Phosphorylation of αB-crystallin in Mitotic Cells and Identification of Enzymatic Activities Responsible for Phosphorylation*

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The immunofluorescence localization of αB-crystallin in U373 MG human glioma cells with an antibody specific for αB-crystallin that had been phosphorylated at Ser-45 revealed an intense staining of cells in the mitotic phase of the cell cycle. Phosphorylated forms of αB-crystallin in mitotic cells were detected in all cell lines examined and in tissue sections of mouse embryos. Increases in the levels of αB-crystallin that had been phosphorylated at Ser-45 and Ser-19, but not at Ser-59, were detected biochemically by isoelectric focusing or SDS-polyacrylamide gel electrophoresis and a subsequent Western blot analysis of extracts of cells collected at the mitotic phase. When we estimated the phosphorylation activity specific for αB-crystallin in extracts of mitotic U373 MG cells, using the amino-terminal 72-amino acid peptide derived from unphosphorylated αB2-crystallin as the substrate, we found that the activities responsible for the phosphorylation of Ser-45 and Ser-19 were markedly enhanced but that the activity responsible for the phosphorylation of Ser-59 was suppressed. The protein kinases responsible for the phosphorylation of Ser-45 and Ser-59 in the amino-terminal 72-amino acid acid peptide were partially purified from extracts of cells that had been stimulated by exposure to H2O2 in the presence of calyculin A. The activities responsible for the phosphorylation of Ser-45 and Ser-59 were eluted separately from a column of Superdex 200 at fractions corresponding to about 40 and 60 kDa, respectively, while the kinase for Ser-19 was unstable. p44/42 mitogen-activated protein (MAP) kinase and MAP kinase-activated protein (MAPKAP) kinase-2 were concentrated in the Ser-45 kinase fraction and Ser-59 kinase fraction, respectively. Recombinant human p44 MAP kinase and MAPKAP kinase-2 purified from rabbit muscle selectively phosphorylated Ser-45 and -59, respectively. The Ser-45 kinase fraction and Ser-59 kinase fraction phosphorylated myelin basic protein and hsp27, respectively. These results suggest that the phosphorylations of Ser-45 and Ser-59 in αB-crystallin are catalyzed by p44/42 MAP kinase and MAPKAP kinase-2, respectively, in cells and that the phosphorylation of Ser-45 by p44/42 MAP kinase is enhanced while the phosphorylation of Ser-59 by MAPKAP kinase-2 is suppressed during cell division.

α-Crystallin, a major structural protein of the vertebrate eye lens, is a polymeric protein with a molecular mass of about 800 kDa. The α-crystallin of the bovine lens is composed predominantly of two types of polypeptide, the A (αA1 and αA2) and B (αB1 and αB2) subunits (1). αA1 and αB1 are the phosphorylated forms of the primary gene products, αA2 and αB2 (2, 3). The molecular mass of each subunit is about 20 kDa, and the similarity between the primary structures of the two subunits is greater than 50% (4). The α-crystallins also share sequence similarity with the small heat shock proteins (hsps) of numerous species (5). Because of the striking similarities among the primary structures of the carboxyl-terminal half of each molecule (the α-crystallin domain), αA-crystallin, αB-crystallin, hsp27, and p20 (6) are considered to be members of the α-crystallin small hsp family in vertebrates (7, 8).

A common feature of small hsps is their formation of large oligomeric complexes such as αA-crystallin and αB-crystallin in the lens. In the skeletal muscle, αB-crystallin, hsp27, and p20 seem to form a large heteropolymer, because the three proteins were copurified from the extract and coimmunoprecipitated with antibodies against each of the three proteins (6, 9). The α-crystallin domain of each small hsp was suggested to be important for this complex formation and the chaperone activity (8). Both αA-crystallin (10) and αB-crystallin (11) are also present in nonlenticular tissues, and the expression of αB-crystallin, but not αA-crystallin and p20, is induced in cells under various stressful conditions, as is hsp27 (12, 13).

