Serine 59 Phosphorylation of αB-Crystallin Down-regulates Its Anti-apoptotic Function by Binding and Sequestering Bcl-2 in Breast Cancer Cells

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The small heat shock protein (sHSP) αB-crystallin is a new oncoprotein in breast carcinoma that predicts poor clinical outcome in breast cancer. However, although several reports have demonstrated that phosphorylation of sHSPs modify their structural and functional properties, the significance of αB-crystallin phosphorylation in cancer cells has not yet been investigated. In this study, we have characterized the phosphorylation status of αB-crystallin in breast epithelial carcinoma cells line MCF7 submitted to anti-cancer agents like vinblastine. We have showed that the main phosphorylation site of αB-crystallin in response to vinblastine is serine 59 and determined a correlation between this post-translational modification and higher apoptosis level. The overexpression of the serine 59 "pseudophosphorylated" mutant (S59E) induces a significant increase in the apoptosis level of vinblastine-treated MCF7 cells. In contrast, overexpression of wild-type αB-crystallin or “nonphosphorylatable” mutant (S59A) result in a resistance to this microtubule-depolymerizing agent, while inhibition of endogenous levels of αB-crystallin by expression of shRNA lowers it. Analyzing further the molecular mechanism of this phenomenon, we report for the first time that phosphorylated αB-crystallin preferentially interacts with Bcl-2, an anti-apoptotic protein, and this interaction prevents the translocation of Bcl-2 to mitochondria. Hence, this study identifies serine 59 phosphorylation as an important key in the down-regulation of αB-crystallin anti-apoptotic function in breast cancer and suggests new strategies to improve anti-cancer treatments.

αB-Crystallin, a member of the small heat shock proteins (sHSP) 5 family, plays a role in many different cellular processes such as cellular growth, transcription, migration, differentiation, and development (1). It has been shown to protect cells from various stress factors, to prevent the aggregation or facilitate renaturation of proteins, and to protect cytoskeletal elements against ischemia injury or depolymerizing agents (2–4). Moreover, it is well established that αB-crystallin negatively regulates apoptosis, modulating several steps in the apoptotic pathway (5, 6). It has been reported that αB-crystallin binds to the pro-apoptotic Bax, Bcl-Xs, and p53 proteins and prevents their translocation to the mitochondria (7, 8). Downstream in this apoptotic pathway, αB-crystallin directly binds to partially processed caspase-3 (p24 intermediate), inhibiting thus the pro-apoptotic function of this protein (9, 10). αB-Crystallin also plays a role in many other different cellular processes such as cellular growth, transcription, migration, differentiation, and development (1).

In cancer cells, αB-crystallin has been found to process resistance to apoptosis, but the mechanisms are not fully understood (11). Indeed, it has been recently shown that αB-crystallin predicts poor clinical outcome in breast cancer and that apoptosis resistance conferred by αB-crystallin contributes to the aggressive behavior of basal-like breast carcinomas (12–16). Moreover, chemotherapy has been shown to enhance the expression of αB-crystallin in glioma and retinoblastoma cells (17, 18), and expression of αB-crystallin protects cancer cells from TRAIL-induced caspase-3 activation and apoptosis in vitro (19). Furthermore, overexpression of wild-type αB-crystallin induces neoplastic-like changes and enhances growth, proliferation, migration, and invasion of mammary epithelial cell lines. Interestingly, these modifications appear to be dependent on the phosphorylation state of αB-crystallin (14, 20). αB-Crystallin was finally identified as specially induced, phosphorylated, and modified with regard to subcellular localization in endothelial cells during tumor angiogenesis (21).

αB-Crystallin is characterized by its capacity to be phosphorylated in response to a variety of stimuli summarized as "stress factors," which could be physiological changes or environmental insults (22–24). This phosphorylation can occur at three serine sites corresponding to residues 19, 45, and 59. At least two pathways are implicated in the αB-crystallin phosphorylation.
tion: the p38 MAPK1/MAPKAPK2 pathway is responsible for the phosphorylation of serine 59 whereas serine 45 phosphorylation appears to be under the control of p42/44 ERK MAPK (25). The kinase responsible of the phosphorylation of serine 19 is still unknown. It has been suggested that phosphorylation induces changes in the conformation of these proteins, which in turn leads to major changes in size and distribution. On the functional level, phosphorylation has been reported to mediate a decrease in the ability of both proteins to act as molecular chaperones and to provide protection against oxidative stress (26). Ectopic expression of a pseudophosphorylated αB-crystallin mutant with the three target serine residues substituted for aspartate did not have any anti-apoptotic activity (10). However, opposite effects have been reported in cardiac myocytes subjected to osmotic or ischemic stress (27). In summary, although a considerable amount of data has been collected, the effect of sHSP phosphorylation is still not fully understood.

