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Degradation of human aquaporin 0 by m-calpain

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Abstract Opacities (cataracts) in the lens of the eye are a leading cause of preventable blindness. Aquaporins function as water channels, and the C-terminus is postulated as a regulatory domain. The C-terminal domain of aquaporin 0 (AQP0) develops numerous truncation sites during lens aging. The purpose of the present experiment was to determine if the calcium-activated protease m-calpain (EC 3.4.22.17) was responsible for truncation of human AQP0. AQP0 was isolated from young human donors, incubated with recombinant m-calpain, and the cleavage sites on the released peptides were determined by on-line electrospray ionization mass spectrometry. We found that four cleavage sites on human AQP0 could be tentatively assigned to m-calpain. This is the first evidence for possible calpain activity in human lens. Because the cause(s) of 17 other cleavage sites was unknown, the data also suggested that other, as yet unknown, proteases or non-enzymatic mechanisms are more active than calpain in human lens.
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1. Introduction

Aquaporins in mammals comprise a group of more than 10 integral membrane proteins [1]. AQP0 is the most abundant membrane protein in lens and is localized to differentiated fiber cells [2]. AQP0 contains six transmembrane α-helical domains with a short 9 amino acid N-terminus and a longer 39 amino acid C-terminus extending into the cytoplasm. Aquaporins function as water channels, and the C-terminus is postulated as a regulatory domain. The C-terminal domain of AQP0 seems especially susceptible to post-translation modifications including deamidation, racemization, phosphorylation, and proteolysis. For example, at least 21 cleavage sites have been reported on the C-terminus of AQP0 in aging human lens [2,3] (summarized in Fig. 1).

Selenite nuclear cataract in the rat also showed, along with 10 other cleavage sites, accelerated truncation of AQP0 at K^{238} [4]. This was also the major cleavage site produced when purified rat AQP0 was incubated with the protease m-calpain. m-Calpain is a member of a group of Ca^{2+} activated, intracellular, cysteine proteases [5]. Lens calpains are active in a wide variety of rodent cataracts [6–13]. Part of the mechanism for selenite cataract was probably loss of water homeostasis due to hydrolysis of AQP0 by m-calpain.

Testing the relevance of the calpain-cataract hypothesis in human lens is important because the mechanism for senile cataracts is unknown. Human lenses contain several types of ubiquitous calpains (m- and u-calpains) as well as calpain isoforms derived from tissue-specific p94 calpain [14,15]. Further, many human cataracts contain elevated calcium levels which could activate calpains [16]. However, no typical calpain cleavage sites have been discovered in human lens crystallins, which comprise the bulk of proteins in the lens.

We do not know if the numerous truncations observed on human AQP0 are produced by calpains. The proteases or non-enzymatic mechanisms causing the cleavage sites are difficult to determine because human lens proteins are extremely long-lived and show no turnover during the lifetime of an individual. Overtime, initial proteolytic sites on lens proteins caused by a specific protease could be masked by other competing proteases or non-enzymatic hydrolysis. The two major cleavage sites at N^{246} and N^{259} are known to be sites for non-enzymatic ring formation and deamidation (Fig. 1), which can lead to spontaneous backbone cleavage [2]. The purpose of the present experiment was to use cleavage site analysis to determine if the naturally occurring cleavage sites observed on AQP0 from human lenses were caused by calpain.

2. Methods

Isolation of AQP0 from human lenses. Human lenses at 1–4 days of age were obtained with informed consent following the Tenets of the Declaration of Helsinki. Decapsulated lenses were homogenized at a ratio of 1 ml homogenizing buffer (20 mM phosphate, 1.0 mM EGTA, pH 7.0) per lens. The insoluble pellet was obtained by centrifugation at $20000 \times g$ for 30 min at 4 °C and washed by resuspending each pellet from one lens in 1.0 ml homogenizing buffer containing 50 mM DTT. After centrifugation, each pellet was resuspended by vortexing and brief sonication in 0.5 ml freshly made 7 M urea in homogenizing buffer without DTT and spun down as before. Ice cold 0.1 N NaOH with 1 mM DTT (0.5 ml) was added to the pellet and vortexed briefly to resuspended. After storage on ice for 15 min, the pellet was spun down as before. The supernatant was carefully removed without disturbing the pellet, which was then resuspended in 250 μl homogenizing buffer without DTT by sonicating briefly to obtain a uniform suspension. Protein concentrations were determined using the BCA assay (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as standard.

