PROTEINASE ACTIVITY IN PURIFIED ANIMAL VIRUSES

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Summary: Highly purified virions of influenza virus, vesicular stomatitis virus and mengovirus were found to contain proteinase activity that degrades viral proteins once the virions are disrupted by nonionic detergent or by heat shock. Prior treatment of purified intact virions with irreversible inhibitors of proteolytic enzymes did not retard autodigestion by subsequently disrupted virions.

After enteroviruses attach to cell receptors a rearrangement of virion capsid proteins takes place which leads to loss of certain viral antigens, ultimate release of genome RNA and possibly triggering of penetration into the cell (1-3). Very little is known about these stages following attachment and although it is clear that penetration and eclipse have a higher temperature coefficient than does attachment, no enzymatic activity has yet been described which explains this temperature requirement.

The enveloped viruses such as influenza virus (4,5) and RNA tumor viruses (6) introduce their nucleocapsid into the cell cytoplasm as a result of fusion of the virion envelope with the cell plasma membrane following attachment. Again, this process has a temperature coefficient suggesting that enzyme activity may be required. Following RNA tumor virus penetration, the cell plasma membrane can be seen to be damaged at the point where virus has fused and penetrated (6). The fusion-penetration function of membrane

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viruses such as influenza virus remains to be defined.

Since enterovirus proteins undergo proteolytic cleavage after synthesis and during virion maturation (7-9) we have been examining the possibility that proteolytic enzyme activity may be an integral part of many kinds of purified virions and serve a vital function in virus maturation, penetration and uncoating.

This preliminary report indicates that highly purified animal virions do contain proteolytic activity, although it remains to be determined whether these enzymes are adventitious proteins or virus-coded.

METHODS

Mengovirus was grown in L cells in Eagles medium with 5% calf serum. It was purified essentially as described by Hall and Reuckert (3) except that the sharp virus band from CsCl was passed through two successive columns of Biogel A-5m to remove cesium salt and remaining small proteins. The Biogel columns were equilibrated with 0.05M Tris HCl, pH 7.5. Influenza virus grown in canine kidney cells and vesicular stomatitis virus (VSV) grown in chick embryo fibroblasts were purified by differential centrifugation, velocity sedimentation banding in 5-30% sucrose gradients, gel filtration on Sepharose 2B, banding in 15-45% tartrate density gradients and 2 successive gel filtrations on Sepharose 2B equilibrated with 0.05M Tris-HCl, pH 7.5.

RESULTS AND DISCUSSION

Preliminary experiments showed that purified mengovirus and poliovirus were stable for days at 37°. However, these enteroviruses can be triggered to undergo capsid rearrangement by heating (10) or by contact with cell surface receptors (1,3). This capsid protein alteration causes antigenic change and initiates release of viral RNA (1-3). When we examined the stability of mengovirus during incubation following such heat triggering, it was found that the virion proteins underwent slow digestion (Fig. 1A, 1B).

Slight loss of protein was detected within one hour and marked autodigestion was always observed within 20-24 hr. Even more rapid autodigestion was obtained with purified poliovirus. Pretreatment of virions in Tris-DTT buffer with the irreversible protease-inhibitors tosylamidophenylalanyl chloromethane (TPCK) and N-α-tosyl-lysyl chloromethane (TLCK) at 0.2 mM failed to prevent autodigestion. Therefore the responsible proteolytic enzyme(s) is either buried within (or a part of) the virion capsid protein, or is not affected by these specific inhibitors of proteases with tryptic (11) and chymotryptic (12) specificities.

Fig. 1C and 1D show that VSV also undergoes autodigestion despite a high degree of purification and pretreatment with TPCK and TLCK. Fig. 1E and 1F show similar autodigestion of influenza virus occurring within 4 hr after its envelope was solubilized by the nonionic detergent NP-40. Note that the nucleoprotein polypeptides of VSV (peak 3) and of influenza virus (peak N) are relatively more stable than the other virus proteins. Autodigestion was carried out in sterile siliconized tubes, where all virus protein label was recovered and over 90% of labeled protein remained acid precipitable in 5% trichloroacetic acid. Bacterial contamination was not a factor since similar results were obtained whether or not poisons such as azide or cyanide were present during incubation. Diluted suspensions of these same virus preparations exhibited a more rapid autodigestion. This concentration independence suggests that the responsible enzymatic activity must be bound to (or is a part of) the purified virions.

