αB-crystallin in the rat lens is phosphorylated at an early post-natal age

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Abstract We determined the developmental changes in the phosphorylation state of αB-crystallin in lenses from rats at various post-natal ages by isoelectric focusing gel electrophoresis or sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a subsequent Western blot analysis of extracts of lenses using antibodies that recognized the carboxy-terminal sequence or each of the three phosphorylated serine residues (Ser-19, Ser-45 and Ser-59) in αB-crystallin. Phosphorylated forms of αB-crystallin were barely detected at birth but they became detectable at 3 weeks of age and reached plateau levels at 8 weeks of age. The phosphorylation of αB-crystallin at Ser-45 was observed preferentially. The active form of p44/42 MAP kinase, which is responsible for the phosphorylation of Ser-45 in αB-crystallin, also increased in a development-dependent manner. Thus we found that the developmental increase of the phosphorylation at Ser-45 of αB-crystallin in the rat lens was due to the developmental activation of p44/42 MAP kinase.

Key words: Lens; Crystallin; Phosphorylation; MAP kinase

1. Introduction

α-Crystallin is one of the major vertebrate eye lens proteins. It exists as a large aggregate that is composed of two types of subunits, αA-crystallin and αB-crystallin [1]. Both crystallins also exist in non-lenticular tissues. αA-crystallin exists in spleen [2] and αB-crystallin exists in a wide variety of tissues including skeletal muscle, heart, kidney and nervous tissues [3,4]. They have similarities with a small heat shock protein, hsp27, in terms of the amino acid sequence. Indeed, αB-crystallin is induced by heat or chemical stress [5]. We previously reported that αB-crystallin in mammalian cells was phosphorylated by extracellular stresses that also increase the phosphorylation of hsp27 [6]. Stress-induced phosphorylation of αB-crystallin occurred at three serine residues, Ser-19, Ser-45 and Ser-59, which were the same sites at which the phosphorylation of αB-crystallin in bovine and human lenses occurs [7–9]. Recently we identified the protein kinases responsible for the phosphorylation of αB-crystallin. Phosphorylation of Ser-45 was responsible for p44/42 MAP kinase and phosphorylation of Ser-59 was responsible for MAPKAP kinase-2 [10]. However, the biological significance of the phosphorylation of αB-crystallin is unknown. In this study, we estimated the development-dependent phosphorylation of αB-crystallin in the rat lens.

2. Materials and methods

2.1. Preparation of tissue extract

Male Wister rats of various post-natal ages were used. Lenses were sampled and frozen at −80°C until analysis. The frozen lenses were homogenized at 0°C with a Physcotron (NS-50, Niti-On, Chiba, Japan) in a 10 volume (v/w) of 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 0.1 M NaF, 10 μg/ml trypsin inhibitor and 0.3 mg/ml PefablocSC (Boehringer Mannheim, Germany) and then sonicated at 0°C for 30 s. Homogenates were centrifuged at 4°C at 125 000×*g* for 20 min and then supernatant fractions were used for analysis.

2.2. Electrophoresis and Western blot analysis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli [11] in a 12.5% polyacrylamide slab gel. Tricine/SDS-PAGE was performed as described previously [10] by the method of Schägger and von Jagow [12] in 16.6% polyacrylamide gels that contained 13.3% glycerol. Isoelectric focusing (IEF) was performed as described previously [6] by the method of O'Farrell [13], using the Protean II system of Bio-Rad (Tokyo, Japan). For Western blot analysis, the proteins in a polyacrylamide gel were transferred electrophoretically to a nitrocellulose sheet and immunostained with affinity-purified antibodies that recognized each of the three phosphorylated serine residues in bovine αB-crystallin [10], the amino-terminal region in αB-crystallin [10] or the carboxy-terminal region in αB-crystallin [4] and peroxidase-labelled antibodies raised in goats against rabbit IgG as second antibodies. For the detection of p44/42 MAP kinase, we used rabbit anti-rat MAP kinase R2 antibody (Upstate Biotechnology, Lake Placid, NY, USA) or rabbit anti-human phospho-p44/42 MAP kinase antibody (New England Biolabs, Bever, MA, USA) as first antibodies. The peroxidase activity on a nitrocellulose sheet was visualized on X-ray film by use of a Western blot chemiluminescence reagent (Renaissance, Dupon NEN, Boston, MA, USA).