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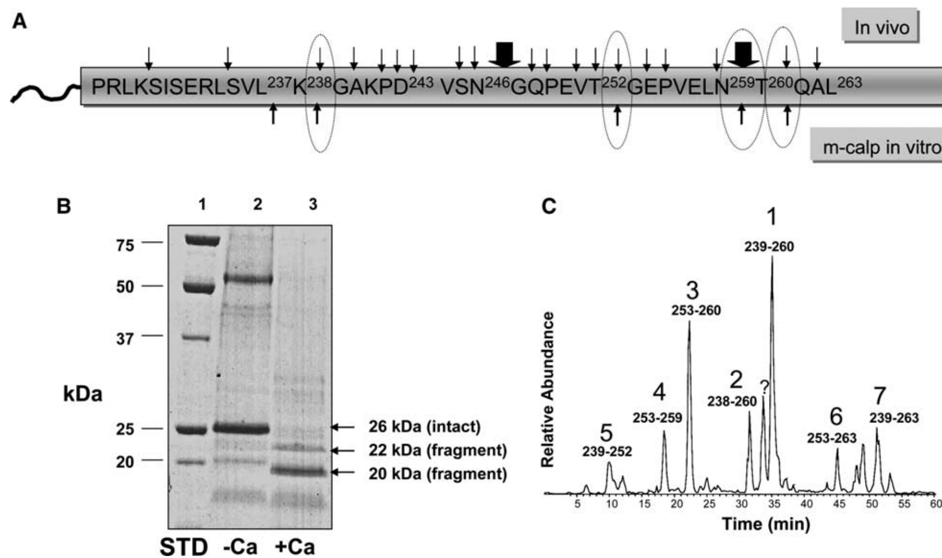

Fig. 1. (A) Diagram of the amino acid sequence in the C-terminus of human AQP0. Arrows from above indicate in vivo cleavage sites observed in aging human lenses [2,3]. Arrows from below indicate the five in vitro calpain cleavage sites observed on AQP0 in the present studies after incubation of partially purified human lens membrane proteins with purified m-calpain. Bold arrows indicate major sites. Dotted ovals indicate identical cleavage sites. (B) Coomassie blue-stained SDS–PAGE of lens membrane proteins from human lenses 1–4 days of age incubated with purified, recombinant m-calpain for 20 min in the absence (lane 2) or presence (lane 3) of 1 mM free calcium. (C) Reverse phase HPLC chromatogram and the amino acid sequence of each major peak derived from mass spectrographic analysis of peptides released after incubation of human AQP0 with purified m-calpain. Range of numbers above each peak correspond to the N- and C- terminal amino acids of each released peptide, which can be located in (A). Peak numbers 1–7 are used for identification (see Section 3). The "?" mark indicates an unidentified peak.

Incubation of AQP0 with m-calpain. AQP0 was resuspended by vortexing in 20 mM Tris, pH 7.4, buffer with 1 mM EGTA. Recombinant rat m-calpain (Calbiochem/EMD Biosciences Inc., San Diego, CA) was added at a ratio of 0.8 units per 10 μg membrane proteins. After a 20 min incubation with or without 1 mM free calcium, the reaction was stopped by addition of excess EGTA. After centrifugation, pellets were diluted in Laemmli buffer without heating, and SDS–PAGE was performed with on 12% polyacrylamide gels using the Mops buffer system (Invitrogen Corporation, Carlsbad, CA).

Mass spectroscopy and sequencing. C-terminal peptides released into the supernatant were separated by reverse-phase HPLC on a 0.5×250 mm C_{18} column (SB-C18, Aglilent, Palo Alto, CA) and analyzed by on-line electrospray ionization mass spectrometry (ESI-MS) using a model LCQ ion trap (ThermoFinnigan, San Jose, CA). Tandem mass spectra of peptides were analyzed by Sequest software using a no enzyme specificity search and human subset of the Swiss-Prot database [17].

3. Results

In vitro proteolysis produced by incubating m-calpain with human AQP0 was visualized by SDS–PAGE (Fig. 1B). In preparations without calcium, intact AQP0 was the major band at 26 kDa (Fig. 1B, lane 2). Activation of m-calpain with calcium caused almost total loss of the intact 26 kDa AQP0 band and formation of two major bands at 22 and 20 kDa (lane 3). The released AQP0 fragments in the samples were separated by reverse phase chromatography into seven major peaks (Fig. 1C). LC–MS analysis of the peptides in the peaks identified five cleavage sites, which are located in Fig. 1A (lower arrows):

Peaks 1, 2 and 3: *T^{260} cleavage site.* These peaks showed the most intense ion currents, and all three peptides contained new C-termini at T^{260}. This cleavage site has also been observed in minor amounts in normal middle-aged human lenses [2] (Fig. 1A, upper arrow). These data suggested that cleavage at T^{260} in AQP0 could be caused by activation of m-calpain in human lens, but that this activity occurred only to a minor extent during normal aging.