The major posttranscriptional modifications of the α-crystallin small hsp family are due to the phosphorylation of serine residues. The phosphorylation of hsp27 by mitogen-activated protein (MAP) kinase-activated protein (MAPKAP) kinase-2 is enhanced when cells are exposed to heat (14, 15) or chemicals (16). p20 in vascular smooth muscles is phosphorylated in association with cyclic nucleotide-dependent vasorelaxation (17). In addition, p20 was phosphorylated in vitro by both cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase (17). Bovine αA-crystallin is phosphorylated at Ser-122 (2), and αB-crystallin is phosphorylated at Ser-19 or Ser-21, Ser-43 or Ser-45, and Ser-59 (18–20). It was believed for some years that αB-crystallin and αA-crystallin in the lens were phosphorylated in a cyclic AMP-dependent manner (2, 21)
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Fig. 1. Specificity of antibodies that recognized each of the phosphorylated serine residues in αB-crystallin. Fifty-ng aliquots of αB1-crystallin, purified from bovine lens, were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Each membrane was then incubated for 2 h at room temperature with antibodies against p19S, p45S, or bovine p59S (0.5 µg/ml) that had been preincubated overnight at 4 °C with or without (None) αB1-crystallin purified from bovine lens (αB1), or with the p19S, p45S, or bovine p59S peptide (10 µg) of αB-crystallin. The membrane was then incubated for 1 h with peroxidase-labeled antibodies raised in goats against rabbit IgG, and the peroxidase activity on the membrane was visualized on x-ray film by use of a Western blotting chemiluminescence reagent (Renai

or by the kinase activity of the proteins themselves (22, 23). However, we demonstrated recently that αB-crystallin in U373 MG human glioma cells is phosphorylated at three serine residues (Ser-19, -45, and -59) in response to various types of stress (24). We raised antibodies in rabbits that recognized αB-crystallin that had been phosphorylated at each of the three serine residues individually (24). In the present study, by using these antibodies, we found that the phosphorylation at each site in αB-crystallin is regulated differently during mitosis, and we obtained evidence suggesting that p44/42 MAP kinase and MAPKAP kinase-2 are responsible for phosphorylation of Ser-45 and Ser-59, respectively, in αB-crystallin in vivo.

Experimental Procedures

Reagents—Affinity-purified fluorescein isothiocyanate-labeled goat antibodies against rabbit IgG were purchased from Bio Source International (Camarillo, CA). Biotin-labeled antibodies against rabbit IgG that had been absorbed with mouse serum and horseradish peroxidase-labeled streptavidin were obtained from Vector Laboratories (Burlingame, CA). Pefablock SC was obtained from Boehringer Mannheim (Tokyo, Japan). Phorbol 12-myristate 13-acetate (PMA), okadaic acid, calyculin A, taxol, thymidine, and lysyl endopeptidase were obtained from Wako Pure Chemicals (Osaka, Japan). Ampholine pH 6–8, ampholine pH 3.5–10, and CNBr-activated Sepharose 4B were obtained from Amersham Pharmacia Biotech. Five mg of protein A (obtained from Nacalai Tesque, Kyoto) were coupled with 1 g of CNBr-activated Sepharose 4B. Recombinant human p44 MAP (Erk1, activated) kinase was obtained from StressGen Biotechnologies Co. (Victoria, Canada). MAPKAP kinase-2 purified from rabbit skeletal muscle was obtained from Upstate Biotechnology (Lake Placid, NY). Nocodazole, myelin basic protein purified from bovine brain, histone (type III-S) from calf thymus, and the catalytic subunit of protein kinase A (PKA) purified from bovine heart were obtained from Sigma-Aldrich (Tokyo). The antibody against p44/42 MAP kinase was obtained from New England Biolabs (Beverly, MA). The antibody against MAPKAP kinase-2 (C-18) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). SB202190 and PD98059 were obtained from Calbiochem.

Culture and Treatment of Cells—U373 MG human glioma cells (obtained from American Type Culture Collection, Rockville, MD) were grown in Eagle’s minimal essential medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal calf serum (ICN Biomedicals, Inc., Aurora, OH) at 37 °C in a CO₂ incubator. The cells were seeded on 90-mm dishes, and the medium was changed every 2 or 3 days. HeLa S3, Swiss 3T3, 3Y-1 B1–6 (25), and NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical Co.) supplemented with 10% fetal calf serum. Mitotic cells were collected by double-block procedures that involved incubations with thymidine and nocodazole (26) and also by mechanical release from standard cell cultures. U373 MG cells at confluent cultures were also exposed to various chemicals for 1 h in the CO₂ incubator. Cells were then washed twice with phosphate-buffered saline (PBS), containing 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na₂HPO₄, and 0.2 g of KH₂PO₄ in 1000 ml of H₂O) and frozen at −80 °C for a few days prior to use. For the Western blot analysis and the assay of phosphorylation activities with the crude extracts of cells, cells were sonicated in 80 mM HEPES-NaOH buffer, pH 7.0, that contained 0.1 mM NaF, 0.3 mg/ml Pefabloc SC, 10 µg/ml trypsin inhibitor, 0.2 µg/ml okadaic acid, and 0.2 µl calyculin A. For the purification of protein kinases responsible for the phosphorylation of αB-crystallin, the frozen cells on each dish were collected and suspended in 20 mM Tris-HCl buffer, pH 7.5, that contained 1 mM EDTA, 1 mM EGTA, 5% glycerol, 1 mM benzamidine, and 0.03% Brij 35 (A) supplemented with 0.3 mg/ml of Pefabloc SC, 10 µg/ml of trypsin inhibitor, 0.2 µg/ml okadaic acid, and 0.2 µl calyculin A. Each suspension was sonicated and centrifuged at 125,000 × g for 20 min at 4 °C to obtain the soluble extracts of cells.

