Proteolytic cleavage of microtubule-associated proteins by retroviral proteinases

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Aspartic proteinases from human immunodeficiency virus type 1 (HIV-1) and avian myeloblastosis virus (AMV) were found to interfere with microtubule assembly. Preincubation of the proteinases with purified brain microtubule proteins (tubulin and microtubule-associated proteins) at low ionic strength (pH 6-8), completely inhibited microtubule assembly. Analysis of microtubule proteins after incubation with proteinase showed no effect on tubulin but extensive cleavage of the microtubule-associated proteins 1 and 2 was observed. The digestion by the two proteinases differed. In the presence of HIV-1 proteinase, a fragment with an M, of approximately 300000 appeared, as well as at least three other new fragments, with M, values of 188000, 124000 and 73000. In the presence of AMV proteinase, the microtubule-associated proteins were extensively digested to many small fragments. The extending microtubule-associated proteins normally seen by electron microscopy on the microtubule surface disappeared after treatment with AMV proteinase. Our results show that retroviral proteinases are not restricted to cleavage of viral polyproteins in vitro. It is suggested that proteolysis of microtubular proteins by viral proteinases is an important step in viral pathogenicity and that it may be part of a mechanism causing degenerative effects in infected cells.

Introduction

Retroviruses contain an aspartic-type proteinase as part of their gag or pol genes (Kräusslich & Wimmer, 1988). This enzyme is required for a post-translational cleavage of the precursor polyproteins of the virus. A defect in this processing results in the production of immature viral particles which lack infectivity (Peng et al., 1989). Retroviral proteinases have, up to now, been considered as highly specific, cleaving only the precursor polyproteins of their own virus. Polyproteins of heterologous retroviruses are not readily cleaved (Darke et al., 1988; Kräusslich & Wimmer, 1988; Kräusslich et al., 1989). Cleavage of non-viral proteins has been shown only for completely denatured substrates (Dittmar & Moelling, 1978).

Viral infections are frequently associated with damage of infected cells, often resulting in lysis of the cell. The cause for this has not yet been explained by a specific mechanism. HIV infection in humans, for example, results in a defective immune system, loss of CD4+ cells and damage of the central nervous system (de la Monte et al., 1987). The reason for these symptoms is not known.

Cathepsin D, which is a non-viral aspartic proteinase, has recently been found to degrade microtubule-associated proteins (MAPs) (Matus & Green, 1987). The proteolysis was suggested to be involved in an age-related degeneration of the brain. Microtubules are a component of the cytoskeleton in all eukaryotic cells (Dustin, 1984). They consist of polymer of α- and β-tubulin dimers and MAPs of which MAP 1 (35000), MAP 2 (270000) and the tau proteins (55000 to 70000) are the best characterized. Tubulin assembles into long, hollow tubes which are structurally stabilized by the MAPs. Being part of the cytoskeleton, microtubules are involved in the structure of the cell as well as in many of its functions. Most notably, they are involved in the formation of the mitotic spindle of dividing cells, in intracellular transport processes such as transport in the neuronal axons, and in the structural maintenance of axons and dendrites (Dustin, 1984).

In view of the important roles of microtubules, and the observed effect of cathepsin D on MAPs, it was of interest to determine whether retroviral aspartic proteinases also can degrade microtubule proteins. Our results show that the substrate specificity of retroviral aspartic
proteases in vitro is not restricted to viral proteins as previously thought, since they also degrade MAPs. Proteolytic breakdown of the microtubule system might be an explanation for the observed damage on infected cells, most notably in the nervous system in patients with AIDS.

Methods

Microtubule proteins. Tubulin and MAPs were prepared from bovine brain in the absence of glycerol as described previously (Wallin et al., 1986).