The fact that breast cancer cells acquire resistance to chemotherapy agents is actually a major clinical problem, and signaling pathways responsible for promoting cell death in response to microtubule inhibitors are under intensive investigations. Inhibitors of microtubule polymerization such as the Vinca alkaloids (vinblastine and vincristine) are used mainly in combination with other drugs for the treatment of Hodgkin’s disease, non-Hodgkin’s lymphomas, testicular cancer, Kaposi’s sarcoma, breast cancer, and other malignancies (28). The cellular effects of vinblastine are mediated preliminary by binding to tubulin subunits, inhibiting microtubule polymerization and leading to the disruption of microtubule dynamics. Evidence suggests that phosphorylation inhibits the anti-apoptotic properties of Bcl-2, initiating the apoptotic program in cancer cells (29). However, whether such modifications are necessary and sufficient for cell death by microtubule inhibitors is not clear.

In the present study, we asked whether modification of αB-crystallin levels of expression, as well as phosphorylation status, modifies the resistance level of mammary epithelial adenocarcinoma cells MCF7 to anti-cancer treatment such as vinblastine. We found that αB-crystallin overexpression lowers the apoptosis level of MCF7 cells in response to vinblastine treatment, while, in contrast, inhibiting its endogenous level with shRNA increases apoptosis. We demonstrate further the crucial role of phosphorylation in the modulation of αB-crystallin anti-apoptotic function in chemotherapy resistance. Moreover, the work described hereafter is the first to illustrate that phosphorylated αB-crystallin preferentially interacts with Bcl-2, preventing its translocation to mitochondria. These results suggest a new mechanism implicated in vinblastine-induced apoptosis through down-regulation of Bcl-2 anti-apoptotic function by αB-crystallin, in a phosphorylation-dependent manner. The finding presented herein should be instrumental in developing future strategies to down-regulate the anti-apoptotic activity of αB-crystallin in cancer cells, as well as increasing the effectiveness of microtubule-interfering agents.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Vinblastine, doxorubicin, paclitaxel, and Bcl-2 monoclonal antibody were purchased from Sigma (Saint-Quentin Fallavier, France). αB-Crystallin monoclonal antibody, specific polyclonal antibodies to phosphorylated αB-Crystallin, polyclonal antibody anti-HA were purchased from Stressgen (Victoria, CA). Monoclonal antibodies to actin were purchased from Abcam (Cambridge, UK). Peroxidase-conjugated antibodies were obtained from Pierce (Rockford, IL) and Alexa Fluor 488 goat anti-rabbit from Invitrogen (Cergy-Pontoise, France). Hoechst 33342 was purchased from Roche Diagnostics (Meylan, France).

**Cell Culture**—MCF7 breast epithelial adenocarcinoma cells were grown in complete DMEM supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml penicillin/streptomycin, and maintained at 37 °C with 5% CO₂.

**Cloning Information**—PCR-directed mutagenesis was used to produce human αB-crystallin with serine-to-glutamate or serine to alanine mutations at serine 19, serine 45, and/or serine 59 for pseudophosphorylated forms, respectively. All relevant data on origin of αB-crystallin cDNAs, plasmid constructs, and their designation, cloning methods, and PCR primers are given in Table 1.

**Western Blot Analysis**—Cellular extracts were resolved by 10% SDS-PAGE, as previously described (32). Reacting bands were detected using enhanced chemiluminescence (Pierce Supersignal West Pico). Quantifications were performed using the ImageJ software (NIH) after scanning of the autoradiograph.