If protease action were important for some early stage(s) of infection (e.g. penetration and/or uncoating) then it might be possible to inhibit virus infectivity with an irreversible protease inhibitor. Since the data presented above (Fig. 1C, Fig. 1D) showed that 2×10^{-4} molar concentrations of TPCK and TLCK did not significantly diminish the proteolytic activity of subsequently disrupted purified virions, we tested the inhibitory capacity of very high concentrations of TPCK and TLCK under conditions known to be favorable for virion proteolytic activity. Table 1 shows that we were able

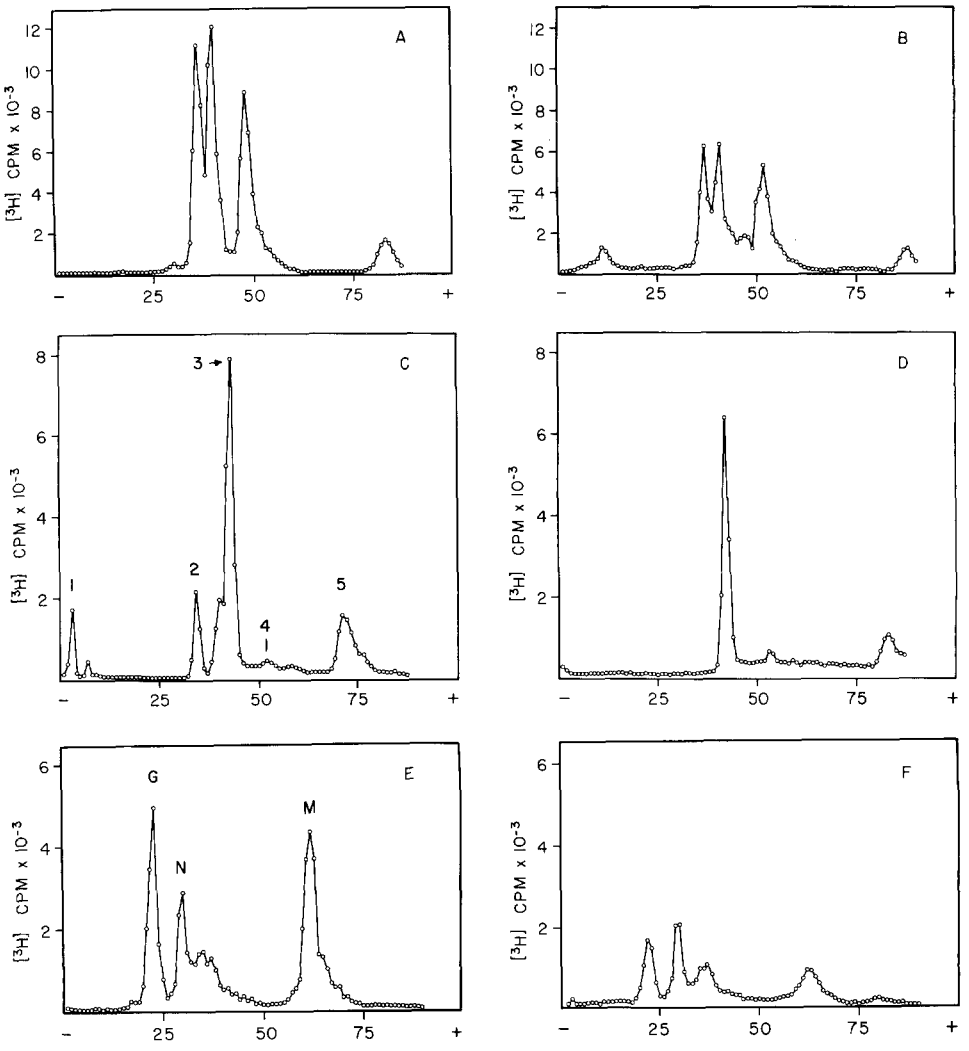

Fig. 1 Autodigestion of highly purified virions of mengovirus, VSV and influenza virus.

