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PROTEASE OF ADENOVIRUS TYPE 2. IN VITRO PROCESSING OF CORE PROTEIN[1]

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SUMMARY

Protease activity associated with temperature sensitive mutant ts3 of adenovirus type 2 was studied. This activity was induced only when ts3 was propagated at 33°C. By *in vivo* and *in vitro* experiments the enzyme was found to cleave main core polypeptide PVII to VII. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis, the protease activity of ts3 was partially characterized. Phenylmethylsulfonyl fluoride (1mM) and SDS (0.5%) inhibited its activity completely. EDTA (10 mM) did not seem to inhibit its activity. Protease activity was completely abolished after 10 min. of incubation at 60°C. Autocatalytic cleavage of PVII to VII was not observed.

INTRODUCTION

Proteolytic processing of precursor polypeptides from numerous animal and bacterial viruses is a known phenomenon (1,2). At least three polypeptides present in adenovirus type 2 are derived by proteolytic cleavages from larger precursor polypeptides (3). The physiological role of these changes is at present unknown. However, there is substantial evidence available which indicates that they occur late in the pathway of virion assembly. It also seems pertinent that these cleavages play some role in the maturation of infectious virus particles (3). The nature and source of the protease involved and the site of their activity, therefore, may elucidate our understanding of the molecular events taking place during assembly. We report here, the *in vitro* processing of PVII to VII, and demonstrate that this cleavage is due to an adenovirus type 2 induced protease activity.

MATERIAL AND METHODS.

CELLS AND VIRUS.

Adenovirus type 2 and its temperature-sensitive mutant "ts3" (4)

[1]This is paper XI in a series. Reference II is number VI in the series.

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were used. Viruses were propagated in HEp-2 cells (Flow Laboratories, Rockville, Md.) grown to confluent monolayer in Dulbecco's modified medium as previously described (3).

PREPARATION OF SUBSTRATE.

Mutant ts3 is defective in the processing of PVII to VII at 39°C (5). Therefore, cell free extract of ^{35}[S] -methionine labelled ts3 infected HEp-2 cells was used as a source of substrate. HEp-2 cells infected with ts3 were incubated at 39°C, and at 17 h post-infection, ^{35}[S]-methionine was added to the cultures (0.1 ml of 250 uCi/ml/pétri dish). The cells were harvested after 42 to 46 h post-infection at 39°C, washed 3 times with 10mM phosphate buffer (pH 7.4) and resuspended in the same buffer. Cell-free extracts were prepared by freeze-thawing the cells 3 times, followed by low-speed centrifugation to remove membranes. This supernatant was used as the substrate.

PREPARATION OF PROTEASE.

Cell free extracts of ts3 infected HEp-2 cells propagated at 33°C and 39°C were used as enzyme source. Growth conditions and preparation of cell free extracts, was exactly the same as described for substrate preparation, except proteins were not labelled. Cell free extract from mock-infected cells were used as control.

DETERMINATION OF PROTEASE ACTIVITY.

Ten to 20 µl of substrate preparation was mixed with the same volume of enzyme, and adjusted to 50 µl with phosphate buffer, and incubated overnight at 37°C. After incubation, the reaction was stopped by adding 50 µl of lysing solution (100mM Tris, pH 6.8; 2% SDS; 20% glycerol; 0.2% mercaptoethanol; 0.002% phenol red). Samples were heated for 3 min. in boiling water. In controls, enzyme was replaced with the same volume of phosphate buffer. Samples were analyzed for enzyme activity by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The conversion of PVII to VII was taken as evidence of protease activity as observed by autoradiography.

THERMAL INACTIVATION AND INHIBITOR STUDIES.

Enzyme samples were heated in a constant temperature water bath for 10 min. at various temperatures, then chilled in cold water and assayed for residual protease activity. In the case of inhibitors, enzyme samples were assayed in the presence of desired concentrations of phenylmethylsulfonyl fluoride (1mM); EDTA (1,5 and 10mM) and sodium dodecyl sulfate (0.05, 0.1, 0.2 and 0.3%) then assayed for the remaining protease activity at 37°C.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS.

Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis was carried out as described previously (5). After electrophoresis, the gels were dried and autoradiographed with Kodak RP-oxomat X-ray film. Gels containing low ^{35}[S]-methionine counts were treated with PPO as described by Bonner and Laskey (6) and exposed at -85°C. Quantitative analysis was carried out as follows:(1) gels were stained for proteins, the bands of PVII and VII were cut out and digested overnight with 0.5ml of 33% of hydrogen peroxide at 68°C. The samples were then cooled to 0°C for half hour and 5 ml of aquasol added, mixed well to obtain a clear solution and counted. (2) In some experiments, X-ray films were scanned and the peaks obtained from the respective bands PVII and VII were extrapolated. The ratio of extrapolated values were used in determining the rate of conversion of PVII to VII as a measure of enzyme activity.

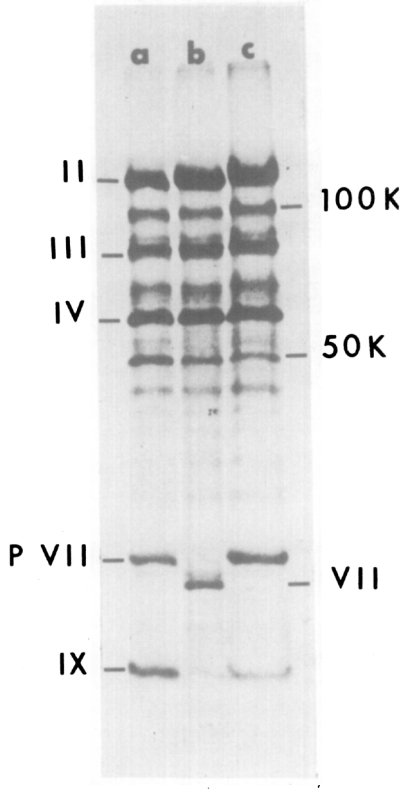

Figure 1. The effect of temperature on the synthesis of protease. Protease activity was determined as described in Materials and Methods. a, control; b, enzyme obtained from ts3 infected HEp-2 cells propagated at 33°C; c, enzyme obtained from ts3 infected HEp-2 cells propagated at 39°C.

RESULTS AND DISCUSSION

In vivo studies have shown that processing of PVII in ts3 takes place at 33°C (5). Pulse chase experiments have confirmed these previous results, and revealed furthermore that processing of PVII could be detected as early as 21 hr post-infection. The effect of temperature on ts3 induced synthesis of protease is illustrated in Fig. 1. Cleavage of PVII to VII was only observed with enzyme prepared from ts3 infected cells at 33°C. No cleavage of PVII was observed when substrate was treated with buffer alone or with enzyme obtained from 39°C infected cells. These results demonstrate the presence of protease activity in ts3 infected cells propagated at 33°C. However, the possibility cannot be ruled out that, this protease activity may be host cell induced at

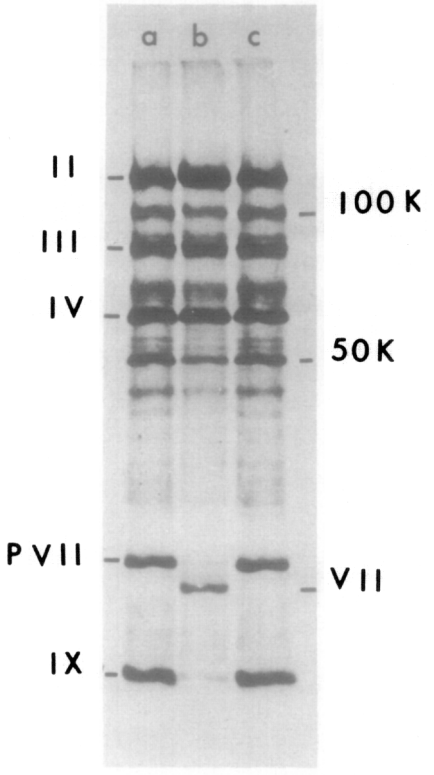

Figure 2. Comparison of protease activity from ts3 and mock-infected HEp-2 cells on the processing of PVII. a, control; b, substrate was treated with enzyme obtained from ts3 infected cells, c, substrate was treated with enzyme from mock-infected cells.

