Communication

Cleavage Efficiency by Adenovirus Protease Is Site-dependent*

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The adenovirus protease cleaves consensus sequences (M/I)LXGX-G and (M/I)LXGX-X. Using purified recombinant protease, we showed that a peptide bearing the GX-G site was hydrolyzed more rapidly than a peptide bearing the GG-X site. The GX-G site was also preferentially cleaved on viral protein pVI which bears both sites of cleavage. Evidence is presented that suggests a biological role for this differential cleavage efficiency.

Adenoviruses, like many other viruses, encode an endoprotease which is required for virus maturation and infectivity (1). The enzyme (AVP)1 is a cysteine protease that normally cleaves seven viral proteins at two consensus sites: (M/I)LXGX-G or (M/I)LXGX-X (2). Other proteins bearing these sequences are also susceptible to AVP, particularly after denaturation (3–6). In addition to its role in virus maturation, the AVP also appears to have a role in the early phase of infection in decapsidation and release from the endosome (1, 7, 8). The AVP also appears to have a role in the early phase of infection (1). The enzyme (AVP)1 is a cysteine protease that normally

RESULTS AND DISCUSSION

As shown by Webster et al. (2) and confirmed by others (reviewed in Ref. 1), the adenovirus protease cleaves two consensus sequences, (M/I)LXGX-G and (M/I)LXGX-X. To compare the relative cleavage efficiency of the two sites, two fluorogenic substrate peptides LYRA2 (ly-AnLRGG-AFSWK-ctmr-R) and LYRA3 (ly-AnLRGA-GFSWK-ctmr-R) were synthesized. The two consensus sites were synthesized. We have previously shown hydrolisis of this type of substrate by the adenovirus protease (AVP (11)). LYRA3 appeared to be cleaved somewhat more rapidly than LYRA2 (Fig. 1). Cleavage efficiency of both peptides increased significantly when the recombinant AVP was first incubated with the enzyme-stimulating peptides pVlc or VEGGS. Again, more LYRA3 was hydrolyzed than LYRA2. These digestions were carried out at low substrate concentrations (5 μM). Under these conditions, LYRA3 was cleaved with an efficiency double that of LYRA2 (Fig. 2). It should be noted that at this substrate concentration the dimethyl sulfoxide solvent is at 66.7%. Tolerance of organic solvents by the AVP was reported by McGrath et al. (14). To be sure that this is not an artifact of the high dimethyl sulfoxide concentration, an experiment with 200 μM final concentration of substrate was digested in a normal reaction volume (300 μl), so the dimethyl sulfoxide concentration is lower (6.7%). The same ratio of difference between the cleavage of LYRA2 and LYRA3 was observed (i.e. 2.5 times more cleavage of LYRA3, result not shown). To clarify this difference we did a time course reaction with both peptides using the same conditions at 5 μM substrate concentration. The results show that LYRA3 was cleaved more efficiently than LYRA2 (Fig. 3). The difference between these two substrates is the permutation of two amino acids in the cleavage site: GG-A for LYRA2 to GA-G for LYRA3. These experiments show that at least in the case of a peptide substrate the GX-G consensus site is cleaved more rapidly than the GG-X consensus site. A peptide with a similar sequence (MSGA-GFSW) was also reported to be cleaved 1.5 times more rapidly than a control peptide bearing the GG-X site (GSGG-AFSW (2)). Enzyme activity was stimulated by the

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‡ The abbreviations used are: AVP, adenovirus protease; PAGE, polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Expression and Purification of Adenovirus Protease—Cloning, expression, and purification of the Ad2 protease using the modified expression vector pRPAd2E3 (pRIT27 from Pharmacia Biotech Inc.), a temperature-inducible protein A gene fusion vector, under the control of the ApR promoter, was done essentially as described before (4). In some experiments, a second expression plasmid, pLPV, which contains 14 amino acids fused to the N terminus of the protease, was also used (10). Protease Assay—Unless indicated otherwise, peptide assays were performed as follows. A total reaction volume of 300 μl contained 5 μM substrate (11), 2–100 pmol of pRPAd2E3 protease, and reaction buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8, 2 mM NaCl). The reaction was incubated at 37 °C for the indicated times. The synthesis and rationale of the LYRA substrates was described previously (11). The peptide sequence in LYRA2 was ly-AnLRGG-AFSWK-ctmr-R and in LYRA3 was ly-AnLRGA-GFSWK-ctmr-R. Protease activity was also measured by the cleavage of viral polypeptide pVI to VI (intermediate form of VI) and VI. The source of pVI was ts1 virions grown at 39 °C, purified, and disrupted with 10% pyridine, and dialyzed with TB buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA), and boiled to denature proteins. Cleavage was detected by staining blots with anti-VI serum (see Western Blotting). Precursor pVI was also prepared by disrupting [35S]Met-labeled ts1 virus particles produced at 39 °C (12). The reaction mixture (40 μl) contained 10 μl of substrate and 10 μl of enzyme in reaction buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). Incubation was at 37 °C for the indicated times.