**Immunofluorescence**—Cells were treated as previously described (32). For αB-crystallin serine 59 phosphorylation (P59) staining, cells were incubated with a specific anti-phospho Ser59 antibody (1/200), and further incubated with Alexa fluor anti-rabbit antibody (1/1000). Nuclei were stained with Hoechst 33342. Serine 59 phosphorylation of αB-crystallin and GFP-positive cells were observed under a fluorescence microscope (Olympus Bx60, Rungis, France). Apoptotic cells were small, dense and frequently fragmented, whereas surviving cells were fatter and well attached to the dish. Images were collected using a CoolSnap fx camera (Photometrics, Evry, France).

**Transient and Stable Transfections**—1 × 10⁶ cells and 6–12 μg of plasmid DNA (Table 1) were used for each transfection. Transfections were performed using Jet-Pei (Qiogen, Montreal, Canada) as described by the manufacturer. Transient transfectants were treated or not with the specified drugs 24 h after transfection. Stable transfectants were selected with 250 μg/ml neomycin (Invitrogen, Cergy-Pontoise, France) for 2 weeks. Isolation was repeated twice to ensure clonality. Two to three independent clones were tested for flow cytometry experiments.

**Cytometric Analysis**—Cell death was measured through mitochondrial potential with 100 nM of the lipophytic dye DIOC₃(3), Molecular Probes, Eugene, OR, and necrosis was quantified by propidium iodide (PI) staining (1 μg/ml) for 30 min at 37 °C. 15,000
The origin of αB-crystallin cDNAs, plasmid constructs, cloning methods, and PCR primers is shown.

| Construct | Plasmid Designation | Insertion site | Primer useda |
|-----------|---------------------|----------------|--------------|
| 1         | pcDNA3-αBCW         | n/a            | b            |
| 2         | pEGFP-αBCW         | n/a            | a, b         |
| 3         | pEGFP-S59A        | Site-directed mutagenesis from 1 Kpn I/Xba I | q, b |
| 4         | pEGFP-3A         | Site-directed mutagenesis from 1 Kpn I/Xba I | q, b |
| 5         | pEGFP-S59E        | Site-directed mutagenesis from 1 Kpn I/Xba I | q, b |
| 6         | pEGFP-3E         | Site-directed mutagenesis from 1 Kpn I/Xba I | q, b |
| 7         | pcDNA3-HA-αBCW    | HA-αBC | o, p |
| 8         | pcDNA3-HA-S59A    | HA-S59A | q, b |
| 9         | pcDNA3-HA-S59E    | HA-S59E | q, b |
| 10        | pcDNA3-HA-S3E     | HA-S3E | q, b |
| 11        | pcDNA3-6His-αBCW  | 6His-αBC | r, s |
| 12        | pcDNA3-6His-S59A  | 6His-S59A | q, b |
| 13        | pcDNA3-6His-3A    | 6His-3A | q, b |
| 14        | pcDNA3-6His-S59E  | 6His-S59E | q, b |
| 15        | shRNA-αBCW       | Sh | u, w |
| 16        | shRNA-Smb         | Smb | v, w |

*Primer used was as follows: a, 5'-aaaaaagcttgaacgcctctcggaattgcagccggattgcagccttttggaaa-3; b, 5'-aaagcttatgcggggttctcatcatcatcatcatcgtgcc-3; c, 5'-cattctctccac gaacccagccgcctc-3; d, 5'-cattctctccac gaacccagccgcctc-3; e, 5'-cattctctccac gaacccagccgcctc-3; f, 5'-cattctctccac gaacccagccgcctc-3; g, 5'-cattctctccac gaacccagccgcctc-3; h, 5'-aaagcttatgcggggttctcatcatcatctcgtgcc-3; i, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; j, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; k, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; l, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; m, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; n, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; o, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; p, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; q, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; r, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; s, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; t, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; u, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; v, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; w, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; x, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; y, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3; z, 5'-aaagcttatgcggggttctcatcatctcgtgcc-3.