Immunostaining—Indirect immunofluorescence staining was performed as described previously (27). All immunostained specimens were examined under a confocal laser scanning microscope (LSM 410; Karl Zeiss, Oberkochen, Germany). Sections of mouse embryonic tissues were prepared, and the immunohistochemical staining of these sections was performed as described previously (28).

Electrophoresis and Western Blot Analysis—SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (29) in 10 or 12.5% polyacrylamide gels. Tricine/SDS-PAGE was performed as described by Schagger and von Jagow (30) in 16.6% polyacrylamide gels that contained 13.3% glycerol. Isoelectric focusing (IEF) was performed as described previously (24) by the method of O’Farrell (31), using the Protean II system from Bio-Rad (Tokyo). For the Western blot analysis, proteins on a gel were transferred electrophoretically to a nitrocellulose membrane, and the membrane was incubated successively for 2 h with primary antibodies and then for 1 h with peroxidase-labeled secondary antibodies. Antigens on the membrane were visualized on x-ray film using a chemiluminescence reagent (Renai

Antigens incubated with antibodies

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Antibodies—Affinity-purified rabbit antibodies against the carboxyl-terminal decapeptide of αB-crystallin were prepared as described previously (11). Rabbit antisera against the amino-terminal dodécapeptide of αB-crystallin was raised in rabbits by injecting the peptide (MDIAHHPPWR-Cys) conjugated with hemocyanin (Sigma) using N-(4-carboxycyclohexylmethyl)maleimide (32), and the antibodies were purified by use of a column of bovine αB2-crystallin-coupled Sepharose as described previously (11). Antibodies that recognize each of the three phosphorylated serine residues in human αB-crystallin (Ser-19, -45, and -59) were prepared as described previously (24). Because the internal sequence of the peptide (APSWFDTG LSE, residues 57–67, p59S) of human αB-crystallin included a residue different from that in bovine and rat αB-crystallin (the Phe-61 in the human sequence is Ile-61 in bovine or rat), antibodies raised with p59S of the human sequence did not react with bovine and rat αB1-crystallin. Therefore, the peptide of the bovine (and rat) sequence (APSWFDTG LSE; bovine p59S) was synthesized, and the antisera was raised in rabbits as described previously (24). The antibodies that recognized the phosphorylated Ser-59S of bovine and rat αB-crystallin were purified with bovine p59S peptide-coupled Sepharose. The binding of anti-p19S, anti-p45S, and anti-bovine p59S to bovine αB1-crystallin was eliminated by preincubation of the various antibodies with bovine αB1-crystallin or with the...
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RESULTS

Immunocytochemical Localization of Phosphorylated Forms of αB-crystallin—Among the three antibodies that recognized the phosphorylated serine residues in αB-crystallin, anti-p45S had relatively high affinity and specificity when crude extracts of U373 MG cells that had been exposed to various types of stress were subjected to IEF and a subsequent Western blot analysis (24). Therefore, we attempted the localization of αB-crystallin phosphorylated at Ser-45 by indirect immunofluorescence staining with antibodies raised against p45S. As shown in Fig. 2A, various lines of cultured cells, including U373 MG human glioma cells, HeLa S3 cells, human skin fibroblasts, Swiss 3T3 and NIH 3T3 mouse fibroblasts, and 3Y-1 B1–6 rat fibroblasts, were immunostained with antibodies against p45S. Prominent staining of phosphorylated Ser-45 in αB-crystallin was apparent in mitotic cells of all cell lines examined. In most cases, staining was evident as fine granular deposits, with staining throughout the cytoplasm of mitotic cells and clearly separate from the chromosomes. In some interphase cells, there was faint granular staining in the cytoplasm. The phosphorylation staining of mitotic cells was confirmed by the treatment of cells with an antimotic agent, taxol. This treatment increased the number of cells that were immunostained with antibodies against p45S (Fig. 2A). The incubation of antibodies with p45S abolished the staining, confirming the specificity of staining (Fig. 2B, d and h). An enhanced phosphorylation of Ser-45 in αB-crystallin seemed to occur during mitosis. Phosphorylation began to appear during prophase and continued until telophase or cytokinesis (Fig. 2C). The phosphorylation of Ser-45 in mitotic cells was observed in vivo as well as in cultured cells, when the mouse embryonic tissues were subjected to immunohistochemical staining with the same antibody (data not shown). Samples of some human malignant tumors were also positive for immunostaining with antibodies against p45S, and only mitotic cells were immunostained in such cases (data not shown).