Western Blotting—Proteins were separated by a 15% SDS-PAGE, electroblotted onto a nitrocellulose membrane (Hybond C-Extra) and reacted with a rabbit polyclonal anti-VI antibody raised against pVI amino acid residues 94–170, generously provided by D. Matthews and W. C. Russell (13). The antigen-antibody complex was detected with 125I-labeled protein A (Amersham).

Reagents—The peptide acetyl-VEGGS-amide (VEGGS) was high performance liquid chromatography purified, and a stock solution (1 mM) was prepared in the reaction buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8). Peptide Fv (GvQLKRRCRF) was purchased from Coast Scientific (San Diego, CA) and prepared as described before (11).

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pVIc peptide as described in several previous reports (11, 15, 16). This peptide is a cleavage product from the C terminus of capsid precursor protein pVI and is likely to be involved in coordinating enzyme activity with capsid assembly and maturation during virus infection (17). The VEGGS peptide was identified as a protease ligand in a phage display library (18). This peptide mimics the effects of pVIc on protease, particularly as measured by enhanced enzyme activity (18).

The Preferred Cleavage Site on Protein pVI Is Also GX-G: Cleavage of the GX-G Site Is Also More Rapid on Protein pVI—
The adenovirus capsid protein precursor pVI is cleaved in vivo by the viral protease at two sites: at a GG-X type site 28 to 33 residues from the N terminus and at a GX-G site exactly 11 residues from the C terminus. The latter cleavage generates the 11-residue pVIc peptide responsible for stimulating enzyme activity (9, 15, 16). Protein pVI is therefore an ideal candidate to test the hypothesis of preferential cleavage of the GX-G site on a viral protein. The experiment was performed with three sources of protein pVIc: (i) recombinant pVI, (ii) [35S]Met-labeled disrupted ts1 virions grown at 39 °C, and (iii) unlabeled, disrupted ts1 virions grown at 39 °C. Cleavage was detected by changes in migration on SDS-PAGE and Western blotting as described in the “Materials and Methods.”

Fig. 1. Is cleavage efficiency substrate site-dependent? The recombinant adenovirus protease (pRPAD2E3, 40 pmol) was preincubated with or without (−) stimulating peptides (40 μM pVIc or 100 μM VEGGS) for 30 min at 20 °C and then 5 μM substrate was added: LYRA2 (ly-AnLRGG-AFSWK-ctmr-R) or LYRA3 (ly-AnLRGA-GFSWK-ctmr-R). Cleavage was determined by measuring the increase in fluorescence using excitation at 420 nm and emission at 520 nm and a 1-cm path length quartz cuvette in a Hitachi F2000 fluorescence spectrophotometer with a band pass of 10 nm for both excitation and emission. The reaction buffer was 10 mM Tris-HCl, pH 8, 1 mM EDTA, and 2 mM NaCl. The reaction was incubated for 1 h at 37 °C. The number of experimental determinations are indicated within the histograms. Student’s t test: *, p < 0.05.

Fig. 2. Effect of high substrate concentration. The reaction was done in a small volume (30 μl) to attain a high substrate concentration. The reaction mixture contained 20 pmol of recombinant enzyme (pRPAD2E3), 40 μM pVIc, and 200 μM LYRA2 or LYRA3. After an incubation time of 90 min at 37 °C, the reaction was stopped by adjusting the volume to 300 μl with TE (10 mM Tris-HCl, pH 8, 1 mM EDTA, and 2 mM NaCl) to be able to use the 1-cm path length quartz cuvette in the fluorescence spectrophotometer.

Fig. 3. Time course of cleavage of LYRA2 and LYRA3. The reaction mixture contained 40 pmol of protease (pRPAD2E3) and 40 μM pVIc and was preincubated for 30 min at 20 °C before adding LYRA2 or LYRA3 and incubating at 37 °C for the indicated time. Cleavage was measured by the increase in fluorescence as described in Fig. 1.

