Activation of Adenovirus-Coded Protease and Processing of Preterminal Protein

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Adenoviruses code for a protease that is essential for infectivity and is activated by a disulfide-linked peptide, derived from the C terminus of the virus structural protein pVI (pVI-CT). The protease was synthesized at relatively high levels late in infection and was detected in both cytoplasmic and nuclear fractions of adenovirus-infected cells. DNA was not found to be a cofactor of the protease, as previously proposed (W. F. Mangel, W. J. McGrath, D. Toledo, and C. W. Anderson, *Nature [London]* 361:274–275, 1993), but a role for DNA in facilitating the activation of the protease by pVI-CT in vivo cannot be ruled out. Adenovirus preterminal protein is a substrate for the virus-coded protease, with digestion to the mature terminal protein proceeding via the formation of two intermediates. Each of the three cleavage sites in the preterminal protein was identified by N-terminal sequencing and shown to conform to the substrate specificity of adenovirus protease, (M,L,I)XGX-X. Functional studies revealed that preterminal protein and intermediates but not mature terminal protein associated with adenovirus polymerase, while only the intact preterminal protein and none of its digestion products bound to DNA. These results suggest that the virus-coded protease may influence viral DNA replication by cleavage of both genome-bound and freely soluble preterminal protein, with consequent alterations to their functional properties.

Virus-coded proteases have been shown to play central roles in the infectious cycles of both DNA and RNA viruses. Many have novel reaction mechanisms and are very selective in terms of the peptide bonds that they are able to cleave, with secondary and tertiary structures of substrates being important elements. The two main processes carried out by virus-coded proteases are the separation of polyproteins into functional units in RNA viruses and maturation cleavages of structural proteins in DNA viruses (16). Nevertheless, they have also been shown to carry out specific cleavages that serve to regulate virus RNA synthesis and the translation of cellular proteins in the cases of the Sindbis virus nsP2 and poliovirus 2A proteases, respectively (7, 50).

The adenovirus protease is coded for by the L3 region of the genome and specifically cleaves at least six virus-coded proteins and the cellular protein cytokeratin 18, late in infection (5, 37, 42). A temperature-sensitive mutant of adenovirus type 2 (Ad2), Ad2*ts1*, that is defective in protease activity at nonpermissive temperatures has been isolated, and the mutation has been mapped to a single base in the protease gene, resulting in a proline-to-leucine substitution in the protein (51). Synthetic peptides have been used to define the substrate specificity of the protease as (M,L,I)XGX-X, and inhibitor studies suggest that it is a member of the subclass of cysteine proteases that are mechanistically related to trypsin (46, 47). Sequence alignments and site-directed mutagenesis of the protease, however, indicate that the enzyme may represent a new subclass of cysteine protease (27). Recently, it has been shown that adenovirus protease is activated by a disulfide-linked peptide derived from the C terminus of the virus structural protein pVI, with the suggested activation mechanism being one of thiol-disulfide interchange (44). In an

independent study, it has been proposed that, in addition to the 11-amino-acid pVI-derived peptide, viral DNA is a cofactor for the protease and that in the absence of DNA, virtually no activity can be detected (19). One of the substrates of the protease, the preterminal protein (pTP), is the protein primer for viral DNA replication and is cleaved via an intermediate to the mature terminal protein (33). There is controversy about the exact location of the cleavage sites that give rise to intermediate and mature terminal proteins (29, 37, 47), and the relevance of proteolytic processing of pTP to DNA replication remains to be established.

Studies of DNA replication in yeasts and of simple genomes in eukaryotic cells, including those of the animal viruses simian virus 40 and adenovirus, have proved invaluable in defining the essential components for replication. These include an origin of DNA replication, specific origin-binding proteins, and auxiliary sequences, binding host proteins, that stimulate initiation of DNA replication (8). In Ad2, the origin-binding proteins are virus-coded DNA polymerase (Adpol) and pTP, which form a stable heterodimer and together bind specifically to bp 9 to 18 of the adenovirus DNA (28, 36). The efficiency of adenovirus DNA replication is enhanced by the specific binding of two cellular factors, nuclear factor I (NFI) and nuclear factor III (NFIII), to auxiliary DNA sequences at bp 19 to 39 and 40 to 51, respectively (23, 26, 39). NFI stimulates binding of Adpol-pTP to the origin through a direct protein-protein interaction with Adpol (3, 4), stabilizing the preinitiation complex (22). The mechanism of action of NFIII appears to be distinct from that of NFI (21), and DNA binding of NFIII is accompanied by structural changes in the origin DNA that may influence the frequency of initiation (40). One other protein, the virus-coded DNA-binding protein, is required for adenovirus DNA replication in vitro and in vivo. Included among the multiple functions of DNA-binding protein are enhancing the affinity of NFI for its recognition site in the origin of DNA replication, increasing the processivity of Adpol during elongation, and acting as a helix-destabilizing protein (6, 18, 20, 34, 52).

