Cysteine Protease Activated by Expression of HIV-1 Protease in Transgenic Mice

MIP26 (AQUAPORIN-0) CLEAVAGE AND CATARACT FORMATION IN VIVO AND EX VIVO*

(Received for publication, October 7, 1996)

Kenneth P. Mitton†, Toshikazu Kamiya§, Santa J. Tumminia, and Paul Russell

From the Laboratory of Mechanisms of Ocular Diseases, National Eye Institute, National Institutes of Health, Bethesda, Maryland 20892 and SKyowa Hakko Kogyo Co., LTD., Tokyo 194, Japan

Transgenic mice, homozygous for HIV-1 protease expression in the eye lens, display degradation of some lens crystallins and cytoskeletal proteins prior to cataract formation on postnatal days 23–25. Alterations to the internal lens hydration state also occur; therefore, the status of the aquaporin protein MIP26 was examined over postnatal days 16–25 to determine if it was altered during cataractogenesis. The MIP was identical in transgenic and control lenses until day 21. By postnatal day 25 (frank cataract), in the lenses obtained from transgenic animals, the 26-kDa band was absent and there was a concurrent increase in the proportion of MIP23. Immunoblotting demonstrated cleavage at the C terminus. Lenses were also maintained in an organ culture system to demonstrate that the cataractogenic process is inherent to the isolated lens and to determine the contribution of cysteine protease action. Organ culture experiments revealed a similar progression to nuclear cataract formation as seen in vivo. Two-dimensional gel analysis of the soluble lens crystallin fraction of organ cultured lenses revealed the same cleavage pattern as occurs in vivo. Organ culture of transgenic lenses with E64, a cysteine protease inhibitor, dramatically delayed cataractogenesis and prevented proteolytic cleavage of both MIP26 and crystallins. HIV-1 protease, while the trigger of cataract formation, does not appear to be the protease responsible for cleavage of MIP or lens crystallins. These results suggest that activation of endogenous cysteine protease activity is involved in the cleavage of these proteins and occurs downstream of HIV-1 protease action.

Replication of the human immunodeficiency virus (HIV),1 leads to severe dysfunction of the infected patient’s immune system (1–3). Patients with acquired immune deficiency syndrome (AIDS) are susceptible to secondary pathologies that can lead to death. Intense research to elucidate the molecular aspects of HIV viral/cell interactions led to the creation of transgenic mice containing the entire HIV-1 genome with a nonfunctional viral reverse transcriptase. Many of these transgenic mice displayed cataract formation within 3 to 6 months of age (4). While AIDS patients are at risk of blindness from uveitis and retinitis caused by secondary viral infections, little information exists regarding possible increased incidence of cataracts. One small study of patients has reported ocular lens opacities in many of the HIV-1-infected patients examined (5).

Transgenic mice (line TG72) expressing the HIV-1 protease under control of the lens-specific αA-crystallin promoter also develop cataracts on postnatal days 23–25 indicating that expression of the protease alone will cause cataracts. Cataract formation is dependent on the HIV-1 protease, because transgenic mice expressing an inactive HIV-1 protease in the lens remain cataract-free as do TG72 mice that are administered HIV-1 protease inhibitors (6). In homozygous TG72 mice, lenses show refractive index changes prior to cataract formation. Lens nuclear alterations appear about postnatal day 21, and by day 23 distinct nuclear cataracts are apparent. The cataractous nuclei of the lenses are displaced posteriorly with increasing age and increase in diameter. Light scattering measurements and scanning electron microscopy have revealed that alterations to the cytoskeleton and membrane components are responsible for much of the opacification, and biochemical data have shown extensive proteolysis of lens proteins. Damage to both lens crystallins and lens fiber cell membranes leads to water influx and a decrease of nonfreezable water. Density fluctuations from cytoplasmic density variation and the possible increased extracellular space cause the light scattering which is seen as cataract (7).