Western Blot Analysis of Extracts of Mitotic Cells—To test the results obtained in our immunocytochemical studies, we collected U373 MG cells at the mitotic phase after successive treatments of cells with thymidine and nocodazole. We subjected extracts of untreated control cells and of mitotic cells to IEF and a subsequent Western blot analysis with antibodies against the carboxyl-terminal peptide of αB-crystallin (Fig. 3A, αB-C) or with antibodies against p45S (Fig. 3A, p45S). The extracts were also subjected to SDS-PAGE and a subsequent Western blot analysis with antibodies against p19S, p45S, p59S, and the carboxyl-terminal peptide of αB-crystallin (Fig. 3B). After IEF, the two bands of the acidic form of αB-crystallin, corresponding to those of αB1-crystallin purified from bovine lens, were clearly detected in the case of mitotic cells after immunostaining with antibodies both against the carboxyl-terminal peptide of αB-crystallin and against p45S. The results of the SDS-PAGE and Western blot analysis revealed that the increases in phosphorylated serine residues in αB-crystallin in mitotic cells corresponded to phosphorylation at Ser-19 and -45, while the levels of phosphorylated Ser-59 tended to be reduced in mitotic cells. These results suggest that the phosphorylation of these three serine residues in αB-crystallin is catalyzed by multiple protein kinases.

The increases in the levels of αB-crystallin that had been phosphorylated at Ser-45 in mitotic 3Y-1 B1–6 cells and in mitotic BRL 3A rat liver cells were confirmed by IEF and Western blot analysis, as shown in Fig. 3C. Phosphorylated αB-crystallin in U373 MG cells was detected after 6 h of exposure to nocodazole, and two bands of phosphorylated αB-crystallin were detected in cells that had been exposed to nocodazole for 12–18 h. However, when cells that had been exposed to nocodazole for 12 h were returned to the standard medium, the phosphorylated αB-crystallin disappeared completely within 6 h (data not shown).

These results confirmed our immunohistochemical finding that the level of αB-crystallin phosphorylated at Ser-45 was elevated in mitotic cells. In addition, the SDS-PAGE and Western blot analyses revealed that the level of αB-crystallin phosphorylated at Ser-19 was also elevated, while that of αB-crystallin phosphorylated at Ser-59 was depressed in mitotic cells.

Activities of Protein Kinases That Phosphorylate αB-crystallin in Mitotic Cells—When bovine αB2-crystallin was incubated with extracts of U373 MG cells in the presence of [γ-32P]ATP and Mg2+, the 32P label was barely incorporated into αB2-crystallin, even when we used extracts of cells that had been exposed to PMA and okadaic acid (Fig. 4A). However, when αB2-crystallin that had been treated with lysyl endopeptidase was used as a substrate, the N-72K peptide was phosphorylated significantly even with extracts of control cells, and the phosphorylation was markedly enhanced with extracts of cells that had been exposed to PMA and okadaic acid (Fig. 4A). These results suggest that the aggregation properties of the purified αB-crystallin might be different from the native form in cells and that protein kinases might be unable to gain access to sites of potential phosphorylation. The treatment of αB2-crystallin with lysyl endopeptidase resulted in the destruction of the α-crystallin domain and the formation of N-72K peptide, which contained each of the three phosphorylation sites in αB-crystallin accessible to protein kinases.

To clarify whether the changes in the levels of αB-crystallin phosphorylated at the various serine residues were the result of changes in the activities of protein kinases that are specific...
for the respective residues, we assayed the phosphorylation activities in extracts of control and mitotic U373 MG cells using the N-72K peptide of αB-crystallin as the substrate. After incubation at 30 °C for 20 min in the presence of ATP and Mg2+, aliquots of each reaction mixture were subjected to Tricine/SDS-PAGE and subsequent autoradiography (Fig. 4C) or a Western blot analysis (Fig. 4B) with the antibodies that recognized each of the three phosphorylated serine residues. As shown in Fig. 4B, the levels of N-72K phosphorylated at Ser-19 and Ser-45 that had been produced during incubations with extracts of mitotic cells were much higher than the levels after incubations with extracts of control cells, while the levels of N-72K phosphorylated at Ser-59 after incubations with extracts of mitotic cells were lower than those after incubations with extracts of control cells. Thus, the extent of incorporation of 32P into N-72K was similar for the extracts of control and mitotic cells, as shown in the autoradiogram in Fig. 4C.