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Following the assembly of these proteins into a preinitiation complex at the viral origin of DNA replication, Adpol catalyzes the transfer of dCMP to Ser-580 of pTP, which serves as the protein primer for DNA replication. Initiation is followed by elongation of the nascent DNA strand by Adpol, with fork movement being accompanied by DNA strand displacement (reviewed in references 14, 31, and 38).

Here we investigate the mechanism by which the viral protease is activated and examine the proposed role of DNA in this process. Sites at which the active protease cleaves the adenovirus pTP have been identified and the functional consequences of this proteolytic processing have been determined.

MATERIALS AND METHODS

Cells and virus. HeLa cells were maintained in suspension in Glasgow S-MEM medium supplemented with 7% newborn calf serum at a density of 3×10^5 to 6×10^5 cells per ml. Ad2 was grown in suspension cultures of HeLa cells, extracted with fluorocarbon, and titrated as described previously (48, 49).

Preparation of Ad2-infected cell extracts. HeLa spinner cells were infected with Ad2 at a multiplicity of infection (MOI) of 10 PFU per cell, and at 0, 6, 12, 18, 36, and 52 h postinfection, 200 ml of cells was collected by centrifugation for 15 min at $1,000 \times g$ and washed with phosphate-buffered saline (PBS). Cytoplasmic and nuclear extracts were prepared from cell pellets (17), with the final volume of each extract being 1 ml for each time point.

Peptide synthesis. Peptides were synthesized and purified as previously described (47).

Preparation of antisera. Antisera were raised against the synthetic peptides MALSVNDCARLTGQSC and CRPNPRN EEICWIEM, corresponding to the N terminus of Ad2 pTP and C terminus of Adpol, respectively. Peptides were coupled to human serum albumin via cysteine residues by using the linkage agent *N*-succinimidyl-3-(2-pyridyldithio)-propionate (45). Rabbits were immunized with peptide-human serum albumin conjugate emulsified in Freund's complete adjuvant and boosted at 14-day intervals with conjugate emulsified in incomplete adjuvant. The preparation of polyclonal antiserum to pTP and of the antiserum specific for the N-terminal amino acids of the adenovirus protease has been described previously (35, 44).

SDS-PAGE and Western (immuno) blotting. Protein samples were denatured and reduced by boiling in the presence of 1.25% sodium dodecyl sulfate (SDS)–0.35 M 2-mercaptoethanol prior to being analyzed by polyacrylamide gel electrophoresis (PAGE) in 10% polyacrylamide (29:1, acrylamide-bisacrylamide) gels containing SDS. Separated proteins were electrophoretically transferred from the SDS gel onto a polyvinylidene difluoride (PVDF) membrane (Sigma) in 48 mM Tris–39 mM glycine–20% methanol at 60 mA for 1 h with a semidry system (Pharmacia LKB). Membranes were blocked for 30 min at 20°C in PBS–0.1% Tween 20–5% nonfat milk powder (PBS-TM buffer) and then incubated for 30 min with primary antiserum. Primary antisera were used at a dilution of 1:4,000 in PBS-TM buffer. Membranes were washed in PBS-TM (twice for 10 min each) and then incubated for a further 30 min with horseradish peroxidase-conjugated protein A (Amersham), diluted 1:2,000 in PBS-TM, prior to two 10-min washes in PBS-TM and two 10-min washes in PBS–0.1% Tween 20. Blots were developed with an enhanced chemiluminescence detection reagent (Amersham) and visualized on X-ray film.

In some cases, PVDF membranes were stripped of antibodies by treatment with 100 mM 2-mercaptoethanol–2% SDS–

FIG. 1. Expression of adenovirus protease in Ad2-infected HeLa cells. Cells were infected at an MOI of 10 PFU per cell, and cytoplasmic and nuclear extracts were prepared at 0, 6, 12, 18, 36, and 52 h postinfection. Proteins in extracts were separated by SDS-PAGE and analyzed by Western blotting with an antipeptide serum raised against the 15 N-terminal amino acids of the Ad2 protease as the primary antiserum. Either 100 or 50 μ g of total protein was applied to gels for cytoplasmic and nuclear extracts, respectively. un, Uninfected control.

62.5 mM Tris-HCl (pH 6.7) for 30 min at 70°C and then washed extensively with PBS–0.1% Tween 20. Membranes were then blocked in PBS-TM and reprobed with antisera, washed, and developed as described above.

Purification of recombinant adenovirus proteins. Adpol, pTP, and protease were expressed and purified from sf9 cells infected with recombinant baculoviruses as described previously (36, 41, 44).