2.3. Assays of the protein kinase activities responsible for phosphorylation of αB-crystallin

The protein kinase assay was performed as described previously [10]. Briefly, the extract of tissue that contained 100 μg protein was incubated at 30°C for 20 min with 5 μg of lysylendopeptidase-treated αB2-crystallin that contained an amino-terminal 72 amino acid (N-72K) peptide, 1 mM ATP, 10 mM $MgCl_2$, 100 nM okadaic acid, 100 nM calyculin A, 0.05 M NaF, 300 μg/ml Pefabloc SC and 10 μg/ml trypsin inhibitor in a reaction mixture made to a final volume of 100 μl with 50 mM HEPES-NaOH, pH 7.0. The reaction was stopped by the addition of an equal volume of sample buffer for tricine/SDS-PAGE and then the phosphorylated amino-terminal 72 amino acid peptides were analyzed by tricine/SDS-PAGE followed by Western blot analysis as described previously [10].

2.4. Quantitation of protein concentrations

Concentrations of soluble proteins in tissue extracts were estimated with a protein assay kit (Bio-Rad) with bovine serum albumin as standard.

3. Results

3.1. Post-natal changes in levels of the phosphorylated form of αB-crystallin in the rat lens

First we analyzed the extracts of rat lenses from birth to 8 weeks of age by IEF followed by Western blot analysis. As shown in Fig. 1, the bands that have the same isoelectric point as αB1-crystallin, the phosphorylated form of αB-crystallin,

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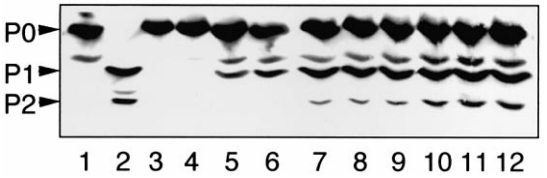

Fig. 1. The post-natal increase in levels of phosphorylated αB-crystallin in the rat lens. Lenses from post-natal 0 day (lanes 3 and 4), 2 week (lanes 5 and 6), 4 week (lanes 7–9) or 8 week (lanes 10–12) old rats were homogenized and 2 μg of each soluble extract obtained by centrifugation was subjected to IEF with subsequent Western blot analysis using antibodies against carboxy-terminal decapeptide of αB-crystallin as described in Section 2. Lane 1, 50 ng of αB2-crystallin purified from bovine lens; lane 2, 50 ng of αB1-crystallin purified from bovine lens. p0, unphosphorylated αB-crystallin; p1 and p2, phosphorylated αB-crystallin.

were barely detected in lenses of rats at birth but they increased rapidly in intensity up to 3 weeks of age and reached a plateau at 4–8 weeks of age. In rats of 4–8 weeks of age, bands corresponding to αB-crystallin phosphorylated at two sites (p2) were detected. To clarify which serine residue is phosphorylated in αB-crystallin in the rat lens during post-natal development, we next employed the antibodies that recognized each of the three phosphorylated serine residues in αB-crystallin. The intensity of the bands corresponding to αB-crystallin phosphorylated at each of the three serine residues increased up to 4 weeks of age although the total level of αB-crystallin changed little (Fig. 2). An increase in levels of αB-crystallin phosphorylated at Ser-45 was evident while the levels of αB-crystallin phosphorylated at Ser-19 and Ser-59 were relatively low (Fig. 2). Preferential phosphorylation of αB-crystallin at Ser-45 was observed only in the lens and similar levels of αB-crystallin phosphorylated at Ser-45 and Ser-59 were detected in other tissues such as diaphragm (Fig. 3), heart and soleus muscle (data not shown).

3.2. *Post-natal changes in the protein kinase activities responsible for phosphorylation of αB-crystallin*

We estimated the protein kinase activities responsible for phosphorylation of αB-crystallin in rat lenses using lysylendopeptidase-treated αB2-crystallin as a substrate. As shown in Fig. 4A, the protein kinase activities responsible for phosphorylation of each of the three serine residues in αB-crystallin increased up to 6 weeks of age although the protein kinase activities responsible for Ser-19 and Ser-59 were much lower as compared with the kinase activity for Ser-45. These results are consistent with the results obtained for the levels of the phosphorylated forms of αB-crystallin (Fig. 2).

3.3. *Post-natal changes in levels of the total and activated form of p44/42 MAP kinase in the rat lens*

Recently, we reported findings that strongly suggested the phosphorylations of αB-crystallin at Ser-45 and Ser-59 were catalyzed by p44/42 MAP kinase and MAPKAP kinase-2, respectively [10]. The present results indicate that the marked increase in αB-crystallin phosphorylated at Ser-45 was due to the increased activity of protein kinase for Ser-45. Therefore we determined the levels of the activated (phosphorylated) form of p44/42 MAP kinase by using antibodies specific for the phosphorylated form of the enzyme. As shown in Fig. 4B, the total level of p44/42 MAP kinase barely changed, however the level of phosphorylated (activated) p44/42 MAP kinase tended to increase after birth to early adulthood. A specific inhibitor of p44/42 MAP kinase kinase (MEK), PD098059 [14], slightly but significantly inhibited the phosphorylation of Ser-45 by extracts of lenses from post-natal 3 weeks old rats (data not shown), suggesting that p44/42 MAP kinase itself was activated by MEK during the incubation period under the conditions shown in Fig. 4A.