Our results indicate that the increased levels of αB-crystallin phosphorylated at Ser-19 and -45 and the decreased levels of αB-crystallin phosphorylated at Ser-59 resulted from changes in the activities of different protein kinases.

Partial Purification of Protein Kinases Responsible for Phosphorylation of N-72K Peptide from U373 MG Cells—To identify the enzymatic activities responsible for the phosphorylation of αB-crystallin, we partially purified the phosphorylation activities from U373 MG cells. Frozen cells from about 30 dishes
that had been exposed to 4 mM H2O2 and 2.5 mM calyculin A at 37 °C for 1 h were collected at 0 °C in 10 ml of buffer A supplemented with 0.3 mg/ml of Pefablock SC, 10 mM MgCl2, 0.2 mM okadaic acid, and 0.2 mM calyculin A. After sonication, the homogenate was centrifuged at 4 °C for 20 min with a final volume of 50 ml. The supernatant was passed through a column with 10 ml of buffer A, and protein kinase activities for Ser-45 by about 2-fold. The activities that phosphorylated Ser-19 (Ser-19 kinase) and Ser-59 kinase were eluted in similar fractions that contained 100 μg of protein were incubated at 30 °C for 20 min with 5 μl of bovine aB2-crystallin (1 mg/ml) or 5 μl of lysyl endopeptidase-digested aB2-crystallin (1 mg/ml) in the presence of 0.5 mM [γ-32P]ATP (2 μCi/tube), 5 mM MgCl2, 100 mM NaF, 100 mM calyculin A, 0.05 mM Na2F, 200 μg/ml Pefablock SC, and 10 μg/ml trypsin inhibitor in a final volume of 50 μl with 50 mM HEPES-NaOH, pH 7.0. The reaction was stopped by the addition of 50 μl of the sample buffer for electrophoresis. Thirty-μl aliquots of the mixture were subjected to Tricine/SDS-PAGE. Thirty-μl aliquots (for detection of N-72K) or 10-μl aliquots (for the detection of phosphorylated serine residues) of the mixture were subjected to Tricine/SDS-PAGE and a Western blot analysis with antibodies that recognized each of the phosphorylated serine residues (p19S, p45S, and p59S) or antibodies against the amino-terminal dodecapeptide of aB-crystallin (aB-N). Bl, incubated without a cell extract. C, extracts of control and mitotic cells that contained 100 μg of protein were incubated at 30 °C for 20 min with 5 μl of lysyl endopeptidase-digested aB2-crystallin (3 mg/ml) in the presence of 1 mM ATP, 10 mM MgCl2, 100 mM okadaic acid, 100 mM calyculin A, 0.05 mM NaF, 200 μg/ml Pefablock SC, and 10 μg/ml trypsin inhibitor in a final volume of 50 μl with 50 mM HEPES-NaOH, pH 7.0. The reaction was stopped by the addition of 100 μl of the sample buffer for Tricine/SDS-PAGE. Two-μl aliquots (for detection of N-72K) or 10-μl aliquots (for the detection of phosphorylated serine residues) of the mixture were subjected to Tricine/SDS-PAGE and a Western blot analysis with antibodies that recognized each of the phosphorylated serine residues (p19S, p45S, and p59S) or antibodies against the amino-terminal dodecapeptide of aB-crystallin (aB-N). Bl, incubated without a cell extract. C, extracts of control and mitotic cells that contained 100 μg of protein were incubated at 30 °C for 20 min with 5 μl of the peptidease-treated aB2-crystallin in the presence of 0.5 mM [γ-32P]ATP and 5 mM MgCl2 in reaction mixture with a final volume of 50 μl, and after terminating the reaction by the addition of the sample buffer for electrophoresis, 30-μl aliquots of the mixture were subjected to Tricine/SDS-PAGE, and the gel was exposed to x-ray film (32P) as described above.
after the chromatography of Superdex 200. These results suggest that each of the three serine residues is phosphorylated by the three different protein kinases.