Protease assays. Purified baculovirus-expressed Ad2 protease (25 ng) was activated by incubation with 2 μ g of GVQSLKRRRCF (pVI-CT) per ml in 25 mM Tris-HCl (pH 8)–1 mM EDTA for 15 min at 20°C, with Ad2 DNA (0.6 μ g/ml), 2 mM dithiothreitol (DTT), or 1 M NaCl being included as indicated. In some experiments, protease was activated in the absence of EDTA and treated with DNase I (final concentration, 10 U/ μ l) in the presence of 5 mM MgCl₂ for 5 min at 20°C. The activity of DNase I was stopped by the addition of EDTA to 20 mM. Following the incubation with pVI-CT, the peptide substrate LSGGAFSW (20 μ g) was added to give a final assay volume of 50 μ l, with incubation for 1 to 30 min at 37°C. Reactions were stopped by removing 5 μ l of incubated sample and adding 10 μ l of 0.1 M phosphate (pH 2.5) and 85 μ l of double-distilled water. Specific digestion of LSGGAFSW to the tetrapeptides LSGG and AFSW was monitored by capillary electrophoresis at 200 nm with a Biofocus 3000 with a coated capillary (24 cm by 25 μ m). Quantitative data were obtained by measuring peak areas.

Digestion of pTP by adenovirus protease. Recombinant Ad2 protease (20 to 400 ng) was activated by preincubation with 2 μ g of pVI-CT peptide per ml for 15 min at 20°C. Purified recombinant pTP (1 to 20 μ g) was incubated with activated protease for 5 min to 12 h at 37°C. The usual assay buffer contained 50 mM Tris-HCl (pH 8), 0.2 M NaCl, and 5 mM EDTA. In some assays, bovine serum albumin (BSA) or Adpol was included at the concentrations indicated. Reactions were stopped by the addition of dithiodipyridine to a final concentration of 0.5 mM.

Immunoprecipitations. Polyclonal antiserum specific for the C-terminal amino acids of Adpol was coupled to protein A-agarose beads (Sigma) by using the cross-linking reagent dimethylpimelimidate (13). Samples of pTP and Adpol or BSA were clarified by centrifugation at $40,000 \times g$ for 15 min and incubated with 10 μ l of antibody-protein A beads for 2 h at 4°C (final volume, 100 μ l). Supernatants were collected following

FIG. 2. Effect of DNA and NaCl on adenovirus protease activity. (A) Cleavage of the peptide substrate LSGGAFSW by Ad2 protease in 25 mM Tris-HCl (pH 8)–1 mM EDTA with and without DNA (0.6 μ g/ml) and in 25 mM Tris-HCl (pH 8)–1 mM EDTA–1 M NaCl with and without DNA (0.6 μ g/ml). The progress of the reactions was monitored by generation of peptide AFSW (nanomoles). Initial rates were determined from slopes of lines during linear phases of reactions. (B) Purified, activated Ad2 protease was incubated in the presence and absence of DNase I (10 U/ μ l) for 5 min at 20°C prior to being incubated for 20 min at 37°C with the peptide substrate LSGGAFSW. The progress of the reactions was monitored as above. (C) Ad2 protease was incubated with activating peptide in low-ionic-strength buffers with and without DNA (0.6 μ g/ml) and in 1 M NaCl buffers as above. DTT was added to 2 mM 0, 2, 5, and 15 min after addition of pVI-CT. Protease samples were then incubated for 15 min with the

centrifugation at 6,500 rpm for 3 min in a microcentrifuge, and the beads were washed three times with 10 ml of 25 mM Tris-HCl (pH 8)–0.3 M NaCl–1 mM EDTA–0.05% Nonidet P-40. Antibody-protein complexes were disrupted by treating the beads with 8 M urea–3% SDS for 5 min at 20°C, and supernatants were collected following low-speed centrifugation as above. Samples were then analyzed by SDS-PAGE and Western blotting.

Gel electrophoresis DNA-binding assay. Two complementary oligonucleotides containing the 1 to 18 sequence from the Ad2 origin of DNA replication (36) were annealed and labeled with the Klenow fragment of DNA polymerase I in the presence of [α - ^{32} P]dATP and [α - ^{32} P]dCTP. The labeled fragment was purified on an 8% polyacrylamide gel (29:1, acrylamide-bisacrylamide), eluted from the gel by passive diffusion into 10 mM Tris-HCl (pH 7.5)–100 mM NaCl–1 mM EDTA, and stored at 4°C.

Purified recombinant pTP (80 ng) was incubated with ^{32} P-end-labeled double-stranded oligonucleotides for 15 min at 20°C in a total volume of 20 μ l containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 7.5), 1 mM DTT, 1 mM EDTA, 20 mM KCl, and 1 mg of BSA per ml. At the end of the incubation period, glycerol was added to 5%, and samples were loaded onto a 6% polyacrylamide gel (44:0.8, acrylamide-bisacrylamide) containing 50 mM Tris-borate and 1 mM EDTA (pH 8.3), which was electrophoresed at 10 V/cm for 60 min at 4°C. Labeled species were visualized by exposure of dried gels to X-ray film at –70°C in the presence of intensifying screens.