HIV proteinase. A synthetic gene for a proteinase of human immunodeficiency virus type 1 (HIV-1) was expressed in Escherichia coli (Symbicon) and cells were lysed in a French press. The lysate was centrifuged for 20 min at 10000 g and 30 min at 100000 g at 4 °C. Initial experiments were performed with these crude lysate preparations. Details of the construction and expression of the synthetic gene will be presented together with the purification of the enzyme (L. Goobar et al., unpublished results). The purity and concentration of the enzyme after the final size exclusion chromatography step was estimated from silver staining of the gels after SDS-PAGE (Blum et al., 1987). The only band observed (approximately 10000) corresponded to the Mr of the HIV proteinase (data not shown). Proteinase activity was measured after overnight incubation at 37 °C with heat-denatured bovine serum albumin (BSA) (0.4 mg/ml in 20 mM-Tris-HCl, 5 mM-EDTA, 1 mM-DTT, 1 mM- NaCl at pH 6). Cleavage was confirmed by SDS-PAGE (Schägger & von Jagow, 1987). It was verified that the proteinase was an aspartic proteinase, as no activity was observed in the presence of 100 µM-pepstatin A.

Proteinase of avian myeloblastosis virus (AMV). AMV, obtained from Life Sciences, was disrupted by ultrasonication in buffer (0.1 g protein/ml, 0.2 mM-Tris-HCl, 0.25 mM-NaCl, 5 mM-DTT, 1% Triton X-100, 1 mM-EDTA at pH 8.8), followed by centrifugation at 40000 g for 50 min, and at 100000 g for 60 min. The high speed supernatant was applied to a Mono-Q anion exchange column (Pharmacia LKB Biotechnology) and the proteinase was eluted with a gradient between 0.15 and 0.35 mM-NaCl in column buffer (20 mM-Tris-HCl at pH 8.0). Fractions with proteinase activity were pooled and concentrated. The protein concentration was measured with a protein assay kit (Bio-Rad) using BSA as a standard. The activity was determined as for the HIV-1 proteinase, except that 1 mM-pepstatin A was used in the inhibition experiment. The purity of the enzyme was checked by SDS-PAGE (Schägger & von Jagow, 1987). No bands were observed after either Coomassie blue or silver staining (Blum et al., 1987), nonetheless the catalytic activity is higher than that observed for HIV proteinase which stains well under these conditions; control proteins from 2.5 to 96K also stained. The lack of a band at 10000 to 15000 representing the proteinase might be caused by an inability of the proteinase to fix in the gel or it may be inaccessible to the stain.

Fig. 1. SDS-PAGE analysis of microtubule proteins incubated in the presence of E. coli lysates expressing HIV-1 proteinase. Microtubule proteins at a concentration of 2 mg/ml were incubated for 25 min in the absence or presence of 60 µl E. coli lysate (total volume 360 µl). Tubulin has separated into two bands, α- and β-tubulin. Lane 1, microtubule protein supernatant (control); lane 2, assembled microtubule pellet (control); lane 3, microtubules assembled in the presence of lysate, supernatant (control); lane 4, microtubules assembled in the presence of lysate, pellet (control); lane 5, microtubules assembled in the presence of lysate and 1 mM-pepstatin A, supernatant (control); lane 6, microtubules assembled in the presence of lysate and 1 mM-pepstatin A, pellet (control); lane 7, microtubules assembled in the presence of lysate with expressed HIV-1 proteinase, supernatant; lane 8, microtubules assembled in the presence of lysate with expressed HIV-1 proteinase, pellet; lane 9, microtubules assembled in the presence of lysate with expressed HIV-1 proteinase and 1 mM-pepstatin A, supernatant; lane 10, microtubules assembled in the presence of lysate with expressed HIV-1 proteinase and 1 mM-pepstatin A, pellet; lane 11, lysate, supernatant (control); lane 12, lysate, pellet (control).
Assemble of microtubules. The assembly of microtubule proteins in assembly buffer (0.1 M-PIPES, 0.5 mM-MgSO4, 1 mM-DTT and 1 mM-GTP at pH 6.8) in a thermostatted cuvette was initiated by raising the temperature from 4 °C to 37 °C. Alternatively, after preincubation of microtubule proteins in assembly buffer without GTP at 37 °C, assembly was initiated by the addition of GTP to 1 mM. The assembly was followed spectrophotometrically by the increase in turbidity, monitored as the apparent absorbance at 350 nm.