Fig. 4. The GX-G site is cleaved more rapidly than the GG-X site in viral protein pVI. Viral substrate pVI was digested as described under “Materials and Methods.” pVI, iVI, and VI were detected by Western blotting using a rabbit polyclonal antibody against protein residues 94–170. These residues are present in pVI, iVI, and VI. At the indicated times (in hours), the reaction was stopped by addition of lysing buffer and boiling the mixture. Lanes a and g are nondigested pVI controls. Lane h, 16-h digestion with protease in the presence of 40 μM pVIc. Lanes i and j, 16-h digestion with inactive mutant proteases C104G and C122G, respectively. A, autoradiogram of densitometric quantitation of autoradiograms as in A. The means of three experiments are expressed as percentages of proteins pVI (open boxes), iVI (hatched boxes), and VI (closed boxes).
The results with the other two sources of pVI were similar. Cleavage of pVI to iVI via the C-terminal G-G site clearly proceeds approximately 9 times more rapidly than cleavage to VI resulting from hydrolysis of the N-terminal GG-X site. This result confirms the results obtained with the peptides above. The larger difference in the rate of hydrolysis of the two sites is presumably due to the sequence and structure context of the sites within the protein. The large aromatic residues in the P2’ and P4’ positions of pVI may ensure enhanced preferential digestion of the C-terminal site which has no such residues (Table I).

The identities of iVI and VI were determined previously (19). In addition, the following factors also support the identity of these cleavage products: 1) identical digestion products were obtained with the purified recombinant pVI protein, 2) the anti-protein VI serum reacted only with digestion products of the expected apparent molecular weight, 3) addition of pVIc significantly enhanced digestion of pVI to iVI and VI (Fig. 4, lane h), 4) incubation with mutant enzymes yielded no products, showing that iVI and VI are not products of degradation or nonspecific digestion (Fig. 4, lanes i and j). Unlike the pVIc peptide, addition of the VEGGS peptide to the enzyme reaction did not enhance the digestion of pVI. This is in contrast to the stimulation observed in the case of the small peptide substrates LYRA2 and LYRA3. Stimulation by VEGGS may be limited by the size or type of the substrate.

It should be noted that most of these experiments were done in the absence of exogenously furnished pVIc peptide. The observed enzyme activity suggests, as we have noted before, that the AVP may have a basal activity in the absence of stimulating peptide (5). Cleavage of pVI to iVI would generate pVIc peptides which could in turn stimulate the enzyme. It is possible that the AVP may have a basal activity in the absence of pVIc peptide (9, 11, 15).

The coding sequence for capsid protein pVI has been determined for a number of adenoviruses. In Table I we assembled the translations of the currently available DNA sequences. A striking degree of sequence conservation even among diverse serotypes is evident: (i) the N-terminal cleavage site is always GG-X, and the C-terminal is always GX-G, (ii) the location of the cleavage sites is precisely 11 residues from the N terminus, (iii) the 11-residue pVIc is a highly conserved consensus sequence, GVKSLKRRCY. These conserved features strongly suggest that the preferential cleavage of the GX-G site by the viral protease and the pVIc-mediated activation mechanism may be general among all adenoviruses.

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### TABLE I

| Protein pVI | P1 | (M/V)LXGG-X | (M/V)LXG-X | Locus |
|-------------|----|------------|------------|-------|
| H2 (250)    | 33 | MSGG-AFSW  | IVGL-GVQSLKRRRCF | PIV6 ADE02 (21) |
| H5 (250)    | 33 | MSGG-AFSW  | IVGL-GVQSLKRRRCF | PIV6 ADE05 (21) |
| H12 (265)   | 33 | LGGN-AFNW  | IVGL-GVKSLKRRRCY | PIV6 ADE12 (22) |
| H31 (260)   | 33 | LNGN-AFNW  | IVGL-GVKSLKRRRCY | HAU14653 |
| H40 (267)   | 33 | LNGN-AFSW  | IVGL-GVKSLKRRRCY | ADRGENOME (23) |
| H41 (266)   | 33 | LGGN-AFSW  | IVGL-GVKSLKRRRCY | HAU14652 |
| MAV1 (237)  | 32 | LHGG-ALGW  | IMGL-GLQPIKRRRCF | PIV6 ADEM1 (24, 25) |
| BAV2 (?)    | 33 | MNGG-AFNW  | — | — |
| CELO (223)  | 28 | LRGG-AINW  | ? | AAVMCPHEX (26) |
| OVA (221)   | 25 | MRGG-FSWS  | LSST-GVATATRRCY | OAU40837 (27) |

a The number of amino acids in the pVI protein of each virus is given in parentheses.
b The locus was obtained from GenBank, and the numbers in parentheses are references.
c Unpublished data.
d B. Harrach, personal communication.
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