Sequence analysis. Sequence analysis of proteins was carried out by the sequencing facility (University of Aberdeen).

Molecular modeling. Structures of peptides were simulated with the CHEM-X software package (Chemical Design Ltd.), using energy-minimized random structure.

RESULTS

Adenovirus protease is expressed at high levels late in infection. HeLa cells grown in suspension were infected with Ad2 at an MOI of 10 PFU per cell and harvested at 0, 6, 12, 18, 36, and 52 h postinfection. Cytoplasmic and nuclear extracts were prepared and analyzed by SDS-PAGE, and Ad2 protease was detected by Western blotting with antiserum specific for the N-terminal 16 amino acids of the protein. The protease is expressed at high levels late in infection (Fig. 1) and is present in both cytoplasmic and nuclear fractions, in keeping with its designation as a late protein and the location of the gene in the L3 region of the genome. Other experiments (data not shown) show that low levels of protease can be detected in the cytoplasm and nucleus at 24 and 28 h postinfection.

DNA and buffers of high ionic strength stabilize the activity of adenovirus protease in the presence of DTT. We have previously reported that the adenovirus protease is activated by a disulfide-linked, virus-coded peptide and have proposed that the mechanism is one of thiol-disulfide interchange (44). Mangel and coworkers (19) suggest that in addition to the peptide, the presence of DNA is essential for protease activity. Here we investigated further the effect of DNA on protease activity. Assays were carried out with the peptide substrate LSGGAFSW, and initial rates of digestion by protease were determined with the standard assay buffer in the presence and

peptide substrate LSGGAFSW, and the digestion to AFSW was compared with that in the control (no DTT) in each case.

absence of 25 pM Ad2 DNA. In contrast to previously reported work (19), protease was found to be active in the absence of DNA; moreover, DNA was found to have no effect on the initial rate of digestion of the peptide substrate (Fig. 2A). The inclusion of 1 M NaCl in the assay buffer, however, was found to increase the initial velocity of the reaction by fivefold, from 0.5 to 2.5 nmol of peptide AFSW per min. As in the case of the low-ionic-strength buffer, the inclusion of Ad2 DNA was found to have no effect on the initial rate of digestion of LSGGAFSW with assay buffer containing 1 M NaCl (Fig. 2A).

To rule out the possibility that the above results (Fig. 2A) are a consequence of the protease preparations being contaminated with DNA, purified protease was treated with DNase I as described in Materials and Methods. Control protease was activated and treated identically except that no DNase I was added. The DNase I-treated and control protease samples were assayed with the peptide substrate LSGGAFSW, and initial rates of digestion by protease were determined as described above. Untreated and DNase I-treated protease samples had indistinguishable initial rates of digestion of the peptide substrate (Fig. 2B), indicating that DNA is not required for catalytic activity of the protease. One explanation for the apparent discrepancy between our results and those of Mangel et al. (19) could be the inclusion of 1 mM DTT in their assay buffers, since DTT has previously been shown to inhibit the activation of the protease by pVI-CT (44).

Adenovirus protease was incubated with pVI-CT in standard low-ionic-strength assay buffer (with and without Ad2 DNA) or in assay buffer containing 1 M NaCl for 15 min at 20°C. DTT was added to a final concentration of 2 mM at 0, 2, 5, and 15 min after the addition of pVI-CT. Peptide substrate, LSGG AFSW (20 µg), in appropriate buffer was added to each protease sample, and digestions were carried out for a further 15 min at 37°C. Rates of digestion of LSGGAFSW were compared with those in the control assay containing no DTT in each case (Fig. 2C). The results show that, in all three buffer systems, if DTT is added 0, 2, or 5 min after pVI-CT, protease activity is markedly reduced compared with the appropriate no-DTT control. If DTT is added 15 min after pVI-CT, protease activity in the standard low-ionic-strength buffer is only 25% of that of the control (no-DTT) value (Fig. 2C, control), while inclusion of DNA in the same buffer results in 70% of the control (no-DTT) activity's being retained (Fig. 2C, +DNA). In the high-ionic-strength buffer, DTT added 15 min after pVI-CT had no inhibitory effect on protease activity (Fig. 2C, 1 M NaCl).

From these data, we can conclude that DNA is not required for protease activity and so is probably not a cofactor, but may serve instead to stabilize the enzyme when reducing agents such as DTT are present.