Curiously, the patterns of proteolysis of the lens crystallin proteins are not those that can be generated by the HIV-1 protease (6). MIP26, a 263-amino acid member of the aquaporin transmembrane family of water channels (8, 9), comprises over 50% of the intrinsic membrane protein of the lens fiber cell. MIP may play a role in regulating the transmembrane flux of water in lens fiber cells and in addition there is evidence to suggest MIP maintains the very small extracellular space between fiber cells through electrostatic association to phospholipid head groups of the opposing fiber cell membrane (10). MIP associates with structural proteins of the lens cytoplasm such as α- and β-crystallins, and the binding of ATP to α-crystallin increases its affinity for binding to MIP (11–13). Limited proteolysis of MIP26 to lower molecular weight species has been reported previously in aging human lenses (14, 15), human senile cataract (16), Philly mouse cataract (17), and the selenite-induced cataract model in the rat (18). This pattern is consistent with the proposed 6-transmembrane and 8-transmembrane models for aquaporin-0 (MIP26) (19–21). Both models propose that a long C-terminal extension of MIP26 extends into the cytoplasmic side of the membrane which would make the C terminus available to cytoplasmic proteases. MIP26 is essential for lens transparency as illustrated by two mutations of MIP26 in the mouse strains Catβ and Lop. Early fiber cell degeneration, similar to that in the TG72 mouse lens, occurs in Catβ and Lop mice (22).

To determine the role that HIV-1 protease may play in the cellular destruction of the lenses of the TG72 animals, the

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Laboratory of Mechanisms of Ocular Diseases, National Eye Institute, National Institutes of Health, Bldg. 6/Rm. 228, Bethesda, MD 20892. Tel.: 301-496-7471; Fax: 301-496-1759; E-mail: kpmilton@helix.nih.gov.

‡The abbreviation used is: HIV, human immunodeficiency virus.
HIV-1 Protease Activates Endogenous Protease

EXPERIMENTAL PROCEDURES

Lenses were obtained from normal FVB/N mice and the mouse line TG72, homozygous for the HIV-1 protease. All experiments strictly adhered to the NIH guidelines on the care and use of animals in research. Lenses were homogenized and fractionated into water-soluble, urea-soluble, EDTA-extractable, and insoluble membrane fractions. The final membrane fraction contained MIP26. Lenses were homogenized directly in 1.5-mL conical plastic tubes containing ice-cold 20 mM Tris-HCl, 1 mM CaCl2, pH 8.0. Homogenates were separated by centrifugation (12,000 × g × 5 min, 5°C) into water-soluble and insoluble fractions. The insoluble fraction was washed sequentially with Tris-Ca buffer (1×), Tris-Ca 7 M urea buffer (2×), Tris-Ca buffer (1×), Tris-2 mM EDTA buffer (2×), 50 mM NaOH (1×), and Tris-EDTA buffer (1×). The final urea insoluble pellets were dehydrated by Speed Vac.

Susceptibility of MIP26 to HIV-1 protease action was tested by incubation of insoluble membrane preparations from normal lenses with exogenous recombinant HIV-1 protease (Bachem Biochem, King of Prussia, PA). Incubations contained 4 mg of resuspended membranes prepared from lenses of normal FVB/N mice and 400 ng of HIV-1 protease in 21 µL of buffer (20 mM Tris-HCl, 1 mM EDTA, pH 7.4). Control incubations contained membrane preparation only. Samples were incubated at 37°C for 20 h in a rotatory shaker. As a positive control for active protease, incubations were carried out with 4 mg of rhesus monkey α-crystallin fraction prepared by gel filtration of monkey lens soluble protein on a CL6B column (90 × 2.5 cm) using 50 mM Tris-HCl, 100 mM NaCl, pH 7.3 buffer.

For organ culture, 18-day-old eyes were enucleated from TG72 animals and opened posteriorly from the optic nerve remnant, avoiding the lens. Pressure was applied to the anterior segment to expel the lens onto paraffin with the assistance of a teflon spatula, followed by immediate transfer into serum-free TC199 media (300 mosm, pH 7.2) with or without 0.50 mM E64 (trans-epoxyoxysuccinyl-l-leucylamido-(4-guanidino)-butane, Sigma). Lenses at this age were transparent. Lens integrity was confirmed by the absence of lens proteins in the culture media after 2 h (23, 24) and was a precondition for use in culture experiments. Culture medium was changed daily.

