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Research article

Spatial distributions of phosphorylated membrane proteins aquaporin 0 and MP20 across young and aged human lenses

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ABSTRACT

In the human ocular lens it is now realized that post-translational modifications can alter protein function and/or localization in fiber cells that no longer synthesize proteins. The specific sites of post-translational modification to the abundant ocular lens membrane proteins AQP0 and MP20 have been previously identified and their functional effects are emerging. To further understand how changes in protein function and/or localization induced by these modifications alter lens homeostasis, it is necessary to determine the spatial distributions of these modifications across the lens. In this study, a quantitative LC-MS approach was used to determine the spatial distributions of phosphorylated AQP0 and MP20 peptides from manually dissected, concentric layers of fiber cells from young and aged human lenses. The absolute amounts of phosphorylation were determined for AQP0 Ser235 and Ser229 and for MP20 Ser170 in fiber cells from the lens periphery to the lens center. Phosphorylation of AQP0 Ser229 represented a minor portion of the total phosphorylated AQP0. Changes in spatial distributions of phosphorylated APQ0 Ser235 and MP20 Ser170 correlated with regions of physiological interest in aged lenses, specifically, where barriers to water transport and extracellular diffusion form.

1. Introduction

At a certain distance from the lens periphery, fiber cells lose their organelles (Bassnett, 2002) and can no longer synthesize new proteins (Faulkner-Jones et al., 2003). As a means of maintaining lens function and transparency, post-translational modifications (PTMs) can alter protein function in a regional manner (Gonen et al., 2004a; Grey et al., 2009; Lin et al., 1998). In order to understand how the effects of PTMs on protein function influence overall lens homeostasis, it is important to determine the spatial distributions of modified proteins across the lens.

Aquaporin 0 (AQP0) and MP20 (also known as LIM2) are two highly modified abundant membrane proteins in lens fiber cells, together constituting over 60% of the membrane protein content (Broekhuyse et al., 1976; Louis et al., 1989). AQP0 functions as a water channel (Chandy et al., 1997; Varadaraj et al., 2005, 1999), an adhesion protein (Gonen et al., 2004a, 2004b; Kumari et al., 2011), and as a structural protein (Al-Ghoul et al., 2003; Lindsey Rose et al., 2006; Shiels et al., 2000), and these functions may be regionally distinct (Ball et al., 2004; Gonen et al., 2004a; Grey et al., 2009). MP20 may play a role in cell adhesion and syncytium formation (Grey et al., 2003; Shiels et al., 2007). Both AQP0 and MP20 are

Abbreviations: AQ, absolute quantity; AQP0, aquaporin 0; AQUA, absolute quantification; AUC, area under the curve; END, endogenous; IS, internal standard; m/z, mass to charge ratio; PTM, post-translational modification; r/a, normalized lens distance; Ser, serine; Thr, threonine; XIC, extracted ion chromatogram.

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known to be phosphorylated (Ball et al., 2004; Ervin et al., 2005; Louis et al., 1985; Schey et al., 2000); however the spatial distribution of phosphorylated forms and the effects of phosphorylation on APQ0 function are not completely understood.

AQP0 is phosphorylated at Ser229 (pSer229), Ser231 (pSer231), and Ser235 (pSer235) (Ball et al., 2004; Schey et al., 2000) in an AKAP2/PKA regulated process (Gold et al., 2012), and a recent lens membrane proteome analysis identified several additional AQP0 phosphorylation sites (Wang et al., 2013). Phosphorylated AQP0 C-terminal peptides showed decreased affinity for Ca^{2+}-calmodulin (Ca^{2+}-CaM) compared to unmodified C-terminal peptides (Gold et al., 2012; Reichow and Gonen, 2008; Rose et al., 2008). At the intact protein level, AQP0 S229D showed reduced affinity for calmodulin compared to wild type AQP0 (Kalman et al., 2008). Furthermore, the S229D mutant resulted in an AQP0 with high water permeability compared to wild type AQP0 (Kalman et al., 2008). Recently, a mechanism was depicted wherein two Ca^{2+}-CaM molecules bind an AQP0 tetramer, resulting in closure of constriction site II by conserved residues and preventing water permeation through AQP0 (Reichow et al., 2013). Additionally, phosphorylation at Ser235 may be important for AQP0 trafficking to the membrane (Golestaneh et al., 2008) as reported for other aquaporins. For example, AQP2 regulation in the kidney is dependent on trafficking of pAQP2 (Wilson et al., 2013); however, there is no published evidence for AQP0 shuttling in lens fiber cells. This type of energy intensive regulation of protein trafficking may be unlikely in the lens given its limited energy budget. The abundance of AQP0 pSer235 has been measured as highest in the inner cortical region of a 34 yr lens (Ball et al., 2004), and Ca^{2+} increases from 300 nM in the lens cortex to 700 nM in the core (Gao et al., 2004). Therefore, regulation of AQP0 permeability may be dependent on regional differences in Ca^{2+} and pSer235 levels.