Inhibition of the Endogenous αB-Crystallin Expression Lowers the Resistance of MCF7 Cells to Vinblastine—To investigate the mechanism of resistance to anti-cancer treatment in breast epithelial carcinoma cell line MCF7, we chose to inhibit the expression of endogenous αB-crystallin and to analyze the survival properties of the cells following treatment with vinblastine, a microtubule depolymerizing agent. For this purpose, we constructed a shRNA vector (19) and stably transfected this construct into MCF7 cells. Two stable clones (Sh19 and Sh22) showed the lowest level of endogenous αB-crystallin level (Fig. 1A). This result was confirmed at the mRNA level by reverse transcription and quantitative PCR (RT-QPCR) (supplemental Fig. S1).
Sh22 treated with 1 μM vinblastine for 24 h showed a significant higher level of apoptosis (72.4 and 60.5%, respectively) compared with untransfected MCF7 or control clone expressing a scrambled sequence (Smb1) (43.2 and 31.0%, respectively) (Fig. 1B). To check that shRNA expression and clonal selection did not result from nonspecific effects, we performed a rescue experiment by transfecting a cDNA coding for the murine αB-crystallin. Because of sequence divergences, murine mRNA is not inhibited by siRNAs designed to knock down human sequences. As cell resistance was restored, we concluded that the specific effect of shRNA was designed to knock down human αB-crystallin expression (supplemental Fig. S2). These results therefore show that high levels of endogenous αB-crystallin significantly protect MCF7 cancer cells from apoptosis after vinblastine treatment.

Vinblastine Induces Serine 59 Phosphorylation of αB-Crystallin in MCF7 Cells—To determine αB-crystallin phosphorylation status on serines 19, 45, and 59 in response to anti-cancer agent vinblastine, Western blotting experiments were performed with anti-α-crystallin and specific antibodies directed against the phosphorylated forms of serine 19, serine 45, and serine 59. Serines 19 and 45 are not or are slightly phosphorylated, respectively, whereas serine 59 (P59) shows a marked induction of its phosphorylation level after 24 or 48 h of vinblastine treatment (Fig. 2A). To evaluate whether treatment by different types of anti-cancer drugs was associated with induction of phosphorylation, we analyzed the levels of phosphorylated serine 59 of αB-crystallin in vinblastine, paclitaxel, a microtubule-stabilizing agent, or doxorubicin-treated MCF7 (Fig. 2B). As shown in Fig. 2B (right panel), both vinblastine and doxorubicin treatments result in a strong induction of serine 59 phosphorylation, whereas cells treated with paclitaxel showed only a small increase in the level of phosphorylation. As data show, the αB-crystallin expression level is unchanged whatever the treatment given to the cells.

Correlation between Vinblastine-induced Apoptosis and αB-Crystallin Phosphorylation State—To determine the subcellular distribution of serine 59-phosphorylated αB-crystallin (Ser-59) and the correlation with apoptosis in vinblastine-treated cells, MCF7 cells were submitted to microtubule-disrupting agent for 48 h. Immunofluorescence studies with anti-phospho-Ser59 and Hoechst 33342 (chromatin condensation state marker) show that a correlation may be established between vinblastine-induced apoptosis, marked by chromatin condensation and phosphorylation on serine 59 of αB-crystallin (Fig. 3A). Indeed, apoptotic cells exhibit a strong Ser-59 phosphorylation staining (arrows). Interestingly, αB-crystallin phosphorylated on Ser-59 presents a cytoplasmic and nuclear localization in apoptotic cells.
**αB-Crystallin and Cancer**

**FIGURE 3. Correlation between Ser-59 phosphorylation and vinblastine-induced apoptosis.**

A, correlation between serine 59 phosphorylation level, monitored by immunofluorescence staining with specific anti-phospho Ser-59 antibody (P59) and apoptosis estimated by chromatin condensation (Hoechst 33342). MCF7 cells were treated with vinblastine (1 μM) for 48 h or left untreated (Control). Most cells showing high levels of serine 59 phosphorylation also display chromatin condensation (arrows); scale bar: 20 μm. B, serine 59 phosphorylation of αB-crystallin enhances vinblastine-induced apoptosis of MCF7. Cells were transfected with GFP-, GFP-αBCWT-, GFP-S59A-, or GFP-S59E-expressing vectors. 24 h after transfection, MCF7 cells were treated or not with vinblastine (1 μM) for 48 h. Subcellular localization of GFP-tagged protein and chromatin condensation were determined as described under "Experimental Procedures." Arrowheads and arrows: Hoechst 33342-stained nuclei of transfected cells, respectively, uncondensed (normal) and condensed (apoptotic); scale bar: 20 μm. C, MCF7 cells were transfected with GFP-, GFP-αBCWT-, GFP-S59A-, GFP-S59E-, GFP-S3A-, or GFP-S3E-, or GFP-S59E-expressing vectors. Apoptotic transfected cells were counted as described under "Experimental Procedures." The mean ± S.E. was calculated from three replicate samples. Statistical significance was determined with a paired Student's t test, where *p < 0.05 was considered statistically significant (*).