Identification of sites in pTP cleaved by protease. pTP and protease were purified from insect cells infected with recombinant baculoviruses as described previously (36, 44). Purified pTP (1 µg) was incubated with protease (20 ng) in the presence of pVI-CT for 0, 5, and 30 min and 2 and 12 h as described in Materials and Methods, and samples were analyzed by SDS-PAGE and Western blotting with a polyclonal antiserum raised against pTP. Figure 3A shows that while the pTP is completely digested to an intermediate form (iTP) within 5 min, digestion to the terminal protein (TP) is much slower, with a 12-h incubation being required for digestion to the TP. In certain gels, the iTP band appeared as a doublet, with the upper band being present only in the 5-min-digested samples, suggesting that the iTP band comprises two species (see Fig. 6B). pTP (20 µg) was incubated with activated protease (400 ng) for 5 and 30 min and 12 h, and samples were separated by

SDS-PAGE, transferred to a nylon membrane, and stained with naphthalene black. N-terminal sequencing of the bands corresponding to the iTP and TP species was then carried out. The results revealed that the iTP band comprised two species, with N termini GVTRMGGRGR (5-min digestion) and GRHLRPNSAA (30-min digestion), with the N terminus of the TP being VGQLRPRENG (12-h digestion). Thus, Ad2 pTP is cleaved by the protease at two sites separated by eight amino acids (MRGF-G and MGGR-G, amino acids 175 to 176 and 183 to 184, respectively) to give iTP and at the MTGG-V site (amino acids 349 to 350) to give TP (Fig. 3C). It is noted that the sites giving rise to both the iTPs have a glycine in the P1' position, while the TP site has the branched amino acid valine at P1'. CHEM-X models of the peptides reveal that the side chain of the valine protrudes across the scissile bond (Fig. 3D). Previous studies have shown that peptides containing amino acids with small side chains, such as glycine and alanine, in the P1' position are cleaved preferentially by adenovirus protease, and therefore the structures of the peptides are consistent with the observation that cleavage to the iTP proceeds at a much faster rate than that from iTP to TP.

pTP genes from five other adenovirus serotypes have been sequenced, and it is noted that, in each case, the TP site and at least one of the iTP sites conform to the substrate recognition motif of the adenovirus protease, (M,L,I)XGX-X (Fig. 3C). Moreover, in each of the iTP sites, there is a glycine or alanine at P1', and in all the TP sites, there is a valine at P1'. We propose that processing of the pTP is a two-step process and that the rapid cleavage to iTP followed by the slower cleavage to TP may serve separate functions in the infectious cycle.

Effect of Adpol on rate of digestion of pTP. In order to participate in initiation of DNA replication, pTP is required to form a stable heterodimer with Adpol and to bind specifically to DNA at the origin of replication. Insertion and deletion mutagenesis studies have been carried out previously with a view to determining which regions of pTP interact with Adpol and DNA (11, 12, 24, 29, 30), but in the main, these results have shown that the protein is very sensitive to such changes, and therefore the Adpol and DNA-binding domains of pTP have not been identified. Here we examined the interactions of the proteolytic fragments of pTP with Adpol and DNA in an attempt to define the critical amino acids involved in binding. A time course digestion of pTP (1 µg) with activated Ad2 protease (20 ng) was carried out for 0, 5, or 30 min or 2 or 12 h, as above, in the presence of 2 µg of either purified Adpol or BSA, as a control. Samples were separated by SDS-PAGE, transferred to a PVDF membrane, and probed with a polyclonal antiserum raised against purified pTP (Fig. 4). In the presence of Adpol, the rate of digestion from pTP to iTP was reduced considerably, with 50% of pTP remaining undigested after 30 min. The presence of BSA had no effect on the rate of digestion of the pTP or iTP, indicating that the inhibitory effect of Adpol is specific. We conclude that either Adpol is binding to pTP in the region of the iTP site or that Adpol binds elsewhere in pTP, altering its conformation in such a way as to reduce the affinity of the protease for the iTP site.

pTP and iTP but not TP bind to Adpol. pTP (1 µg) was incubated in the presence and absence of activated Ad2 protease (100 ng) for 10 min and 2 h, and digestion was stopped by the addition of dithiodipyridine to 0.5 mM, after which each sample was incubated in the presence of 2 µg of purified Adpol or BSA for a further 15 min at 37°C. Samples were diluted in 25 mM Tris-HCl (pH 8)–0.3 M NaCl–1 mM EDTA–0.05% Nonidet P-40 to a final volume of 100 µl prior to clarification by centrifugation. Protein complexes were immunoprecipitated with the Adpol C-terminal antipeptide