Regional changes in AQP0 water permeability are likely to affect lens homeostasis, as predicted by the lens internal circulation system (Mathias et al., 2007, 1997). At around 50 years of age, in a region 6–8 mm from the lens center, a barrier forms that prevents water transport into the lens core (Moffat et al., 1999). It is possible that a decrease in AQP0 water permeability in this region could contribute to the formation of a barrier; however, the phosphorylation state of AQP0 in this region of aged lenses is unknown. Determining the spatial distribution of AQP0 pSer235 will reveal the location within the lens where phosphorylation peaks and the changes that occur with age, thereby enhancing our understanding of AQP0 water permeability regulation throughout the lens.

MP20 is phosphorylated at Ser170 (pSer170) and Thr171 (pThr171) (Ervin et al., 2005); however, the functional effect of MP20 phosphorylation remains to be determined. In the rat lens, MP20 insertion into the fiber cell membrane correlates with the formation of a barrier to extracellular transport (Donaldson et al., 2004; Grey et al., 2003). Recently, a similar barrier was detected in a 69 year old human lens at a normalized lens distance (r/a) of approximately 0.91 where (a) represents the lens radius and (r) represents the distance from the lens core to the region of interest (Lim et al., 2009). It is not known whether MP20 plays a role in the diffusion barrier that forms in aged human lenses, but it is possible that a change in the phosphorylation state of MP20 contributes to its barrier formation.

The aim of this work was to map the distributions and to quantitate the extent of AQP0 and MP20 phosphorylation. This was achieved in both young and aged lenses by careful dissection of lens regions followed by quantitation using AQUA isotopically labeled peptide internal standards and targeted LC-MS/MS. The results demonstrate that the distributions of AQP0 and MP20 phosphorylation change across the lens with age and correlate with regions of physiological interest.

2. Methods

2.1. Lens dissection and tissue preparation

Human lenses of various ages (16, 18, 19, 23, 51, 54, and 60 years) were dissected into 5–7 regions based on developmental fiber cell age (Garland et al., 1996). To compare the location of dissected regions from different lenses, the midpoint distance of each region from the lens center (r) was determined and normalized as a function of the lens radius (a), to yield normalized lens distance (r/a), where 1.0 represents the lens periphery and 0.0 the lens center (Jacobs et al., 2004). For example, a region dissected from the lens periphery (7.5–8 mm) of an 8 mm diameter lens, has a midpoint radial distance (r) of 7.75 mm, and a normalized lens distance (r/a = 7.75 mm/8 mm) of 0.97.

Pairs of lenses, young and aged, were homogenized and washed in parallel for the enrichment of membrane proteins, in a manner similar to that described previously (Ball et al., 2004). Membrane protein pellets were suspended in 180 μL of 50 mM NH_4HCO_3, and 20 μL of acetonitrile (MeCN). Trypsin (1–2 μg) was added and the samples were digested for 24 h at 37 °C. After digestion, samples were spun at 16,000 × g at 4 °C for 10 min. To assure that equal amounts of digested lens protein were loaded in each experiment, BCA protein assays of the digested samples were carried out using digested, bovine serum albumin (BSA) as a standard.

2.2. AQUA peptides

AQUA phosphopeptides (Gerber et al., 2003) were synthesized by Sigma Aldrich, with an isotopically labeled arginine ($^{13}C_6$ $^{15}N_4$, Table 1). Each peptide was reconstituted in 10% formic acid (FA) and diluted to 5 pmol/μL (4–6 ng/μL) with 0.1% FA. Individual AQUA peptides were mixed, and this mixture, which included 3.7 ng of AQP0 pSer235 AQUA peptide, 1.4 ng of AQP0 pSer231 AQUA peptide, and 1.2 ng of MP20 pSer170 AQUA peptide, was combined with approximately 420 ng aliquots of lens protein. Spiked lens samples were dried down and stored at −20 °C.

2.3. Mass spectrometry

Prior to injection, samples were solubilized in 50 μL of 0.1% FA. All samples were analyzed on a Thermo Scientific LTQ XL Orbitrap mass spectrometer in line with an Eksigent nanoLC 2D HPLC pump. Samples (approximately 42 ng) were loaded onto a C_{18}, 100 μm id, 6 cm trap column in 98% mobile phase A (100% H_2O, 0.1% FA) and 2% mobile phase B (100% MeCN, 0.1% FA) for 10 min, separated on a C_{18}, 100 μm id, 20 cm analytical column over a 23 min gradient from 2 to 25% mobile phase B, and ionized by nanospray ionization. Mass spectrometry analysis occurred in two stages – identification and quantitation. For identification, MS scans (mass-to-charge ratio (m/z) 250–1200) were performed in the Orbitrap followed by collision-induced dissociation (CID) of selected ions and MS/MS acquisition in the ion trap. For quantitation, MS scans were carried out in the ion trap. Ions of m/z 275–475 were detected from 0 to 25 min, and ions of m/z 350–800 were detected from 25 to 35 min.

Table 1
AQUA internal standard phosphopeptide

Peptide	Sequence[a]
AQP0 229–238 pSer235	SISE^{I}RLpSVLK
AQP0 227–233 pSer231	LKSIpSE^{I}R
MP20 168–173 pSer170	RLpSTP^{I}R

[a] Isotopically labeled arginines ($^{13}C6 \cdot {}^{15}N4$) are designated by "***I***", and "p" indicates a phosphorylated residue.