Characterization of the Protein Kinases That Phosphorylate Ser-45 and Ser-59—We previously observed (24) that the phosphorylation of Ser-45 was preferentially stimulated in U373 MG cells that had been exposed to PMA and that the phosphorylation of Ser-59 was stimulated in cells exposed to arsenite. The phosphorylation induced by PMA was selectively suppressed in the presence of SB202190, whereas Ser-59 kinase and MAPKAP kinase-2 phosphorylated Ser-59 in the N-72K peptide. The bands weakly stained at Ser-19 and Ser-45 by the Ser-59 kinase preparation were probably due to the contaminated activities of Ser-19 kinase and Ser-45 kinase kinase. After 1 h, the cells were collected, sonicated, and centrifuged to obtain the soluble extracts as described under “Experimental Procedures.” Each extract containing 100 μg of protein was incubated with the lysyl endopeptidase-digested αB-crystallin at 30 °C for 20 min, and the phosphorylated serine residues in N-72K peptide were analyzed using antibodies against p19S, p45S, and p59S, as described in the legend to Fig. 5.

The phosphorylated Ser-45 and Ser-59—

FIG. 5. Elution profiles of enzymatic activities for the phosphorylation of the three serine residues in the N-72K peptide from a column of Superdex 200, and the Western blot analysis of each fraction with antibodies against p44/42 MAP kinase and MAPKAP kinase-2. A, the fractions from the chromatography on a column of DEAE-5PW that contained the phosphorylation activities for the three serine residues were pooled and concentrated. Five hundred-μl aliquots of the concentrate were applied to a column of Superdex 200, and the eluate was collected in 0.5-ml fractions as described under “Results.” Ten-μl aliquots of each fraction were employed for the assay of the phosphorylation of Ser-19 (p19S), Ser-45 (p45S), and Ser-59 (p59S) in N-72K peptide. After 20 min of incubation, 2-μl aliquots (for the detection of N-72K), 5-μl aliquots (for the detection of phosphorylated Ser-45 and Ser-59), or 15-μl aliquots (for the detection of phosphorylated Ser-19) of the mixture were subjected to Tricine/SDS-PAGE and a Western blot analysis of the phosphorylated serine residues, as described in the legend to Fig. 4. Autoradiogram of the phosphorylated N-72K peptide assayed with 10-μl aliquots (for the detection of phosphorylated Ser-19), 5-μl aliquots (for the detection of phosphorylated Ser-45), and 15-μl aliquots (for the detection of phosphorylated Ser-59) are indicated on the top with arrowheads. B, 20-μl aliquots of each fraction were subjected to SDS-PAGE and a subsequent Western blot analysis with antibodies against p44/42 MAP kinase (MAP K) and MAPKAP kinase-2 (MAPKAP K2).

FIG. 6. Phosphorylation of the N-72K peptide by extracts of cells that had been exposed to arsenite or PMA in the presence or absence of SB202190 and PD98059. U373 MG cells were exposed at 37 °C to 200 μM arsenite with (lane 1) or without (lane 2) 10 μM SB202190, an inhibitor of p38 MAP kinase or to 1 μM PMA with (lane 5) or without (lane 4) 50 μM PD98059, an inhibitor of p44/42 MAP kinase kinase. After 1 h, the cells were collected, sonicated, and centrifuged to obtain the soluble extracts as described under “Experimental Procedures.” Each extract containing 100 μg of protein was incubated with the lysyl endopeptidase-digested αB-crystallin at 30 °C for 20 min, and the phosphorylated serine residues in N-72K peptide were analyzed using antibodies against p19S, p45S, and p59S, as described in the legend to Fig. 5.

shows the phosphorylation results. The N-72K peptide detected by antibodies against the amino-terminal peptide of αB-crystallin; Lane 1, untreated control cells.