affected with GFP-tagged αB-crystallin WT (GFP-αBCWT), S59A- or S59E-expressing vectors and treated for 48 h with vinblastine (1 μM). In MCF7 cells transfected with a “pseudophosphorylated” mutant (GFP-S59E), GFP-tagged proteins show a cytoplasmic and nuclear distribution in contrast to cells transfected with GFP-αBCWT or a “nonphosphorylatable” mutant (GFP-S59A), which are localized in the cytoplasm (Fig. 3B). To confirm that the phosphorylation of αB-crystallin was associated with a strong amount of apoptosis, MCF7 cells were transfected with the constructs described above, and, in addition, with a “nonphosphorylatable” mutant (GFP-3A with the three serine residues 19, 45, and 59 mutated) or a “pseudophosphorylated” mutant (GFP-3E), treated with vinblastine and the number of apoptotic GFP-labeled cells were counted after Hoechst staining (Fig. 3C). MCF7 transfected with GFP-S59E and GFP-S3E showed a significant increase of vinblastine-induced apoptosis level (62.8% ± 6.83 and 64.7% ± 1.05 respectively) compared with control vector (pEGFP) (44% ± 4.05). In contrast, cells transfected with GFP-αB-crystallin WT (GFP-αBCWT), or “nonphosphorylatable” GFP-S59A and GFP-3A constructs, presented a lower apoptotic level (22.3% ± 0.8, 9% ± 3.8 and 17% ± 3.6 respectively), indicating that these constructs generate a strong resistance to vinblastine treatment. Interestingly, the nonphosphorylatable αB-crystallin mutant GFP-S59A shows the strongest resistance level (Fig. 3C). To confirm these results, the same experiment as above was performed with HA-tagged constructs. The results obtained confirmed our first set of experiments (data not shown).

Serine 59-phosphorylated αB-Crystallin Enhances the Apoptosis in MCF7 Cells: a Flow Cytometry Study—To confirm the previous results, we prepared stable clones expressing phosphorylation mutants of αB-crystallin. In this experiment, cell death was measured through mitochondrial potential (∆Ψm) with the lipophylic dye DiOC₆(3), and necrosis by propidium iodide staining assays, with flow cytometry. We used stable clones because they expressed levels of αB-crystallin constructs similar to the endogenous one, in comparison to transiently transfected cells that express higher levels. In addition, the use of DiOC₆(3) dye allowed to monitor early apoptotic events at the mitochondrial level compared with nuclear condensation showed in the previous results. The effect of αB-crystallin WT, S59A, and S59E expression on vinblastine-induced apoptosis of cells was quantified, and one set of data is presented (Fig. 4). Cells distribution analysis clearly showed an increase of DiOC₆(3)-negative S59E cells following treatment with vinblastine (Fig. 4E). In contrast, for αB-crystallin WT and S59A clones, the majority of cells remained positive for DiOC₆(3) staining (Fig. 4, C and D, respectively). The results of three independent experiments measuring cell apoptosis are summarized in panel F. There was an increase in cell apoptosis frequency in S59E-expressing cells (32.3%) compared with cells expressing αB-crystallin WT (15.6%). Confirming the results presented above, αB-crystallin WT or S59A-transfected cells displayed resistance to vinblastine (11.2% and 8.8%, respectively) compared with the control level (56.8%).