SDS–PAGE analysis of assembled microtubules. Microtubule samples (0.3 ml) were withdrawn from the assembly mixture (above) after 25 min and centrifuged in an Airfuge (Beckman) for 5 min at full speed at room temperature to pellet assembled microtubules. The pellets were redissolved in 0.5 ml sample buffer and the supernatants were diluted with an equal volume of sample buffer for SDS–PAGE according to Laemmli (1970). The samples were run on a 5 to 12% linear polyacrylamide gel and stained with Coomassie blue.

Electron microscopy of assembled microtubules. Samples were withdrawn from the assembly mixture after 25 min and negatively stained directly, or assembled microtubules were isolated by centrifugation (see above), embedded, sectioned and stained (Wallin et al., 1986).

Results

Proteolysis of microtubule proteins

Incubation of microtubule proteins with lysates from E. coli expressing HIV-1 proteinase showed degradation of MAPs (Fig. 1). MAP 1 and MAP 2 were completely degraded in both the assembled microtubules (pellet) and the non-assembled microtubule proteins (supernatant). Pepstatin A is known to be a specific inhibitor of aspartic proteinases. Both AMV and HIV-1 proteinases were inhibited by 1 mM-pepstatin A (Hansen et al., 1988; Kotler et al., 1989). MAP degradation was inhibited in the presence of pepstatin A. Furthermore, in controls with lysates from bacteria which do not express the enzyme, no degradation of the MAPs was observed (Fig. 1).

Assembly of microtubules

To determine the significance of the proteolysis of MAPs under these conditions, the assembly of microtubules was studied in the presence of either purified HIV-1 or AMV proteinase. Microtubule assembly in vitro was inhibited by both HIV-1 and AMV proteinases (Fig. 2a, b). Without preincubation no effect was seen in the presence of the HIV-1 proteinase (data not shown). After 10 min preincubation of microtubule proteins with 3 µg/ml of HIV-1 proteinase at 37 °C in the absence of GTP (to avoid assembly of microtubules), the GTP-induced assembly of microtubules was approximately 88% that of the control (Fig. 2a). Complete inhibition of assembly was obtained after a 30 min preincubation with a higher concentration, 8 µg/ml, of HIV-1 proteinase in the absence of GTP. Inhibition of assembly was not reversed upon the addition of 1 mM-GTP. Assembly could be induced when 20 µM-taxol was added. However, the increase in turbidity was higher than in the control assay without taxol (Fig. 2a). A similar complete inhibition of the GTP-induced assembly was also observed after 12 min preincubation with AMV proteinase (Fig. 2b). This inhibition could also be reversed by the addition of 20 µM-taxol.

Unfortunately pepstatin A itself induced aberrant forms of microtubules (data not shown) and therefore could not be used in the assembly assay.

Analysis of microtubule proteins

After assembly of microtubule proteins, the assembled microtubules (pellet) were separated by centrifugation from the proteins which did not assemble (supernatant),
and analysed by SDS-PAGE. The gels revealed that all
the high Mr MAPs, MAP 1 and MAP 2, in both the
supernatant and the microtubule pellet, were hydrolysed
(Fig. 3 and 4) in the presence of either HIV-1 or AMV
proteinase. The HIV-1 proteinase cleaved MAP 1 into a
stable form with an approximate Mr of 300 000, interme-
tiate to that of MAP 1 and MAP 2 (Fig. 3). Only
a small amount of this fragment associated with
assembled microtubules. At least three new fragments
(188 000, 124 000 and 73 000) remained associated with
the assembled microtubules. When microtubule assem-
bly was first completely inhibited by treatment with
HIV-1 proteinase, and thereafter reassembled by the use
of taxol, no high Mr MAPs (MAP 1, MAP 2 or the MAP
1-derived fragment) were found associated with
the assembled microtubules (Fig. 3, lane 5). However, the
same three new fragments as found in the presence of a
lower amount of HIV-1 proteinase remained associated.
AMV proteinase hydrolysed the MAPs to a greater
extent than HIV-1 proteinase, as was evident from SDS-
PAGE results (Fig. 4) showing only small fragments with
Mr values less than that of tubulin. After preincubation
with AMV proteinase an even more extensive proteolysis
was seen (data not shown).

Tubulin itself was apparently unaffected by treatment
with either HIV-1 or AMV proteinase, since both α- and
β-tubulin had the same Mr on 12% SDS–polyacrylamide
gels (data not shown).