The phosphorylation induced by PMA was selectively suppressed in the presence of SB202190, whereas Ser-59 kinase and MAPKAP kinase-2 phosphorylated Ser-59 in the N-72K peptide. The bands weakly stained at Ser-19 and Ser-45 by the Ser-59 kinase preparation were probably due to the contaminated activities of Ser-19 kinase and Ser-45 kinase kinase. The activity for Ser-19 kinase was barely detected in the presence of SB202190, whereas Ser-59 kinase and MAPKAP kinase-2 phosphorylated Ser-59 in the N-72K peptide. The bands weakly stained at Ser-19 and Ser-45 by the Ser-59 kinase preparation were probably due to the contaminated activities of Ser-19 kinase and Ser-45 kinase in the Ser-59 kinase preparation. The activity for Ser-19 kinase was barely detected in the Ser-59 kinase preparation. The activity for Ser-19 kinase was barely detected in the Ser-59 kinase preparation.
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The phosphorylation of αB-crystallin in U373 MG human glioma cells is induced by exposure of the cells to various types of stress, such as arsenite, PMA, okadaic acid, anisomycin, H$_2$O$_2$, sorbitol, NaCl, and heat (24). All three serine residues (Ser-19, -45 and -59) are phosphorylated to some extent in mitotic cells upon exposure to PMA plus okadaic acid revealed no characteristic differences from that in control cells (data not shown), but it did reveal that all mitotic cells were intensely immunostained with antibodies against p45S, independently of the treatment of cells with chemicals. The intense staining of mitotic cells with antibodies against p45S was observed in all cell lines examined, as well as in sections of mouse embryos. The increases in the levels of αB-crystallin phosphorylated at Ser-45 in mitotic cells were confirmed biochemically by IEF or the SDS-PAGE of cell extracts and subsequent Western blot analysis with antibodies against p45S or against the carboxy-terminal peptide of αB-crystallin (αB-N) for the detection of the N-72K peptide as described in the legend to Fig. 5. Lanes 1 and 6, incubated without protein kinase, B, the same amounts of Ser-45 kinase (Ser-45 K), Ser-59 kinase (Ser-59 K), p44 MAP kinase (MAP K), or MAPKAP kinase-2 (MAPKAP K2) as described above were incubated for 5 min with 5 μg each of myelin basic protein (lane 1), αB2-crystallin (lane 2) and its lysyl endopeptidase-digest (lane 3), and hsp27 purified from human muscle (lane 4) and its lysyl endopeptidase-digest (lane 5) in the presence of [γ-32P]ATP and Mg$^{2+}$, and the reaction mixture was subjected to Tricine/SDS-PAGE and subsequent autoradiography as described in the legend to Fig. 4. Molecular mass markers (in kDa) are indicated with arrowheads.

FIG. 7. SDS-PAGE and Western blot analysis of a Ser-45 kinase fraction and a Ser-59 kinase fraction. A, a Ser-45 kinase fraction (lane 1; 2 μg) and a Ser-59 kinase fraction (lane 3; 5 μg) were subjected to SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. Lane 1, molecular mass markers in kDa. B, human recombinant p44 MAP kinase (lane 1; 2 μg), a Ser-45 kinase preparation (lane 2; 2 μg), MAPKAP kinase-2 purified from rabbit skeletal muscle (lane 3; 0.1 units) and a Ser-59 kinase fraction (lane 4; 5 μg) were subjected to SDS-PAGE and a subsequent Western blot analysis with antibodies against p44/42 MAP kinase (lanes 1 and 2) or against MAPKAP kinase-2 (lanes 3 and 4).

FIG. 8. Characterization of partially purified Ser-45 kinase and Ser-59 kinase and phosphorylation of the N-72K peptide derived from αB2-crystallin by p44 MAP kinase and MAPKAP kinase-2. A, 10-μl aliquots of the Ser-45 kinase (lanes 4 and 5) or Ser-59 kinase (5 μg; lanes 9 and 10) fractions separated by chromatography on a column of Superdex 200, recombinant human p44 MAP kinase (1 μg; lanes 2 and 3), or MAPKAP kinase-2 purified from rabbit muscle (0.1 units; lanes 7 and 8) were incubated at 30 °C for 10 min (lanes 2, 4, 7, and 9) or 20 min (lanes 3, 5, 8, and 10) with the lysyl endopeptidase-digested αB2-crystallin as described in the legend to Fig. 4, and aliquots of each reaction mixture were subjected to Tricine/SDS-PAGE and a Western blot analysis with antibodies against p19S, p45S, and p59S for the detection of phosphorylated serine residues and against the amino-terminal peptide of αB-crystallin (αB-N) for the detection of the N-72K peptide as described in the legend to Fig. 5. Lanes 1 and 6, incubated without protein kinase, B, the same amounts of Ser-45 kinase (Ser-45 K), Ser-59 kinase (Ser-59 K), p44 MAP kinase (MAP K), or MAPKAP kinase-2 (MAPKAP K2) as described above were incubated for 20 min with 5 μg each of myelin basic protein (lane 1), αB2-crystallin (lane 2) and its lysyl endopeptidase-digest (lane 3), and hsp27 purified from human muscle (lane 4) and its lysyl endopeptidase-digest (lane 5) in the presence of [γ-32P]ATP and Mg$^{2+}$, and the reaction mixture was subjected to Tricine/SDS-PAGE and subsequent autoradiography as described in the legend to Fig. 4. Molecular mass markers (in kDa) are indicated with arrowheads.
increased significantly in mitotic U373 MG cells and 3Y-1 cells that had been collected after exposure to nocodazole (data not shown). However, it remains to be clarified whether the induced synthesis of αB-crystallin in cells exposed to nocodazole is related to the phosphorylation states of αB-crystallin in the same cells.