Phosphorylated αB-Crystallin Interacts with Bcl-2 in MCF7—We hypothesized that serine 59-phosphorylated αB-crystallin might influence the apoptotic process by interacting with apoptotic-related proteins. Then, we conducted co-precipitation and immunoprecipitation assays to identify the possible interactions between Ser-59-phosphorylated αB-crystallin with the pro- and anti-apoptotic proteins, Bax, Bcl-Xs, and Bcl-2, respectively. We present here the most relevant results, obtained with Bcl-2. Expression vectors coding for 6his-S59A or 6his-S59E were transfected into MCF7 cells. Proteins interacting with S59A or S59E αB-crystallin were precipitated from cell extracts with Ni-NTA beads. Results in Fig. 5A show that
Bcl-2 is co-purified with His<sub>6</sub>-tagged S59E/H<sub>9251</sub>B-crystallin from cell lysate. In contrast, a very low level of Bcl-2 is found in a co-precipitation experiment performed with 6his-S59A/H<sub>9251</sub>B-crystallin (Fig. 5A). Also, it appears that serine 59 phosphorylation of <em>H</em><em>B</em>-crystallin strongly enhances the affinity of <em>H</em><em>B</em>-crystallin to Bcl-2. The converse analysis was performed. MCF7 cells were transfected with HA-S59E or HA-S59A and co-immunoprecipitates using anti-Bcl-2 antibody were further probed with an anti-HA antibody. Serine 59-phosphorylated <em>H</em><em>B</em>-crystallin (HA-S59E), but not the nonphosphorylatable mutant (HA-S59A), was co-immunoprecipitated from MCF7 cells with Bcl-2 (Fig. 5B). Thus these results indicate that <em>H</em><em>B</em>-crystallin interacts with Bcl-2 in a phosphorylation-dependent manner.

Endogenous Bcl-2 Co-precipitate only Serine 59-phosphorylated <em>H</em><em>B</em>-crystallin—The previous experiment (Fig. 5) demonstrated that Bcl-2 preferentially bind to <em>H</em><em>B</em>-crystallin phosphorylated on serine 59. As these results were obtained using transfected constructs, we wondered whether endogenous phosphorylation of <em>H</em><em>B</em>-crystallin strongly enhances the affinity of <em>H</em><em>B</em>-crystallin to Bcl-2. The converse analysis was performed. MCF7 cells were transfected with HA-S59E or HA-S59A and co-immunoprecipitates using anti-Bcl-2 antibody were further probed with an anti-HA antibody. Serine 59-phosphorylated <em>H</em><em>B</em>-crystallin (HA-S59E), but not the nonphosphorylatable mutant (HA-S59A), was co-immunoprecipitated from MCF7 cells with Bcl-2 (Fig. 5B). Thus these results indicate that <em>H</em><em>B</em>-crystallin interacts with Bcl-2 in a phosphorylation-dependent manner.

FIGURE 4. αB-crystallin phosphorylation induces cell death in MCF7: a flow cytometry study. MCF7 cells were stably transfected with the pcDNA3 vector (CTL), HA-αBC (WT), HA-S59A (S59A), or HA-S59E (S59E) constructs. A, immunoblot showing the level of expression of αB-crystallin (αBC) in the CTL, WT, S59A, and S59E cells. Panels B, C, D, and E show cell sorting results from CTL, WT, S59A, and S59E cells, respectively. Cell death was determined 24 h after vinblastine treatment (1 μM) by reduced DIOC6(3) staining (quadrant R4), and necrosis by PI staining (left upper quadrant). F, quantification of apoptosis (DIOC6(3) negative indicating mitochondrial potential (ΔΨm) loss and PI-negative cells) in stable transfectants, treated (■) or not (□) with 1 μM of vinblastine for 24 h. The mean ± S.E. was calculated from three replicate samples. Statistical significance was determined with a paired Student’s t test, where p < 0.05 was considered statistically significant (*, p < 0.05; **, p < 0.01).