Electron microscopy

Morphological changes of microtubule structure were
seen after treatment with AMV proteinase. The AMV
proteinase-treated microtubules differed from the con-
trol microtubules as seen on embedded and sectioned
samples, since no extending MAPs were seen on the
surface (Fig. 5). The microtubules were also much more
closely aligned than the control microtubules, further
indicating the absence of high Mr MAPs.

In negatively stained samples no obvious morphologi-
cal alterations of the assembled microtubules were found
in the presence of the HIV-1 proteinase after a short
preincubation time, although the MAPs were partially
degraded. A longer preincubation of microtubule pro-

![Fig. 3. SDS–PAGE analysis of microtubule proteins assembled in the presence of HIV-1 proteinase. Lane 1, assembled microtubules
pellet (control); lane 2, microtubule proteins, supernatant (control); lane 3, microtubules assembled in the presence of HIV-1 proteinase
(3 μg/ml), pellet; lane 4, microtubules assembled in the presence of HIV-1 proteinase (3 μg/ml), supernatant; lane 5, taxol (20 μM)-
induced microtubules after preincubation for 30 min at 37 °C with HIV-1 proteinase (8 μg/ml), pellet; lane 6, taxol (20 μM)-induced
microtubules after preincubation for 30 min at 37 °C with HIV-1 proteinase (8 μg/ml), supernatant; lane 7, Mr standard proteins.]
Proteolysis of MAPs by viral proteinases

In the present study we have found that the retroviral proteinases of HIV-1 and AMV are not as strictly selective for their own polyprotein substrates in vitro as previously believed. The proteinases were able to affect microtubules by cleavage of the MAPs. Although both proteinases are aspartic proteinases, they did not affect microtubules similarly. This is probably due to differences in substrate specificity. It has been shown that an AMV substrate is not cleaved by HIV proteinase (Kräusslich et al., 1989). However, this does not exclude the possibility that certain other sequences can function as a substrate for both enzymes. It has also been shown that the specificity of AMV proteinase is dependent on the salt concentration (Kotler et al., 1989). The conditions under which the present experiments have been performed were optimized for the study of microtubule assembly. They differ in certain respects to what is optimal for AMV and HIV proteinase catalysis, most notably in pH and ionic strength. The catalytic properties for the enzymes may therefore be affected. However, the conditions here are probably more representative of those in the cell, compared to those optimal for the proteinase activities.

In the presence of a low concentration of the HIV-1 proteinase, the extent of assembly was only slightly affected, despite proteolysis of the MAPs. Many new fragments were found, some of which remained bound to assembled microtubules of normal morphology. MAP 1 was digested to a fragment with an approximate Mr of 300,000, a fragment found between MAP 1 and MAP 2 on SDS-polyacrylamide gels. A similar stable MAP 1 fragment has been found in the presence of another aspartic proteinase, cathepsin D (Matus & Green, 1987), indicating that there are similarities between retroviral and animal aspartic proteinases.

Upon prolonged incubation with the HIV-1 proteinase, MAP 2 was degraded extensively, as found also after digestion of microtubule proteins with cathepsin D. Microtubule assembly was completely inhibited upon prolonged preincubation with the proteinase. However, assembly could be reversed by addition of the assembly-stimulatory drug taxol, which is able to induce the assembly of tubulin independently of the presence or

Discussion

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Fig. 4. SDS-PAGE analysis of microtubule proteins assembled in the presence of AMV proteinase. Lane 1, microtubule protein supernatant (control); lane 2, assembled microtubule pellet (control); lane 3, microtubules assembled in the presence of AMV proteinase (0.1 μg/ml), supernatant; lane 4, microtubules assembled in the presence of AMV proteinase (0.1 μg/ml), pellet.