Samples of membrane preparations or membrane/protease incubations were separated on 12.5% polyacrylamide SDS-gels using the Pharmacia PhastGel system (Pharmacia Biotech Inc.). Washed membrane pellets or membrane/protease incubation mixtures were dissolved in sample buffer (2.5% SDS, 2% Nonidet P40, 5% Pharmalite 3-10 ampholytes. Pharmacia IEF 3-9 PhastGels were equilibrated in the same solution for 30 min, and nonidet P40 was added to the membrane/protease incubation mixtures. Membrane pellets or membrane/protease incubation mixtures were dissolved in sample buffer (2.5% SDS, 2% Nonidet P40, 5% Pharmalite 3-10 ampholytes. Pharmacia IEF 3-9 PhastGels were equilibrated in the same solution for 30 min, and Nonidet P40 was added to the membrane/protease incubation mixtures. Membrane pellets were resuspended in buffer (20 mM Tris-HCl, 1 mM EDTA, pH 7.4), and the samples were separated by electrophoresis in the first dimension. Water-soluble fractions were dissolved in 8 M urea, 2% Nonidet P40, 5% Pharmalite 3-10 ampholytes. Pharmacia IEF 3-9 PhastGels were equilibrated in the same solution for 30 min, and samples were focused according to the manufacturer’s protocol. Second dimensions were separated on 12.5% polyacrylamide SDS-gels at 250 V for 60 V-h (15°C). Proteins were visualized with silver staining.

RESULTS

MIP26 was the major intrinsic protein in membrane preparations of normal and younger transgenic TG72 lenses as visualized in SDS gels of the membrane fractions (Fig. 1A). Although there was a small amount of variation between animals, alterations of MIP appeared to start about postnatal day 20 and were complete by day 25. Increased amounts of the 25-kDa and 23-kDa polypeptides were detected in the lenses of TG72 mice by 21 days of age compared to control lenses from the normal mice. By 25 days of age, MIP26 was essentially absent in lenses from TG72 mice, and only the 23-kDa polypeptide remained. This pattern of degradation paralleled the formation of nuclear cataract.

Incubations of lens membrane preparations from control lenses with recombinant HIV-1 protease did not resemble the proteolysis of MIP26 in vivo. MIP26 was resistant to the recombinant HIV-1 protease even in membrane preparations substantially free of many associated extrinsic proteins (Fig. 1B). A positive control for recombinant HIV-1 protease activity was provided by incubations with monkey α-crystallin fractions under the same conditions. Proteolysis of α-crystallins resulted in lower molecular weight products, demonstrating that the recombinant HIV-1 protease was active under the incubation conditions employed.

Transparent lenses from 18-day-old TG72 mice, removed prior to cataract formation, went on to develop nuclear cataract after 2 days of organ culture in medium TC199. The nuclear opacity increased in diameter to involve more of the lens cortex throughout 6 days of culture. This progression was very similar to the cataract formation in TG72 mice in vivo. Lenses were also incubated in medium containing 0.5 mM E64, a cysteine protease inhibitor. E64 at this concentration did not inhibit recombinant HIV-1 protease activity when used with HIV protease substrate III (Bachem Biochem) in vitro (data not shown). TG72 lenses cultured in medium TC199 containing E64 were free of nuclear cataract after 2 and 4 days of culture in contrast to TG72 lenses cultured without E64 (Fig. 2A). E64-treated lenses developed nuclear cataract after the 6th day of organ culture. Besides the delay in progression of cataract, the diameter of the opacities were smaller than seen in untreated lenses.