FIGURE 5. Specific interaction of serine 59-phosphorylated αB-crystallin with Bcl-2. A, 6his-S59A- and 6his-S59E-expressing vectors were transfected into MCF7 cells. 48 h later, cell extracts were isolated. The protein complex with 6his-S59A or 6his-S59E was precipitated (P) on Ni-NTA beads and analyzed on immunoblots (IB) with an anti-Bcl-2 antibody, or an anti-αB-crystallin antibody. αBC and Bcl-2 show, respectively, the initial expression level of endogenous (lower band) and transfected (upper band) αB-crystallin and Bcl-2 before precipitation (Lysate). B, HA-S59A- and HA-S59E-expressing vectors were transfected into MCF7 cells. Following immunoprecipitation (IP) with the anti-Bcl-2 antibody, αB-crystallin phosphorylation mutants bound to Bcl-2 (HA: initial levels of transfected HA-αB-crystallin; Bcl-2: initial level of cellular Bcl-2) were analyzed on immunoblots (IB) with the anti-HA antibody (HA-αBC), and the anti-Bcl-2 antibody to check reproducibility of results for the three conditions (Bcl-2).
aB-Crystallin and Cancer

When vinblastine (Vinp) treatment with phosphatase (lanes 3, 6, 8) or not (lanes 2, 7, 9) were immunoprecipitated with the anti-Bcl-2 antibody for 2 h at 4 °C, run on an IEF gel, and further analyzed after blotting with the anti-aB-crystallin antibody (aBC). The only band revealed is at the lower position (lane 1), which identifies the phosphorylated aB-crystallin. Controls (IP controls) were done without anti-Bcl-2 antibody (lane 3), pretreatment with phosphatase (lane 4), or without extracts (lane 5). Loading controls corresponding to lanes 1 and 2 are shown lanes 6 and 7, respectively. The same extracts were stained with anti-serine 59-phosphorylated aB-crystallin (P59), which identifies the lower band as the phosphorylated aB-crystallin.

The proper localization of Bcl-2 to the Mitochondria—The proper localization of Bcl-2 in the intracellular membranes, notably into the mitochondrial outer membrane, is required for its anti-apoptotic function (30). Therefore, inhibition of the subcellular localization of Bcl-2 could lead to the inhibition of its death-suppressor activity. To test whether Ser-59-phosphorylated aB-crystallin affects mitochondrial localization of Bcl-2, MCF7 cells were transfected with HA-S59A or HA-S59E expressing vectors, or left untransfected, and treated 24 h later with vinblastine. Then, mitochondrial and cytosolic proteins were isolated from the cells and immunoblotted using antibodies against Bcl-2 or aB-crystallin. As shown in Fig. 7, in S59A aB-crystallin-expressing cells as well as in control untransfected cells, Bcl-2 was mainly detected in the mitochondrial fraction. In contrast, Bcl-2 was detected in the cytosolic fraction but barely detectable in the mitochondrial fraction of MCF7 cells transfected with the “pseudophosphorylated” mutant S59E. These results suggest that the interaction between “pseudophosphorylated” aB-crystallin and Bcl-2 mainly occurs in the cytoplasm of MCF7 cells and that Ser-59 phosphorylated S59E aB-crystallin could prevent the translocation of Bcl-2 to mitochondria. Interestingly, analysis of the subcellular localization of aB-crystallin showed that the “pseudophosphorylated” mutant S59E has an equally distribution in mitochondrial and cytosolic fraction while the “nonphosphorylatable” mutant S59A was nearly undetectable in the mitochondrial fraction.

DISCUSSION

In the cancer field, the primary reason for treatment failure is drug resistance, which causes cancer cells to become insensitive to chemotherapy. Recently, it has been demonstrated that the small heat shock protein aB-crystallin is a novel oncogenic protein that protects cancer cells against apoptosis induced by diverse stimuli (14), including chemotherapeutic drugs and TNF-related apoptosis-inducing ligand (TRAIL) (19), by interacting with pro-apoptotic proteins or inhibiting caspase-3 activation (7–9, 31). Our data corroborate these results because inhibition of endogenous aB-crystallin expression with shRNA lowers cell resistance to vinblastine pro-apoptotic treatment (Fig. 1). We confirmed these results obtained with stable clones in siRNA transient transfection assays (supplemental Fig. S4). In addition, we checked that siRNA used have no off-target effects in a rescue experiment with a murine aB-crystallin cDNA that is not inhibited by siRNAs, because of murine/human sequence divergence (supplemental Fig. S5). Consequently, efforts to understand the oncogenic mechanisms of aB-crystallin in response to chemotherapeutic treatments may provide therapeutic insight to selectively overcome the apoptosis-resistance of cancer.
The role of phosphorylation of αB-crystallin in protecting cells is still poorly understood and the influence of phosphorylation and the structure-function relationships of αB-crystallin remain controversial. In this study, we have used breast epithelial adenocarcinoma cells MCF7 to determine the role of αB-crystallin phosphorylation in the response to microtubule-interfering agents. We analyzed the expression and phosphorylation state of αB-crystallin after vinblastine treatment, and showed a strong and major phosphorylation on serine 59 (Fig. 2). We found similarly to our previous work (32) that p38 MAPK is necessary for the induction of αB-crystallin phosphorylation on serine 59 by vinblastine in MCF7 cells (data not shown). These data are corroborated by a study showing that vincristine regulates the anti-apoptotic properties of HSP27 proteins in a phosphorylation-dependent manner in breast cancer cells (33). In addition, attenuated phosphorylation of HSP27 was recently correlated with tumor progression in patients with hepatocarcinoma (34). The results obtained using vinblastine, paclitaxel, and doxorubicin in MCF7 cell line demonstrate that phosphorylation of αB-crystallin is a specific mechanism depending on the drug used (Fig. 2, C and D). It is interesting to note that vinblastine, an inhibitor of microtubules polymerization, and doxorubicin, a DNA intercalating agent, exhibit different mechanisms of action to increase tumor cell killing (28).

