Activation of the Adenovirus Protease Requires Sequence Elements from Both Ends of the Activating Peptide*

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The adenovirus protease requires activation by an 11-residue peptide, GVQSLKRRRCF, to achieve maximum proteolytic activity. Derived from the C terminus of the viral protein pVI, the activating peptide (pVI-CT) forms a disulfide bond with cysteine 104 of the protease and causes a conformational change that accompanies the development of proteolytic activity. Results presented here show that the interaction of pVI-CT with the protease is dependent not only on the cysteine 10 but also on glycine 1 and valine 2. Removal of these residues, acetylation of the N-terminal glycine, or mutation of the valine to alanine or threonine significantly reduces or abolishes activation. Peptides lacking Gly-1 and Val-2 still form a disulfide bond with the protease but do not cause a conformational change in the protease also they are not effective inhibitors of activation as the interaction is readily reversed by full-length pVI-CT. These results suggest that pVI-CT causes activation by binding to two distinct regions of the protease and in doing so stabilizes the catalytic site. The reversible nature of the activation, suggested by the results presented here, may well reflect an in vivo regulatory mechanism.

The protease coded by adenovirus plays an essential role in the replication cycle of the virus (1) and has distinctive properties that make it of intrinsic scientific interest and an attractive target for antiviral therapy. It is known to cleave several capsid proteins (2) suggesting that it has a role in virion maturation; it cleaves the preterminal protein (pTP), the protein primer for DNA replication, thereby altering the affinity of that protein for the viral polymerase (3). It has also been reported to cleave the cellular protein cytokeratin 18 (4) raising the intriguing possibility that it has a role in the escape of the mature virus from the cell.

Its properties are distinctive in several ways. It has an unusual substrate specificity (5, 6) that depends primarily on recognizing a hydrophobic residue (M, L, or I) in the P4 position (7) and accepting only a glycine in P2. Although it appears to be a cysteine protease (8), the catalytic histidine and cysteine (His-54 and Cys-122) are in the reverse order of that found in the archetypal cysteine protease, papain (Cys-25 and His-159), which has led to them being classified in separate families within the category of cysteine protease (9). Perhaps the most interesting facet of its mode of action, however, is that in contrast to most other proteases it does not require proteolytic activation (10). The development of significant proteolytic activity depends on the participation of an 11-residue peptide (GVQSLKRRRCF), which is derived from the C terminus of the viral capsid protein pVI (11, 12). There have also been reports that viral DNA is involved in the catalytic mechanism (12, 13), but other reports (3) suggest that DNA is not necessary for catalysis but may help to stabilize the protease in vitro and could enhance the interaction of protease and substrates in vivo.

Previous studies (11) have shown that the cysteine residue of the activating peptide (pVI-CT) is essential and that the activation may be caused by the disulfide-bonded dimer of pVI-CT in a mechanism that involves disulfide interchange. However, the truncated peptide KRRRCF did not activate the protease indicating a role for the N-terminal half of the molecule. Jones et al. (14) have provided some insight into the mechanism by which pVI-CT operates by showing that it causes a conformational change in the protease through its interaction with one of the two conserved cysteines in the protease, Cys-104, leaving the other conserved cysteine, Cys-122, as the active site nucleophile. The crystal structure of the protease in combination with pVI-CT has recently been solved (15), and this shows that pVI-CT is covalently bound forming an additional strand to a b-sheet structure within the protease stabilized by a disulfide bond with Cys-104.

In this paper we provide more information on the structural parameters of pVI-CT that are important to the activation process and suggest a mechanism by which pVI-CT is able to induce the conformational change necessary for the development of catalytic activity by the protease.

MATERIALS AND METHODS

Peptide Synthesis—Peptides were synthesized as described previously (8) and purified by reversed phase chromatography on a C18 column equilibrated in 0.1% (v/v) trifluoroacetic acid. Peptides were eluted by increasing concentrations of acetonitrile. The peptide masses were verified by mass spectrometry, and the sequences were confirmed using an Applied Biosystems Procise Protein Sequencer.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out essentially as described (16).

Expression and Purification of the Ad2 Protease—The protease was expressed in and purified by Escherichia coli BL21(DE3) as described previously (6, 17). Protein concentrations were determined by using absorbance at 280 nm assuming an extinction coefficient of 0.28 for a 0.1 mg/ml solution (concentration verified by amino acid analysis) or by densitometry of Coomassie Blue-stained bands following SDS-polyacrylamide gel electrophoresis using known concentrations of soybean trypsin inhibitor (Sigma) as standard. Both methods gave identical results.