Examination of MIP26 in membrane preparations from organ-cultured TG72 lenses revealed progressive cleavage of MIP26 concomitant with nuclear cataract formation as seen in SDS gels (Fig. 2B). Degradation products appeared at 25 kDa and 23 kDa, as seen in vivo. Higher molecular mass bands...
HIV-1 Protease Activates Endogenous Protease

Fig. 2. Delay of cataractogenesis and MIP26 cleavage by E64 in organ culture. A, transparent lenses from 18-day-old TG72 mice developed nuclear cataract after 2 days of organ culture. The nuclear opacity increased in diameter throughout 6 days of culture and was similar to cataractous TG72 lenses in vivo. Lenses treated with E64 (0.5 mM) were clear on day 2, and formation of the characteristic nuclear opacity was significantly retarded compared to lenses that did not receive E64 treatment on days 4 and 6. B, examination of MIP in membrane preparations from organ-cultured TG72 lenses revealed progressive cleavage of MIP26 coincident with nuclear cataract formation. MIP26 was preserved in lenses treated with E64 compared to untreated lenses.

attributed to glycosylated forms of MIP were also detectable (18). Degradation of MIP26 was more progressive after the 4th and 6th days of organ culture. However, proteolysis of MIP26 was inhibited in organ cultured TG72 lenses treated with E64. This inhibition was effective in preventing the appearance of the 25-kDa and 23-kDa polypeptides throughout the culture period. Intact MIP26 was immunoreactive with rabbit antisera to the N-terminal (anti-MIP-(1–8)), as well as the C-terminal region (anti-MIP-(252–59), anti-MIP-(256–63)) (Fig. 3). The 25-kDa and 23-kDa bands only displayed reactivity to the N-terminal directed anti-MIP-(1–8). Degradation of the MIP26 in organ culture was from the C terminus and mimicked the degradation in vivo.

Two-dimensional gel analysis of soluble proteins from organ-cultured TG72 lenses detected many crystallins including α- and β-crystallins, with progressive proteolysis of αA-crystallin, βA1-crystallin, βB2-crystallin, and βB1/βB3-crystallins occurring with nuclear cataract formation (Fig. 4). Crystallin modifications were not observed in organ-cultured TG72 lenses treated with E64. The pattern observed with cataract formation in organ culture was strikingly similar to the one reported for these lens proteins in vivo (6).

DISCUSSION

The expression of HIV-1 protease in the lens leads to alteration in the membrane aquaporin MIP26 prior to nuclear cataract formation, but apparently not in a direct manner. Gel analysis of lens membrane preparations revealed that MIP26 is cleaved in lenses of transgenic TG72 mice resulting in the progressive formation of 25-kDa and 23-kDa polypeptides. Cleavage of MIP26 was at the C terminus. That HIV-1 protease does not directly cleave MIP26 was suggested by incubations of membrane preparations from control mouse lenses with recombinant HIV protease in vitro. The pattern of MIP26 cleavage is similar to that seen with other rodent cataract models suggesting common mechanisms in the process of opacification regardless of the initiating event (17, 18). Percachia and Girsch (25, 26) have shown that the C terminus of MIP26 is most likely the channel gate. The permeability of liposomes reconstituted with MIP26 is altered by Ca^2+ in the presence of calmodulin which interacts with MIP25–241 in the C-terminal region (27–29). Ehring et al. (30, 31) have shown that the presence of the C-terminal region is required to stabilize MIP in the closed state. Phosphorylation of this region of MIP, as well as cleavage of the C terminus, opens the channel (32, 33). The abnormal increase in the fraction of MIP in the open state as a result of proteolysis is consistent with the alterations to water distribution in the TG72 lens. The changes to water distribution occur at the same time as the cleavage of MIP26, and both precede eventual destruction of fiber cell membranes in the lens nucleus at 23–25 days of age.

