed when the same Adpol fragments, either produced by in vitro transcription and translation or as glutathione-S-transferase fusions in bacteria were tested for pTP binding after separation by SDS–PAGE and binding to PVDF. Adpol fragments 2, 3 and 4 bound pTP while Adpol fragment 1 failed to bind pTP (data not shown).

Thus regions of Adpol that contact pTP are distributed over all but the N-terminal fifth of the Adpol molecule. These data are consistent with previous reports based on mutational analysis implying multiple interaction sites with pTP on Adpol.

Interaction with pTP protects Pol from endolys C digestion

If there are multiple contacts between Adpol and pTP spread over the Adpol molecule it would be expected that interaction with pTP would afford Adpol some degree of protection from protease attack. Thus Adpol was incubated either alone or in the presence of pTP and subjected to partial proteolytic cleavage with endolys C. Adpol digestion products were resolved by SDS–PAGE and detected by western blotting with an Adpol specific antiserum. Prior incubation with subsaturating levels of pTP (pTP:Adpol molar ratio of 1:2) partially protected Adpol from endolys C digestion under standard conditions (compare 0 pTP and pTP:pol 1:2 in Fig. 6). The pattern of Adpol digestion at the highest concentration of protease is similar to that in the absence of pTP. At lower protease concentrations clear protection from digestion to the 50 and 32/53 kDa species is observed and the 98 kDa species accumulates (Fig. 6). If the concentration of pTP is raised to a 2-fold molar excess, protection of Adpol from digestion to all except the highest molecular weight fragment is observed at all concentrations of endolys C tested (Fig. 6, pTP:pol 2:1). Thus, interaction of pTP with Adpol appears to protect the Adpol from endolys C cleavage at multiple sites. This is consistent with pTP forming multiple contacts spaced over the Adpol molecule.

DISCUSSION

DNA replication in adenoviruses involves the participation of Adpol in a variety of multiprotein/DNA complexes. The precise nature of these protein–protein and protein–DNA interactions
must alter to progress through preinitiation, initiation, elongation and termination of replication. The Adpol molecule is required for every stage of the replication process but few functions have been mapped to particular Adpol polypeptide sequences. Here we have shown that, despite the amino acid sequence containing many potential endoproteinase lys C cleavage sites, proteolytic digestion of Adpol with this enzyme produces only a few discrete species (Fig. 1). It is likely that these species arise by cleavage of those parts of the polypeptide chain exposed on the surface of the protein and are not part of well defined secondary structure elements such as α-helices or β-sheets. Thus the cleavage sites may represent regions of poorly ordered polypeptide chains that act as linkers between domains in Adpol or exposed loops that connect buried elements of the protein core.

Structural analysis of DNA polymerase enzymes from diverse sources has indicated that these molecules share a similar domain arrangement that has been compared with the shape of a hand with subdomains consisting of ‘thumb’, ‘palm’ and ‘fingers’ (49). Recently the structure of a pol α family member, which shares some sequence similarities to Adpol, was determined and revealed a molecule with the overall shape of a disc with a hole in the centre (50). Residues surrounding the central hole are directly involved in catalytic activity and three deep grooves, formed by five domains, converge on this point.

Mutational analysis of Adpol has identified the catalytic carboxylates present in the DTD sequence of conserved region I (44) but has failed to shed light on the domain organisation of Adpol. Unique to adenovirus DNA polymerases are two putative zinc finger domains (Fig. 2A). This class of sequence is implicated in the DNA binding of a number of proteins and indeed mutation of the two zinc binding motifs in Adpol has a profound effect upon DNA binding and initiation of replication (43). In this context it is worth noting that the more N-terminal of the two zinc finger motifs (Zn1 in Fig. 2A) is located at a region of susceptibility to endolys C (major cleavage sites 236 and 295) while the other (Zn2 in Fig. 2A) is found toward the end of the molecule (C-terminal half of fragment 4). This region cannot be ruled out as protease sensitive as species produced by endolys C cleavage at this point might be too small to be retained in the 10% polyacrylamide gels used in this study. Thus, it seems probable that at least one and possibly both of the putative zinc finger domains lie on the surface of the polymerase molecule which would be consistent with a role in DNA binding or interaction with other proteins.

It is likely that regions of a protein accessible to proteases and forming antigenic determinants would be on the surface of the molecule and thus available for interaction with other proteins. We have shown that indeed endolys C generated fragments of Adpol denatured and immobilised on a solid phase can interact with pTP in solution (Figs 3 and 4). Having expressed these fragments individually, in a form that should allow them to fold into independent domains, we have demonstrated that interaction with pTP occurs at a number of points over all but the most N-terminal fifth of the Adpol (Fig. 5) and interaction with pTP protects Adpol from digestion to these fragments (Fig. 6). Although the pol 3 fragment (521–1019) generated by in vitro translation interacts with pTP in solution the same fragment does not appear to interact with pTP when it is first denatured in SDS and immobilised on PVDF. In this instance the region of Adpol recognised by pTP may be required to adopt a folded conformation that can be attained after in vitro translation but not after SDS–PAGE and electrophoretic transfer onto PVDF. Thus, it appears that pTP interacts with a number of polypeptide domains spread over Adpol. This is entirely consistent with the mutagenesis data where a single pTP binding domain on Adpol cannot be demonstrated. In the solid phase interaction assays Adpol is initially denatured in SDS and it seems likely that in each fragment pTP is interacting with short linear sequences of Adpol.

During the adenovirus DNA replication process Adpol and pTP are involved in a number of different protein/protein and protein/DNA interactions. Indeed Adpol and pTP themselves interact in two distinct fashions. As well as forming the Adpol–pTP heterodimer in solution Adpol binds pTP already bound to DNA at the 5’ ends of replication intermediates (33). The availability of a number of sites on the Adpol molecule for interaction with pTP may allow for variation in the strength of such binding, depending on which particular sites are used and in what combination. Such changes in protein/protein interaction may facilitate the orderly sequence of conformational changes and interactions required in the progress of adenovirus DNA replication from preinitiation complex formation at the origin, to initiation, to elongation and termination. It seems probable that interactions of Adpol with other replication proteins may be of a similar nature and we have observed that NFI binds at least two Adpol endolys C digestion products denatured and immobilised on a solid phase (unpublished). Similarly, recent data suggests a number of contact points on the pTP molecule for Adpol (46). The availability of a range of possible interactions between replication proteins may allow precise control of the adenovirus DNA replication process by enabling the conformational changes required for transition from one stage to the next.

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