FIG. 3. Sequencing of sites cleaved in pTP by Ad2 protease. (A) Western blot showing the digestion of pTP to TP via iTP. pTP was incubated with Ad2 protease for 0, 5, or 30 min or 2 or 12 h, and digestion products were separated by SDS-PAGE prior to analysis by Western blotting with a polyclonal antiserum raised against pTP. (B) pTP was incubated with protease for 5 or 30 min or 2 h, samples were transferred to a nylon membrane, and iTP and TP species were sequenced. The sequences corresponding to the N termini of the two iTP proteins and the TP are shown. (C) Schematic diagram of Ad2 pTP, showing the positions of the sites cleaved by the protease, giving rise to iTP and TP. Listed below are the sequences from preterminal proteins from the other serotypes sequenced to date. Sequences were obtained with the Genetics Computer Group sequence analysis package (9). (D) CHEM-X models of amino acids surrounding two iTP and one TP cleavage site (MGGRGRHL, MRGFGVTR, and MTGGVFQ, respectively). The position of the cleaved bond is marked by an arrow in each case.

serum coupled to protein A-agarose beads, and the resulting samples were separated by SDS-PAGE. pTP and proteolytic fragments were detected in Western blots with polyclonal anti-pTP and pTP N-terminal antipeptide sera. The blots in Fig. 5A reveal that following 10-min and 2-h incubations with protease, all pTP was digested to iTP and TP, respectively. Note that these assay mixes included 100 ng of protease, explaining the shorter digestion times than in Fig. 4. The Western blot of immunoprecipitations probed with polyclonal

FIG. 4. Digestion of pTP, alone or in association with Adpol, by the adenovirus protease. pTP was incubated with BSA or Adpol for 15 min at 37°C to allow heterodimer formation. Samples were then incubated with activated Ad2 protease for a further 0, 5, or 30 min or 2 h or 12 h at 37°C. Proteins were separated by SDS-PAGE, and digestion to iTP and TP was determined by Western blotting and probing with the polyclonal antiserum raised against pTP.

anti-pTP serum reveal that only pTP and iTP, not TP, bind to Adpol. In control samples to which no Adpol was added, no pTP or digestion products were precipitated, ruling out the possibility that the proteins were binding nonspecifically to the beads (Fig. 5B).

It was previously noted that the polyclonal antiserum raised against recombinant pTP reacted only with TP and not the N-terminal fragments released by the protease (44). To determine whether the N-terminal 188 amino acids remain associated with iTP following digestion to iTP by adenovirus protease, the PVDF membrane in Fig. 5B was stripped of antibody and reprobed with pTP N-terminal peptide antiserum. The N-terminal fragment was precipitated in the presence of Adpol (Fig. 5C), indicating that it remains associated with Adpol-bound iTP under the conditions used in this assay (0.3 M NaCl and 0.05% Nonidet P-40). The same N-terminal fragment, however, was not coprecipitated with Adpol when all pTP and iTP had been cleaved by protease to TP (Fig. 5C). Taking these results together with those above, we conclude that the integrity of the TP cleavage site is critical for the pTP-Adpol interaction.

Only the pTP and not any of the proteolytic fragments binds to DNA. pTP has been shown to bind weakly but specifically to the DNA at the origin of replication (36). To determine which region of the protein is responsible for DNA binding, pTP (2 µg) was digested with activated Ad2 protease (20 ng) for 0, 5, or 30 min or 2 h or 6 h, digestion was stopped by the addition

FIG. 5. Association of Adpol with pTP and digestion products. pTP was incubated with Ad2 protease for 10 min or 2 h at 37°C in either the absence or presence of pVI-CT. Digested samples were then incubated with BSA (–pol) or Adpol (+pol) for a further 15 min at 37°C to allow heterodimer formation. Proteins were immunoprecipitated with a polyclonal antipeptide serum raised against the C-terminal amino acids of Adpol. Supernatants and precipitated proteins were then separated by SDS-PAGE and analyzed by Western blotting with either polyclonal antiserum raised against pTP or a peptide corresponding to the N-terminal 15 amino acids of pTP. Controls in which pTP was incubated with inactive protease (no pVI-CT) are included for each sample (lanes C). (A) Supernatants (Snts.) of immunoprecipitated samples probed with polyclonal antiserum raised against pTP. (B) Precipitated proteins (IPs) probed with polyclonal antiserum raised against pTP. (C) Filter from panel B stripped and reprobed with polyclonal antipeptide serum reacting with the N terminus (N-term) of pTP.

of dithiodipyridine at a final concentration of 0.5 mM, and the progress of the reaction was monitored by SDS-PAGE and Western blotting as described previously (Fig. 6B). Equivalent amounts of protein from each digestion point (80 ng) were incubated with a double-stranded oligonucleotide containing the terminal 18 bp of the Ad2 origin of DNA replication (bp 1 to 18), and DNA-protein complex formation was examined by a gel electrophoresis DNA-binding assay (Fig. 6A). DNA-binding activity was detected only in the zero-time sample containing the pTP. Digestion to the iTP abolished DNA binding, indicating that only intact pTP, not iTP, TP, or other proteolytic fragments generated by the adenovirus protease, binds to DNA. Incubation of pTP with either unactivated protease or pVI-CT (2 µg/ml) for 5 to 120 min at 37°C did not affect the ability of the protein to bind to DNA (results not shown). These results indicate that either the DNA-binding