Peak 4: *N^{259} cleavage site.* This site was previously identified as a major cleavage site on AQP0 from aging human lenses [2]. Asparagine-259 undergoes, non-enzymatic deamidation, leading to ring formation and spontaneous cleavage. Residue 259 in AQP0 from rats is a lysine instead of asparagine, and incubation with m-calpain also cleaves at the K^{259} site [4]. Thus, a proportion of the proteolysis at this site could be due to m-calpain, but in long-lived human lenses, the major human cleavage at N^{259} was most likely due to non-enzymatic truncation.

Peaks 5, 4, 3, and 6: *T^{252} cleavage site.* The peaks contained new N and C-termini consistent with m-calpain-induced proteolysis between T^{252} and G^{253}. Minor amounts of this cleavage site have been observed in AQP0 during normal aging of human lenses [3], indicating possible action of m-calpain.

Peaks 1, 5, and 7: *K^{238} site.* Previous studies [4] showed that the K^{238} cleavage site was the dominant fragment produced by action of m-calpain on rat AQP0, and production of cleavage at this site was accelerated in the selenite-induced cataract in rats. K^{238} is believed to be a major in vitro cleavage site produced by m-calpain on human AQP0. Cleavage at this site has been observed at very low abundance in human lenses (Dr. Kevin L. Schey. Medical University of South Carolina, personnel communication).

Peak 2: *L^{237} cleavage site.* The L^{237} cleavage site has not been described in aging human lens.

4. Discussion

Normal aging in human lenses is associated with the formation of at least 21 cleavage sites on the 39 amino acid sequence

on the C-terminus of AQP0 (Fig. 1A). By far, the most abundant truncations were at N^{246} and N^{259} [2,3]. The present in vitro study attempted to determine which of the 21 sites were produced by m-calpain.

We found that incubation of isolated human AQP0 with purified m-calpain produced five truncations. The in vitro truncation at L^{237} has not been described in human lenses. The in vitro truncations at T^{260}, T^{252}, and K^{238} have been observed in minor amounts in human lenses [2,3]. The fifth in vitro truncation site produced by a 20-min incubation with m-calpain occurred at N^{259}. This is also the most prominent truncation observed in aging human lenses [2]. Over a 96-h incubation without m-calpain, approximately 1% of human AQP0 spontaneously truncated at N^{259} (2), opening the possibility of slow non-enzymatic deamidation and spontaneous truncation at this site in vivo. Our data showed that m-calpain also rapidly produces the N^{259} truncation in vitro and suggested that m-calpain could contribute to in vivo truncation if calcium levels were elevated in the lens. Note that the other site for non-enzymatic deamidation and truncation at N^{246} was not produced in the current studies where AQP0 was incubated with m-calpain. The N^{246} is a major site of deamidation and truncation in aging human lenses [2]. Since no peaks with this truncation site were observed in the present studies, our data support the idea that truncation at N^{246} in human AQP0 may be due to deamidation and non-enzymatic cleavage and/or other proteases. In summary, our data implicate m-calpain as a potential contributing protease for 4 of 21 cleavage sites naturally occurring during aging of human AQP0 (Fig. 1A, dotted ovals). To our knowledge, these are the first data showing tentative calpain cleavage sites on a human lens protein.

We do not know the truncation sites on human lenses exposed to the high calcium concentrations found in cataracts [16]. Since calpains are activated by calcium, possibly some of the other in vitro sites, such as the abundant truncation at K^{238}, might then be found in human AQP0 from human cataracts. Alternatively, because of the abundant levels of endogenous calpain inhibitor calpastatin in human lenses, m-calpain activity might continue to be suppressed even in cataracts. We recently found very little proteolysis of crystallins in human lenses incubated in calcium ionophore A231897 (Nakajima, E., Ma, H., Shearer, T.R. and Azuma, M., unpublished), supporting the idea that calpain activity on AQP0 may be minimal in human lenses even when lenticular calcium levels are elevated.

Since our data do not explain the cause of truncations at the other 17 sites observed on AQP0 during human aging, our data provide deductive evidence for the activation of other unknown proteases against AQP0. The lens contains several other protease systems including: calcium-activated transglutaminase [18], the caspase cascade [19], the proteosome complex [20], a trypsin-like protease [21], various peptidases [22], and a membrane associated protease [23]. Understanding which proteases and non-enzymatic cleavage mechanisms are responsible for cleavage of AQP0 in human lens membranes will be a difficult but important challenge. It is important because the integrity of membranes in human lens is related to nutrient regulation, and ultimately, susceptibility to cataract.

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