These results suggest that the post-natal increase in levels of αB-crystallin phosphorylated at Ser-45 is due to the activation of the p44/42 MAP kinase cascade.

4. Discussion

Post-translational modifications of α-crystallin have been studied extensively by using the adult human or bovine lens and many modifications have been found, including phosphorylation, glycation, glycosylation and deamidation [15]. Among these modifications, we are interested in the phosphorylation of αB-crystallin, because hsp27, a member of the α-crystallin small hsp family as is αB-crystallin, is also phosphorylated under conditions of stress. Previously, we have found that αB-crystallin was phosphorylated by extracellular stimuli in cultured cells and by hyperthermia in rat tissues at three serine residues, Ser-19, Ser-45 and Ser-59 [6]. We reported here the developmental increase in levels of phosphorylated αB-crystallin, preferentially at Ser-45, in the rat lens, which was catalyzed by the developmentally increasing activity of p44/42 MAP kinase.

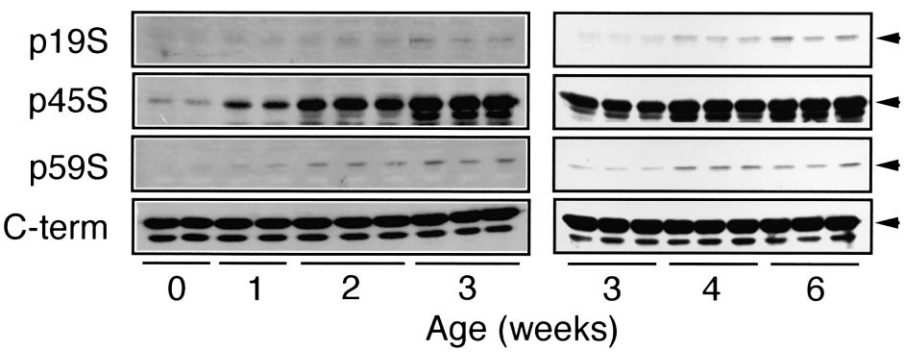

Fig. 2. Post-natal changes of phosphorylation at three serine residues in αB-crystallin in the rat lens. 2 μg of each of the soluble extracts of lenses from various aged rats was subjected to SDS-PAGE with subsequent Western blot analysis using antibodies against carboxy-terminal decapeptide of αB-crystallin (C-terminal) and antibodies that recognized phosphorylated Ser-19 (p19S), phosphorylated Ser-45 (p45S) or phosphorylated Ser-59 (p59S) in αB-crystallin.

The phosphorylation of αB-crystallin in lenses of vertebrates seems to occur at an early post-natal age. Carver et al. revealed an age-related increase in the proportion of the phosphorylated form of αB-crystallin in the bovine lens up to 3 years [16] and Ma et al. found that the phosphorylation of αB-crystallin increases from the fetal stage to 3 years of age in the human lens [17] by using mass spectrometry. However they gave no information about the phosphorylation site in αB-crystallin. We found here that the levels of phosphorylated forms of αB-crystallin increased rapidly within a few weeks of birth and reached a plateau at about 6 weeks of age by using Western blot analysis (Figs. 1 and 2).

The major phosphorylation site in αB-crystallin during the early post-natal stage in rats was Ser-45 (Fig. 2) and the protein kinase activity responsible for phosphorylation of Ser-45 also increased markedly during the first few weeks after birth (Fig. 4A). Expression of the activated form of p44/42 MAP kinase, which is considered to catalyze the phosphorylation of αB-crystallin at Ser-45 [10], increased rapidly after birth in the rat lens (Fig. 4B). It is reported that phosphorylation of αB-crystallin in the bovine lens occurs most actively in the lens epithelial cells and there is no net accumulation of the phosphorylated form of αB-crystallin during differentiation of the fiber cells [18]. The mechanism for the activation of p44/42 MAP kinase in the rat lens during the first few weeks after birth might be affected by the numerous changes in physiological conditions during post-natal development. Many growth factors which activate the MAP kinase cascade play important roles in post-natal development [19,20]. After birth, the lens may be exposed to UV irradiation and oxidative stress from the surrounding environment. It is reported that p44/42 MAP kinase in HeLa cells can be activated by oxidative stress [21] and UV irradiation [22]. p44/42 MAP kinase is well known for its various functions in cell vitality [23–25] but few reports have mentioned the developmental activation of p44/42 MAP kinase in mammalian tissue after birth and this is the first report to reveal the activation of p44/42 MAP kinase during post-natal development in the rat lens.