FIG. 6. Binding of pTP and digestion products to DNA. pTP was incubated with activated Ad2 protease for 0, 5, or 30 min or 2 or 6 h at 37°C, and digestion to iTP and TP was monitored by SDS-PAGE and Western blotting with polyclonal antiserum raised against pTP (B). Protein samples (80 ng) from each time point were incubated for 15 min with a ³²P-labeled double-stranded DNA probe corresponding to bp 1 to 18 of the Ad2 origin of replication. Binding of pTP and digestion products to DNA was analyzed on a nondenaturing polyacrylamide gel (A).

site in the pTP resides across the iTP cleavage sites or that following cleavage at iTP site, the protein undergoes a conformational change that renders it inactive in DNA binding.

DISCUSSION

It has been proposed that adenovirus protease is only active following assembly of virus particles and that its primary function is to carry out limited proteolysis of virus structural proteins to convert immature virus into virions (43). Here we show that Ad2 protease is expressed at high levels late in infection and can be detected in both cytoplasmic and nuclear fractions. This is in keeping with its designation as a late protein, being coded for by the L3 region along with the hexon and pVI; the latter, significantly, is required to activate the protease (19, 44). Given that there are only about 10 copies of protease per virion (1), the high levels present outside virus particles late in infection may be significant with regard to cellular substrates, particularly given the recent observation that cytokeratin 18 is cleaved, specifically by Ad2 protease, late in infection and the suggestion that the resulting disruption of the cytoskeletal network facilitates the release of virions (5). This, along with the finding that pTP is cleaved to iTP in insect cells coinfecting with recombinant baculoviruses expressing Ad2 protease and pTP (44), demonstrates clearly that, when expressed at high enough levels, adenovirus protease activity can be detected outside virions, indicating that other cytoplasmic or nuclear proteins could be substrates of the protease. The presence of the protease in the nucleus also raises the question of whether it carries its own nuclear localization sequence or is transported to the nucleus in association with another protein. No obvious nuclear localization sequence exists in the protease, but the requirement for pVI for activity makes this virus structural protein a possible candidate for transporting protease to the nucleus.

Given the fact that the protein primer for DNA replication, pTP, is processed by adenovirus protease, the suggestion that DNA is a cofactor of the protease (19) was investigated; but unlike Mangel and co-workers, we found that DNA had no

effect on the initial rate of digestion of peptide substrates unless the reducing agent DTT was included in the assays and only then when DNA was added prior to the DTT. This is in agreement with our previous data showing that DTT inhibits the activation of the protease but has no inhibitory effect on active protease (44). The inhibitory effect of DTT on protease activation is consistent with the observation that it is the disulfide-linked, dimeric form of pVI-CT and not the reduced, monomeric peptide that activates the protease. Our results show that DNA is not required for protease activity and so cannot be a cofactor in the true sense of the word. The inclusion of 1 M NaCl in the assays was found to have a similar but more pronounced effect than DNA on protease activity, with a fivefold enhancement of initial rates of digestion of peptide substrates being observed in buffers of high versus low ionic strength. It is important to note that NaCl concentrations above 0.2 M have previously been reported to inhibit Ad2 protease, but this was found to be a function of the conformation of the substrate, pVII, in high salt (2). Thus, peptide substrates, which are more flexible in the conformations that they can adopt, may have advantages over protein substrates for certain mechanistic studies. Like DNA, the presence of 1 M NaCl in the assays was found to prevent the inhibitory effect of DTT, but only when protease was incubated with pVI-CT for at least 15 min prior to addition of the reducing agent.

One interpretation of these results is that, following activation of protease by pVI-CT, an equilibrium is set up between inactive and active protease and that the assay conditions used determine the proportion of each present. When assay conditions favor the active protease, for example, in the presence of DNA or 1 M NaCl, then DTT exerts no inhibitory effect following activation. In low-ionic-strength buffers, protease must be constantly reactivated, and so the presence of DTT is inhibitory even when it is added 15 min after the activating peptide. As discussed above, two *in vivo* situations in which adenovirus protease is active outside virus particles have been identified (5, 44), observations which are not consistent with the notion that viral DNA is a cofactor of the enzyme (19). Furthermore, it is noted that the effects of DNA on protease activity observed (19) were not specific for virus DNA, since other polyanions, including polyglutamic acid and heparin, were found to substitute for it. Nevertheless, given the tight association of the protease with viral cores (1), the possibility that DNA has some influence on protease activity in the context of the virus particle cannot be ruled out. The protease binds weakly to DNA (44), and pVI-CT is a very basic peptide, so polyanions, including DNA, could serve to colocalize the enzyme and activating peptide. Increased local concentrations of the protease and pVI-CT could facilitate protease activation. This explanation is consistent with the observation that optimal DNA concentrations are required and that increasing the concentration of DNA above 50 pM decreased protease activity in the presence of DTT *in vitro* (19).