Protease Assays—Protease assays were carried out essentially as described previously (17). In a typical assay, 10 ml of purified recombinant protease (20 pmol) were incubated with 10 ml (7.4 nmol) of GVQSLKRRRCF (or variant thereof) and 25 ml of 50 mM Tris/HCl, 10 mM EDTA, 2 mM 2-mercaptoethanol, pH 8.0, for 10 min at 37 °C. The
reaction was started by the addition of 5 μl (120 nmol) of LSGAGFSW, and the initial rate was followed by removing 5-μl aliquots of the reaction mixture into 5 μl of 1% trifluoroacetic acid to stop the reaction, diluting to 50 μl with water, and quantitating the formation of the specific digestion products LSGA and GFSW by capillary electrophoresis using a Bio-Rad BioFocus 3000 with a 17 cm × 25 μm coated capillary tube. The activity of the protease in this assay was typically 9.1 nmol of GFSW min⁻¹ nmol⁻¹ protease. In some assays the peptide Ac-LRGAGRSR, which was cleaved by the protease at the A–G bond, was used in an otherwise identical manner.

**Fractional Activation of pVI-CT**

**Mechanism of Activation of the Adenovirus Protease**

In order to investigate the mechanism by which pVI-CT operates, a series of peptides progressively shortened by one amino acid from the N terminus were synthesized and tested for their ability to activate the adenovirus protease. Fig. 1 shows that when compared to the activity achieved with the full-length peptide GVQSLKRRRCF, removal of the N-terminal glycine caused a significant reduction in substrate cleavage while removal of the valine as well essentially abolished proteolytic activity. As would be expected, a peptide lacking the first 4 residues was also inactive (results not shown) while previous work (11) has demonstrated that the peptide KRRRCF was also inactive.

Further evidence for the importance of the N-terminal region of the peptide came from experiments where the N terminus was acetylated resulting in a reduction in the initial reaction rate to 46% of that achieved with the non-acetylated pVI-CT (Table 1) and from peptides where the branched chain hydrophobic residue, valine, was replaced by a smaller hydrophobic residue (alanine) or by a branched chain but more hydrophilic residue (threonine). Both these mutations resulted in a similar reduction in activity.

**Derivatives of pVI-CT That Do Not Activate the Protease Do Not Cause a Conformational Change**

We have previously shown (14) that interaction of pVI-CT with the protease causes a change in the tryptophan fluorescence of the protease indicating a conformational change. Moreover, this conformational change mirrored the increase in proteolytic activity as the activating peptide was titrated in, both fluorescence and proteolytic activity reaching a maximum at around a 100-fold molar excess of peptide, suggesting that this conformational change was a prerequisite for the development of activity.

The importance of the N-terminal region of pVI-CT was further emphasized by an investigation into the ability of the pVI-CT variants, truncated at the N terminus, to influence the intrinsic tryptophan fluorescence of the protease. The results shown in Fig. 3 demonstrate that GVQSLKRRRCF and VQSLKRRRCF both cause an increase in the tryptophan fluorescence and a red shift in the wavelength of maximum emission. On the other hand, the peptides QSLKRRRCF and SLKRRRCF, neither of which were effective in causing activation of the

**Table 1**

| Peptide Relative activity |
|--------------------------|
| GVQSLKRRRCF 100 |
| Ac-GVQSLKRRRCF 46 ± 10 |
| GAQSLKRRRCF 46 ± 9 |
| GTQSLKRRRCF 39 ± 11 |

activity was measured as the initial rate of cleavage of the peptide substrate LSGAGFSW and is expressed as a percentage of the initial rate obtained with the full-length peptide GVQSLKRRRCF. Values are given as mean ± S.E. where the number of determinations was at least 12.
Mechanism of Activation of the Adenovirus Protease

5637

**DISCUSSION**

The results presented here show that both the N- and C-terminal regions of the activating peptide are essential for productive interaction between pVI-CT and the adenovirus protease. The data are in agreement with the structure presented by Ding et al. (15) in showing that full-length pVI-CT forms a 1:1 complex with the protease. In addition, the mass spectrometry results (Fig. 2) show that peptides lacking the N-terminal 2 or 3 residues bind to the protease, a conclusion that is supported by the ability of the larger peptide (QSLKRRRCF) to inhibit (albeit weakly) the development of proteolytic activity.

There is also good evidence that the formation of the pVI-CT-protease complex is dependent on the N-terminal regions of the peptide. The ability to activate the protease is decreased significantly by removal of the N-terminal glycine and effectively abolished if the 2nd residue (valine) was also missing (Fig. 1). Additional evidence of the importance of these 2 residues is presented in Table I. Acetylation of the N-terminal residue decreases the ability of pVI-CT to activate indicating that the free amino group is important for binding. The size and hydrophobicity of the valine residue at position 2 is also important. Peptides where the valine is substituted by the smaller hydrophobic alanine or by the similarly sized branched chain hydrophilic threonine are less efficient activators of the protease. These results are in accord with existing sequence data. An alignment of the C-terminal 15 residues from pVI sequences in the SWISSPROT data base (release 33, April 1996) and sequences translated from nucleic acid sequences obtained from the “Updates” data base of recent releases (July 1996) held at the SEQNET facility (Daresbury, United Kingdom) is shown in Fig. 4. This alignment shows that the protease cleavage site and thus the length of activating peptide is conserved, as is the position of the cysteine residue. The glycine in position 1 is also completely conserved, and valine is found in all cases except one incidence of a conservative replacement by leucine. This alignment supports the contention that the cysteine residue and the GV sequence separated by a fixed distance are critical parameters for interaction.