33°C. Therefore, to distinguish between host or virus induced protease activity the following experiments were performed. Enzyme preparations were obtained from ts3 and mock-infected cells. Their effect on the proteolytic processing of substrate proteins was examined. Figure 2 depicts the specificity of enzyme obtained from ts3 and mock-infected cells propagated at 33°C on the processing of PVII. Once again cleavage of PVII was observed in substrate treated with enzyme obtained from ts3 infected cells. No detectable cleavage was obtained with enzyme from mock-infected cells. These results confirm that, this proteolytic activity is virus induced.

Having established with reasonable certainty the virus-directed processing of PVII, we can now inquire whether, the proteolytic factor responsible

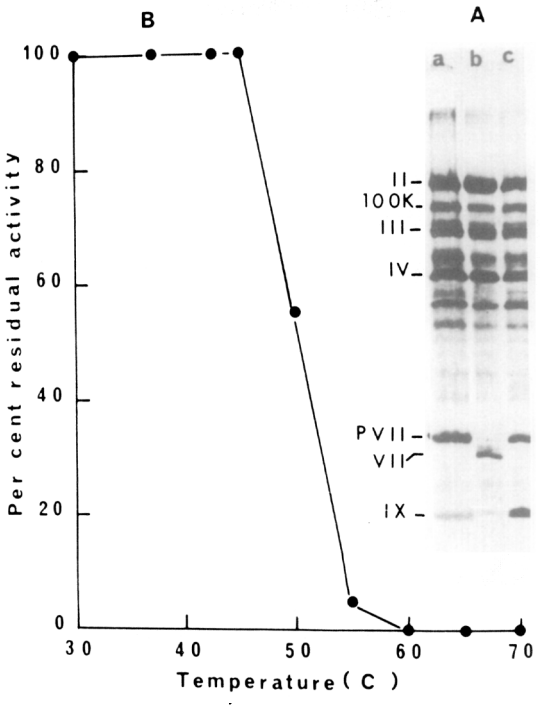

Figure 3. Thermal stability of protease activity.
A. Autoradiogram of protease activity after heating at 100°C.
a, control; b, substrate was treated before and c, after heat treatment. B. Stability of protease at different temperatures. For details see Materials and Methods.

for the formation of VII is an enzyme. Chemical processing of PVII has been reported (7). Therefore, in order to clarify the possibility of chemical cleavage we have examined the following parameters of protease activity. Phenylmethyl sulfonyl fluoride at 1mM concentration inhibited protease activity of ts3, thus suggesting that the enzyme is a seryl protease. EDTA has been found to inhibit certain proteases (8). In the present study EDTA, even up to 10mM concentration, did not inhibit proteolytic cleavage of PVII. These data suggest that the ts3 induced protease activity is due to non-metaloprotease. Unlike proteases from Rous sarcoma virus (9) and polyoma virus (10), the protease of ts3 was inhibited by sodium dodecyl sulfate. Enzyme preparation obtained from wild type adenovirus type 2 propagated at 33 and 39°C processed PVII to VII. Whereas, similar to ts3, other temperature sensitive mutants (3, 11) gave enzymatically active extracts

when propagated at 33°C but not at 39°C. Failure to detect any autocatalytic cleavage in the control suggests that protease activity is due to a distinct protease protein moiety rather than protease activity associated with PVII.

Figure 3A shows the effect of heat on protease activity. Heating of enzyme for 3 min. in boiling water completely inhibited its proteolytic activity, indicating that the cleavage of PVII to VII is probably due to the putative protease enzyme (12) rather than to chemical decomposition. The thermal stability of the enzyme was further investigated. Heat treatment of the enzyme at various temperatures indicated that it retained full activity to 45°C, but lost its activity almost completely at 60°C. Loss of enzyme activity at 50 and 55°C was 45 and 95% respectively (Fig. 3B). In some experiments, even prolonged incubation of enzyme at 43 and 45°C did not show detectable loss of activity. These results suggest that the protease is quite stable at 45°C. Various enzyme preparations retained their full enzyme activity up to 2-3 weeks, when stored at 4°C. The present study has clearly, and specifically demonstrated that the in vitro formation of VII from its precursor PVII is a virus-induced enzymatic function.

ACKNOWLEDGEMENTS

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