We have shown unequivocally that Ad2 pTP is cleaved at three sites: MRGF-G and MGGR-G (amino acids 175 to 176 and 183 to 184, respectively) separated by 8 amino acids, to give iTP, and MTGG-V (amino acids 349 to 350) to give TP. These sites were predicted previously from the results of synthetic peptide studies and are in accord with the substrate specificity of the adenovirus protease (47). Furthermore, sites giving rise to both iTP and TP are conserved in all serotypes for which pTP has been sequenced to date, suggesting that the cleavage of pTP via an intermediate to TP plays a role in the infectious cycle. Perhaps of significance in this regard is the observation that the rate of cleavage of pTP to iTP is consid-

erably greater than that of iTP to TP, raising the possibility that cleavages at the two sites serve distinct functions.

The major approach to mapping the functional domains of pTP has been linker insertion mutagenesis, and although this approach has provided some useful information, the major conclusion appears to be that the virus is very sensitive to mutations in pTP, with changes throughout the 671 amino acids of pTP proving lethal (11, 12, 29). In terms of activity in *in vitro* replication assays, pTP is fractionally more tolerant to mutagenesis, but it has been shown that a number of regions spanning the N-terminal 250 amino acids are absolutely required for initiation of DNA replication (24, 30). In this study, we have used the natural processing of pTP by adenovirus protease to define the regions of pTP that bind to Adpol and the DNA at the origin of replication. Immunoprecipitations show that pTP and iTP but not TP bind to Adpol, suggesting that amino acids critical for the Adpol interactions may reside between the iTP and TP sites. Further evidence that Adpol binds to pTP in proximity to iTP sites comes from the observation that the rate of digestion to iTP is much slower for the Adpol-pTP complex than for free iTP. Studies on the DNA-binding properties of pTP and its digestion products show that only the pTP binds to DNA, indicating that the region of the iTP cleavage sites is important for DNA binding. The importance of the amino acids in the region of the iTP cleavage sites in DNA replication is emphasized by studies of a mutant, *in179*, in which an insertion has been made 48 amino acids toward the N terminus of the iTP site. It has been shown that the mutant virus has a delayed onset of DNA replication and that it has a *trans*-dominant negative effect in that *in179* pTP inhibits wild-type DNA replication in a dose-dependent manner (12).

In considering the role of pTP processing by the protease in DNA replication, free pTP and pTP/TP which is covalently attached to the 5' end of DNA should be regarded as distinct functional entities. Clearly, the role of free pTP is to act as the protein primer for DNA replication, while the significance of the covalently attached pTP/TP remains to be fully elucidated, but it has been shown in transfection experiments that the infectivity of TP-DNA is much greater than that of naked DNA (15).

Roles that have been suggested for the covalently attached pTP/TP include protection of viral DNA from exonucleases, attachment of virus DNA to nuclear matrix, unwinding of the DNA duplex at the origin of replication, and binding to the incoming pTP-pol heterodimer (25, 32). To date, however, few attempts have been made to distinguish between TP-DNA and pTP-DNA, and in most cases the terms have been used interchangeably. This is perhaps surprising, given that *in vivo*, the template for early transcription and the first round of DNA replication is TP-DNA, while the template for subsequent rounds of replication will be pTP-DNA irrespective of whether infections are carried out with wild-type Ad2 or Ad2*ts1* at permissive or nonpermissive temperatures. One role for the digestion of pTP-DNA to TP-DNA by the protease that has been suggested is that the cleavage is required to release the TP-DNA from the nuclear matrix prior to assembly within the virus particles (10). The fact that pTP-DNA is packaged within virus particles during Ad2*ts1* infections at nonpermissive temperatures would tend to argue against this (42). It cannot be ruled out, however, that the nuclear matrix-binding properties of TP-DNA and pTP-DNA are distinct and that this has some bearing on the transcription and replication sites of viral DNA. Another possibility is that the purpose of the processing of pTP-DNA to TP-DNA is to create a different template for either early transcription or the first round of DNA replication.

Taking into account the data from this study showing that although pTP binds to DNA and Adp_{ol}, the mature TP does not, it would not be surprising to find differences between the properties of TP-DNA and pTP-DNA as templates for DNA replication.

Thus, we have used Ad2 protease to map regions of pTP required for interaction with DNA and Adp_{ol} in the assembly of the preinitiation complex and envisage that through future studies on the TP-DNA and pTP-DNA templates, a clearer understanding of the role of terminal proteins in both transcription and replication can be gained. Furthermore, we have shown that in Ad2, virus-coded protease is expressed at high levels late in infection and that although DNA is not a cofactor of the protease, as previously reported, under certain assay conditions it can stabilize the enzyme in an active form.

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