There is evidence from previous work that the protease does have some proteolytic activity in the absence of pVI-CT. Measurable rates of activity have been obtained in the absence of pVI-CT (13) while cleavage of pTP in insect cells, simultaneously infected with recombinant baculoviruses expressing protease and pTP, has been demonstrated (11). Mangel et al. (13) further showed that addition of pVI-CT had little effect on \( k_{cat} \) but caused a significant increase in \( k_{cat} \). This suggests that the substrate-binding site is relatively intact in the absence of pVI-CT, and the consequence of its addition is to stabilize the catalytic apparatus of the protease.

The crystal structure of the pVI-CT/protease complex (15) shows that Cys-104 is on the surface of the molecule and Cys-10 of pVI-CT is disulfide linked to this cysteine whereas residues 3–9 are in a \( \beta \)-strand conformation and lie alongside a \( \beta \)-strand formed from the residues subsequent to Cys-104. The N-terminal region of pVI-CT is enclosed in a loop region of the protease in the region of residues 135–140 with contacts between the N-terminal glycine of pVI-CT and aspartate residue at position 142. This loop region immediately follows a region of helix in the protease where Cys-122 lies at the far end. Thus it is entirely plausible that the effect of pVI-CT is to “tie together” two distinct regions or minidomains of the protease, which in its absence are able to move independently. The effect of this would be to stabilize the catalytic residues in their optimum orientation and to achieve this, interaction from both ends of pVI-CT would be required. This model would also explain why...
the protease displays some activity in the absence of activating peptide, as the dynamic nature of the protein would allow a small proportion of the protease molecules to be in the active conformation at any one instant.

The reduction of activation caused by acetylation of pVI-CT raises the question of how pVI-CT is produced from pVI in the first instance. Previous work (11) indicated that pVI is capable of activating the protease, and we have shown that the peptide IVGLGVQLKRRRCF, which has the same preceding 4 residues as pVI from Ad2, effectively activates the protease and is very rapidly cleaved to the 11-residue pVI-CT. The analogues IVALGVQLKRRRCF and VVGLGVQLKRRRCF that do not contain the consensus sequence for cleavage also activate the protease and were, as expected, not cleaved. Secondary structure predictions suggest the presence of a turn region associated with the cleavage site, and this must allow the GV region to fit into its complementary site in the protease.

The inability of the truncated activating peptides to act as effective inhibitors, in spite of their ability to bind to the protease, was surprising but is indicative of the importance of the GV dipeptide region. It is also possible that the disulfide bond formed between Cys-104 of the protease and pVI-CT is prone to disulfide exchange; certainly there is evidence from the mass spectrometry (Fig. 2) that cysteine-containing truncated versions bind less effectively. The pVI-CT sequences from different serotypes presented in Fig. 4 show that there is a conserved aromatic residue (phenylalanine or tyrosine) in position 11, next to the cysteine, and at least two arginines close by. Both of these factors may serve to destabilize disulfide bonds involving the activating peptide, an effect that may be further enhanced by the presence of an arginine (also well conserved) at position 103 in the protease. This would suggest that the binding of the

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**TABLE II**

| Peptide          | Activity % |
|------------------|------------|
| GVQLKRRRCF       | 100        |
| QSLKRRRCF        | 72 ± 11    |
| SLKRRRCF         | 104 ± 16   |
| LKRRRCF          | 108 ± 6    |
| KRRRCF           | 109 ± 6    |
| GVQSL            | 109 ± 6    |
| GV               | 96 ± 4     |

**FIG. 3.** Only GVQLKRRRCF and VQSLKRRRCF affect the tryptophan fluorescence of the protease. Fluorescence emission spectra of the protease alone (solid lines) and in the presence of GVQLKRRRCF (a), VQSLKRRRCF (b), QSLKRRRCF (c), and SLKRRRCF (d). Excitation wavelength was 280 nm.

**FIG. 4.** Alignment of the C-terminal 15 residues of pVI from different human and animal adenovirus serotypes. Sequences were taken from release 33.0 of the SWISSPROT data base or translated from nucleic acid sequences in the Updates version of the EMBL nucleic acid data base held at the SEQNET facility, Daresbury, UK. The accession number is given where references are not available. The arrow indicates the putative site of cleavage by the adenovirus protease.
intact pVI-CT may be reversible, and the need for a large molar excess to achieve effective activation of the protease (11, 12, 14) may reflect this reversibility and the requirement to push the consequent equilibrium in the direction of complex formation. The reversibility of the activation procedure may represent an aspect of the in vivo regulation of the protease.

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