Fig. 5. Electron microscopy of embedded microtubules. Microtubules were assembled in the presence or absence of AMV proteinase as described in the legend to Fig. 2. Assembled microtubules were pelleted and embedded, sectioned and stained for electron microscopy. (a) Assembled microtubules, control. (b) Microtubules assembled after treatment with AMV proteinase. Bar marker represents 0.1 μm.
absence of MAPs (Schiff & Horwitz, 1981). These microtubules consisted mainly of tubulin, but several fragments of both high and low Mr values were present. Although these fragments bind to assembled tubulin, they were not able to promote assembly. The microtubules had an altered morphology, but this was most probably caused by taxol and not the digestion of the MAPs, since undigested microtubules assembled in the presence of taxol have an altered morphology (Wallin et al., 1986).

Microtubule assembly in the presence of AMV proteinase (without preincubation) was only partly inhibited, although the assembled microtubules consisted mainly of tubulin and a small amount of low Mr fragments of degraded MAPs. However the digestion of the MAPs was reflected by the absence of extending projections normally consisting of MAP 1 and MAP 2.

The assembly of microtubules is dependent on assembly-promoting MAPs, and it is therefore puzzling that AMV proteinase-digested samples could assemble. However at least two explanations exist as to why AMV proteinase-treated microtubule proteins can assemble, although less efficiently, even when the high Mr MAPs were digested. First, it has been shown that tubulin self-assembles after proteolytic cleavage by subtilisin (Serrano et al., 1984). However, after proteolysis of tubulin by subtilisin the regulatory domain in the C terminus was removed, altering its electrophoretic mobility and inducing an altered morphology of the assembled tubulin (Serrano et al., 1984). Such a change in the mobility of tubulin was not induced by the AMV proteinase and no aberrant forms were found. The only morphological effect found was that the extending projections, usually consisting of MAP 1 and MAP 2, were lost. The effects of AMV proteinase are thus most probably not due to hydrolysis of the regulatory domain of tubulin.

Secondly, mild proteolytic cleavage of MAPs with trypsin results in small Mr 35000 fragments which possess the ability to stimulate assembly (Vallee & Borisy, 1977). After incubation with the AMV proteinase, no such fragments were found. However the possibility exists that smaller fragments, not visible in the present SDS-PAGE system, might stimulate assembly. Recently it was suggested, based on sequence data on the assembly-promoting MAP 2 and tau proteins, that these proteins share a small microtubule-binding motif (Lewis et al., 1988). It has been postulated that three repetitive sequences constitute the microtubule-binding site. The three MAP 2 repeats have been synthesized, but only one of them was capable of stimulating microtubule assembly (Joly et al., 1989). This means that a small region, constituting approximately 1% of MAP 2, is enough to promote assembly. The presence of a small fragment, probably including this region, might therefore explain the observed ability of microtubule proteins treated with AMV proteinase to assemble. These assembly-stimulatory fragments are probably further digested upon preincubation with the AMV proteinase, since the assembly then was completely inhibited.

The interaction between cathepsin D and microtubules involves proteolysis of MAP 1 and MAP 2 (Matus & Green, 1987). Our results show that the viral aspartic proteinases were also able to affect microtubules in vitro by cleavage of the MAPs. Disruption of the MAPs, which are part of the cytoskeleton, by the viral proteinases may be an important part of viral pathogenesis. This suggestion is supported by the recent finding that patients with AIDS have defective natural killer cells with an abnormal distribution of tubulin (Sirianni et al., 1988). Studies are now in progress to determine whether other infected cells contain destroyed microtubules.

Viral proteinases are found in most types of viruses and it should be of interest to investigate whether they have a general ability to destroy the cytoskeleton of infected cells and thereby cause morphological changes or lysis. Inhibition of aspartic proteinases has been suggested as an attractive approach for controlling retroviral infection by preventing retroviral particle formation. Our results show that this could possibly also be a useful means of limiting the damage of the immune system and neuronal function in AIDS or other degenerative retroviral diseases.

We are indebted to Mrs Ann Britt Källström, Inger Holmquist and Liselotte Öhberg for technical assistance. Taxol was a generous gift of Dr M. Sufness at the National Institutes of Health, Bethesda, Md., U.S.A. This research was supported by grants from the Swedish Natural Science Research Council, Riksforeningen mot Cancer, Magnus Bergvalls Stiftelse (M.W.) and the Swedish National Board for Technical Development (H.D. and L.G.).

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(Received 23 January 1990; Accepted 24 May 1990)