This first use of an organ culture system of a precataractous lens has increased the understanding of cataract formation in these animals. Degradation of MIP26 and lens crystallins in organ-cultured lenses appeared to be identical to the process in vivo. This suggests that activation of endogenous proteases by the HIV-1 protease had a similar mechanism. Cataract formation in organ culture coincides with the proteolysis of MIP26 and the appearance of 25-kDa and 23-kDa polypeptides as seen in vivo.
HIV-1 Protease Activates Endogenous Protease

Immunoblotting demonstrated that cleavage of MIP26 was at the C terminus, again consistent with the proteolysis of this protein in vivo. The time course of cataract formation appears to be accelerated somewhat in organ culture, which is likely the result of inevitable trauma associated with the dissection of the lens from the globe and initiating culture. All lenses were initially checked for integrity using the very sensitive protein leakage technique, but it is logical that some alterations in the lens occurs as a result of the manipulations necessary to start organ culture.

The pattern of crystallin cleavage in organ-cultured lenses of TG72 mice was also very similar to the pattern previously reported in vivo where βB1, βB3, and βA3-crystallins were degraded rapidly with the onset of nuclear cataract (6). As shown here for MIP26, the cleavage pattern of soluble lens crystallins in vivo cannot be reproduced by incubations of normal mouse lens crystallins with recombinant HIV-1 protease in vitro (6). This infers the hypothesis that other endogenous proteases can become activated by the action of expressed HIV-1 protease. Inhibition of cysteine protease activity in organ-cultured TG72 lenses with E64 dramatically preserved the MIP26 and reduced the formation of MIP26-derived polypeptides at 25 kDa and 23 kDa. Likewise, the formation of nuclear cataract was inhibited in E64-treated organ cultured lenses (6).

Inhibition of cysteine protease activity in organ-cultured TG72 lenses with E64 dramatically preserved the MIP26 and reduced the formation of MIP26-derived polypeptides at 25 kDa and 23 kDa. Likewise, the formation of nuclear cataract was inhibited in E64-treated organ cultured lenses although this inhibitor is ineffective against the HIV-1 protease. Examination of lens crystallins from organ-cultured TG72 lenses on two-dimensional gels also demonstrated that E64 prevented the characteristic proteolysis of αA-crystallin, βB1-crystallin, βB3-crystallin, and βA3-crystallin that occurs with nuclear cataract formation in these animals. Calpain was a logical candidate for the activated cysteine protease activity in the TG72 lens. Two classes of Ca2+-dependent cysteine proteases, calpain I (μ-calpain) and calpain II (m-calpain), and the endogenous inhibitor polypeptide calpastatin are found in the mammalian lens (34). Calpain II is the more abundant form in the lens compared with calpain I. Both calpains have the same regulatory 30-kDa subunit, and become fully activated by micromolar (calpain I) or millimolar (calpain II) Ca2+ concentrations. Calpastatin (mass 110 kDa) directly binds and inhibits calpain I and II (35). It was recently shown by Eto et al. (36) that calpain can translocate to the membrane and degrade substrates at that location. Two possible mechanisms for calpain activation in the TG72 mouse lens would be that HIV-1 protease causes the lens fiber intracellular Ca2+ concentration to increase or that HIV-1 protease might activate calpain by proteolysis directly or by degrading calpastatin.

The cleavage of the MIP26 appears to be an accelerating factor in the opacification of the lens. As more of this important membrane protein is degraded, cellular integrity and particularly ion balance within the cell may be compromised. Subsequent activation of ion-dependent proteases would intensify the destruction of the cells. The similarity of cataract formation in organ culture with that in vivo suggests that the same mechanism of cytoskeletal and membrane structural damage occurs in both.

Acknowledgments—We thank Dr. Larry Takemoto (Kansas State University) for provision of antisera to MIP26 peptides and Dr. J. Samuel Zigler, Jr., for critical reading of the manuscript.

REFERENCES

Cysteine Protease Activated by Expression of HIV-1 Protease in Transgenic Mice: MIP26 (AQUAPORIN-0) CLEAVAGE AND CATARACT FORMATION IN VIVO AND EX VIVO

Kenneth P. Mitton, Toshikazu Kamiya, Santa J. Tumminia and Paul Russell

doi: 10.1074/jbc.271.50.31803

Access the most updated version of this article at http://www.jbc.org/content/271/50/31803

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 7 of which can be accessed free at http://www.jbc.org/content/271/50/31803.full.html#ref-list-1