When bovine αB2-crystallin was incubated with extracts of U373 MG cells in the presence of [γ-32P]ATP and Mg2+, the radiolabel was barely incorporated into αB2-crystallin, even when we used extracts of cells that had been extensively exposed to stress (Fig. 3A). These results suggest that the aggregation properties of αB2-crystallin, separated from αA-crystallins and αB1-crystallin in the presence of 6 M urea (33), might be different from the native form that is found in cells and that protein kinases might be unable to gain access to sites of potential phosphorylation. Therefore, we treated αB2-crystallin with lysyl endopeptidase to destroy the α-crystallin domain and to prepare the N-72K peptide, which contained each of the three phosphorylation sites in αB-crystallin. Using N-72K peptide as the substrate, we were able to detect the activities of protein kinases that phosphorylated αB2-crystallin in a cell-free system. The activities of protein kinase that phosphorylated Ser-19 and -45 were enhanced, and the activity of the kinase that phosphorylated Ser-59 was depressed in mitotic cells, as expected from the results of the Western blot analyses of phosphorylated forms of αB-crystallin. We obtained results similar to those presented above with naturally mitotic cells that had been prepared without chemical treatment (data not shown), an indication that the observed phenomena were not due to direct effects of thymidine and nocodazole on cells.

By using the N-72K peptide, we found that the phosphorylation of each of the three serine residues in αB-crystallin is catalyzed by three different protein kinases. The enzymatic activity that phosphorylates Ser-45 was eluted from a column of Superdex 200 at fractions corresponding to about 40 kDa with an elution profile similar to that of p44/42 MAP kinase, and the activities that phosphorylated Ser-19 and -59 were located in fractions corresponding to about 60 kDa with an elution profile similar to that of MAPKAP kinase-2. However, the phosphorylation activity for Ser-19 was low and unstable. Human recombinant p44 MAP kinase and the Ser-45 kinase fraction selectively phosphorylated Ser-45 in N-72 K peptide, and MAPKAP kinase-2 purified from rabbit muscle and the Ser-59 kinase fraction selectively phosphorylated Ser-59 in N-72K peptide. The Ser-45 kinase fraction was able to phosphorylate myelin basic protein, as was recombinant p44 MAP kinase, and the Ser-59 kinase fraction was able to phosphorylate hsp27, as was MAPKAP kinase-2. The antibodies against p44/42 MAP kinase and against MAPKAP kinase-2 immunoprecipitated only some portions (20–30%) of enzymatic activities, under our conditions, for phosphorylation of Ser-45 and Ser-59, respectively, in extracts of cells as well as in solutions of recombinant p44 MAP kinase and MAPKAP kinase-2 purified from rabbit skeletal muscle, respectively (data not shown). However, the present results differ from the results of previous studies, together with the inhibitory effects of PD98059 and SB202190 on the activation of Ser-45 kinase and Ser-59 kinase, respectively, in cells, strongly suggest that the protein kinase responsible for the phosphorylation of Ser-45 is p44/42 MAP kinase and that responsible for the phosphorylation of Ser-59 is MAPKAP kinase-2.

The phosphorylation of αB-crystallin in U373 MG cells was induced in response to various stimuli with a cyclic AMP-independent process (24). However, it was reported that αB- and αA-crystallins in the bovine lens were phosphorylated in a cyclic AMP-dependent manner in vitro (2, 21) or by their own autokinase activity (22, 23). Therefore, we tested whether PKA would phosphorylate αB-crystallin and hsp27 or their fragments prepared by digestion with lysyl endopeptidase under the present conditions. As shown in Fig. 9A, the catalytic subunit of PKA purified from bovine heart phosphorylated the protease-treated αB2-crystallin and hsp27, but it barely phosphorylated αB2-crystallin and hsp27, although hsp27 was phosphorylated by the Ser-59 kinase fraction and MAPKAP kinase-2 (Fig. 9B). The serine residue in N-72K peptide that had been phosphorylated by PKA was limited to Ser-59 (Fig. 9B), as were the Ser-59 kinase fraction and MAPKAP kinase-2. These results indicate that PKA can catalyze the phosphorylation of Ser-59 in N-72K peptide as can MAPKAP kinase-2. Since the reaction medium for the assay of the phosphorylation with N-72K peptide did not include cyclic AMP, PKA in extracts of cells seemed to be inactive and was unable to phosphorylate N-72K peptide under the present conditions. However, these results suggest that the previous in vitro finding of the phosphorylation of αB-crystallin by PKA in the presence of cyclic AMP were the phosphorylation at Ser-59 in αB-crystallin.

Although the biological significance of the enhanced phosphorylations of Ser-19 and -45 and the suppressed phosphorylation of Ser-59 in αB-crystallin during cell division remains to be clarified, the present results suggest that αB-crystallin might be involved in nuclear functions.

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