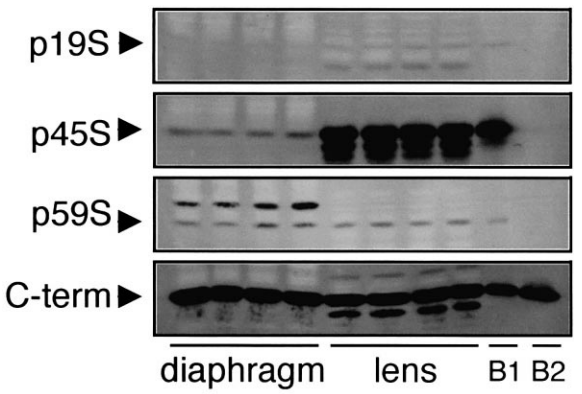

Fig. 3. Ser-45 in αB-crystallin is preferentially phosphorylated in the rat lens. 20 μg of each of the soluble extracts of the diaphragm and 2 μg of each of the soluble extracts of lenses from four rats of 4 weeks of age were subjected to SDS-PAGE with subsequent Western blot analysis using antibodies against carboxy-terminal decapeptide of αB-crystallin (C-terminal) and antibodies that recognized phosphorylated Ser-19 (p19S), phosphorylated Ser-45 (p45S) or phosphorylated Ser-59 (p59S) in αB-crystallin. B1, 20 ng of αB1-crystallin purified from bovine lens; B2, 20 ng of αB2-crystallin purified from bovine lens.

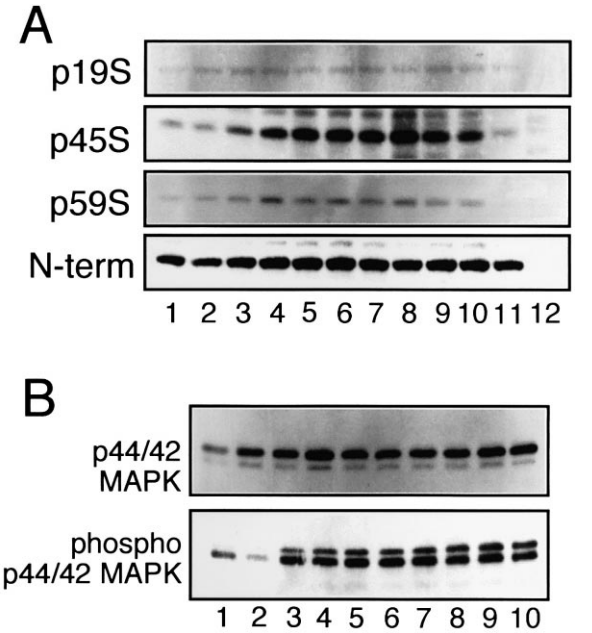

Fig. 4. (A) Developmental change of the protein kinase activity responsible for the phosphorylation of each of the three sites in αB-crystallin. Soluble extracts of lenses from post-natal 0 day (lanes 1 and 2), 1 week (lanes 3 and 4), 3 week (lanes 5–7) or 6 week (lanes 8–10) old rats were incubated with ATP, $MgCl_2$ and lysylendopeptidase-digested αB2-crystallin that contained N-72K peptide at 30°C for 20 min. The reaction was terminated by adding the sample buffer for tricine/SDS-PAGE. 2 μl (for detection of N-72K peptide) or 5 μl (for detection of phosphorylated serine residues) aliquots were subjected to tricine/SDS-PAGE with subsequent Western blot analysis as described in Section 2. Lane 11, no extract control; lane 12, no substrate control. (B) The post-natal change in levels of total and the activated form of p44/42 MAP kinase in the rat lens. Soluble extracts which contained 25 μg protein of lenses from post-natal 0 day (lanes 1 and 2), 1 week (lanes 3 and 4), 3 week (lanes 5–7) and 6 week (lanes 8–10) old rats were subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against p44/42 MAP kinase or phospho-p44/42 MAP kinase.

The physiological significance of phosphorylation of αB-crystallin is unclear. Nicholl and Quinlan reported that αB-crystallin was co-immunoprecipitated with an intermediate filament, vimentin, from soluble extracts of the bovine lens and αB-crystallin inhibited the in vitro assembly of glial fibrillary acidic protein and vimentin [26]. However the inhibition was independent of the phosphorylation of αB-crystallin [26]. Wang et al. reported that phosphorylation of α-crystallin in the rat lens has no effect on the chaperone activity [27]. Recently we reported that phosphorylation of αB-crystallin at Ser-19 and Ser-45 was enhanced in mitotic cells [10]. These phenomena suggest that the phosphorylated forms of αB-crystallin play important roles in the proliferation and differentiation of cells.

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