A. Proteins of control, untreated mengovirus.
B. Same as in A after incubation for 48 hours at 37°. Before incubation in Tris-DTT buffer the virions were heated at 75°for 45 seconds to disrupt the capsid structure.
C. Proteins of control VSV virions. For three hours immediately prior to electrophoresis the virions were treated with a mixture of 0.2 mM TPCK and 0.2 mM TLCK in Tris-DTT buffer at 25°.
D. Same as in C except that following the TPCK, TLCK treatment the virions were incubated for 20 hours at 37°in Tris-DTT buffer containing 0.1% Nonidet P40.
E. Proteins of control, untreated influenza virions.
F. Same as in E except the virions were incubated for 3 hours at 37° in Tris-DTT containing 0.1% Nonidet P40.

All incubations were carried out in sealed, siliconized, sterile test tubes containing 0.1 ml of Tris-DTT buffer (0.01 M dithiothreitol, 0.05 M Tris-HCl pH 7.5). 38.5 µg mengovirus, 29 µg VSV or 15 µg influenza virus were employed. Identical amounts of labeled, control virus and labeled, treated virus were loaded onto the gels.

TABLE 1. INACTIVATION OF VIRUS INFECTIVITY BY PROTEOLYTIC ENZYME INHIBITORS

Virus	Inhibitor*	Incubation medium**	% inactivation of PFU***
mengovirus	TPCK, TLCK 1.5 mM	Tris-DTT	65, 94, 48
influenza virus	TPCK, TLCK 1.5 mM	0.15 M NaCl	90, 86, 56
	TPCK, TLCK 1.5 mM	Tris-DTT	90
	TPCK, TLCK 1.5 mM	Eagles Medium + 5% calf serum	0, 0
vesicular stomatitis virus	TPCK, TLCK 1.5 mM	Tris-DTT	99.3, 90, 96, 78, 99
	TPCK, TLCK 1.5 mM	Tris-DTT + 5% calf serum	0, 10, 62

* 0.05 M stock solutions of TPCK and TLCK were made up in 100% ethanol. Fresh inhibitor solution was diluted into incubation medium containing virus. Equivalent amounts of ethanol were diluted into control virus solutions in the same incubation medium. There was no loss of infectivity due to the incubation medium and alcohol. 1.5 mM TPCK tended to form cloudy aqueous solution and partially precipitate during incubation.

** Tris-DTT incubation medium contained 0.01M DTT, 0.05 M Tris-HCl, pH 7.5. All incubations were carried out for 20 minutes at 25°C.

*** Where several values of plaque forming unit (pfu) inactivation are shown, the data are from different experiments.

to inactivate the infectivity of mengovirus, influenza virus and VSV by 20 minutes of room temperature incubation with mixtures of 1.5 mM each of TPCK and TLCK. This loss of virus infectivity varied quantitatively in different experiments ranging from only several-fold loss of plaque forming ability to several orders of magnitude loss. The presence of serum or other proteins during incubation usually prevented or diminished inactivation but could never reverse it following incubation. Lower concentrations of the inhibitor mixture, or of TPCK or TLCK alone usually caused less extensive and less reproducible inactivation of infectivity. It is not yet clear whether these inhibitors inactivate viruses in a nonspecific manner (e.g. alkylation of virion protein) or whether they actually are interfering with virion proteinases essential for biological function.

Pereira and Skehel (13) recently reported protein degradation upon long

term storage of purified adenovirus and its subunits, and they suggested that it was probably due to contaminating enzymes. Despite the purity and diversity of the viruses employed in the present studies, much more work will be necessary to determine whether these proteinases are virus-coded or firmly bound cellular contaminants. We are currently trying to purify virion proteinases and to obtain conditional virus mutants blocked in proteinase activity. Regardless of the source of these virion proteinases it is obvious that they must be considered in biochemical studies of virions, particularly those involving virion protein chemistry. They are very likely a contributing factor in the many contradictory reports regarding the numbers and proportions of proteins in purified myxoviruses and DNA and RNA tumor viruses.

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