Results from immunofluorescence (transient transfections) and flow cytometry (stable clones) experiments further demonstrated that serine 59 phosphorylation of αB-crystallin is an important key for apoptosis control (Figs. 3 and 4). We obtained similar results, although the method for measuring apoptosis was different: flow cytometry analysis was based on mitochondrial potential failure, which corresponds to earlier pre-mitochondrial steps, while counting nuclear condensation reveal later post-mitochondrial events (Fig. 3). Indeed, overexpression of pseudophosphorylated αB-crystallin mutants S59E induces a significant increase in the apoptotic level of vinblastine-treated cells while overexpression of αB-crystallin WT or nonphosphorylatable αB-crystallin reduces chemotherapy sensibility to vinblastine. Interestingly, nonphosphorylatable αB-crystallin S59A induces the highest survival level. This result may seem counterintuitive, but one explanation is that short-time effects of Ser-59 phosphorylation of αB-crystallin are protective, whereas long-lasting effects could result in pro-apoptotic signals.

To further understand how phosphorylation of αB-crystallin might influence the apoptotic process, we have analyzed potential differential interactions between phosphorylated and nonphosphorylated αB-crystallin and members of the Bcl-2 family. Hsp27 has been shown to interact or to modulate the functions of cytochrome c, Smac/Diablo, Daxx, Bax, and to inhibit pro-caspase 3 and 9 activation (35, 36). αB-crystallin binds to pro-apoptotic Bax, Bcl-Xs, and p53 (7, 8), but the modulating effect of phosphorylation on these interactions has not yet been investigated, except for inhibition of Daxx-mediated apoptosis by Hsp27 (37). Our results demonstrate for the first time that serine 59-phosphorylated αB-crystallin can directly and preferentially bind to Bcl-2 (Figs. 5, A and B and 6). Other molecules tested such as cytochrome c, Bax, and Bcl-X did not give significant results (data not shown). The pseudophosphorylated mutant S59E displayed a strong affinity to the anti-apoptotic protein Bcl-2 in contrast to the nonphosphorylatable mutant S59A. The serine 59-phosphorylated αB-crystallin markedly sequestered Bcl-2 in the cytosol and prevented its translocation into mitochondria during vinblastine-induced apoptosis (Fig. 7). In contrast, the anti-apoptotic protein Bcl-2 was mainly detected in the mitochondrial fraction of S59A-expressing MCF7. As a result of this interaction, phosphorylation of αB-crystallin can down-regulate the anti-apoptotic activity of Bcl-2 and then allow downstream apoptotic events to be turned on. It is possible that the phosphorylated αB-crystallin binds to other pro-apoptotic members of the Bcl-2 family (7). We propose that there may exist a competition between these molecules for αB-crystallin, which can be modulated by phosphorylation, to specifically sequester the translocation of pro- or anti-apoptotic partners during stress-induced apoptosis. Thus, our results provide a novel mechanism responsible for promoting cell death in response to microtubule inhibitors. It may be interesting to modify the level of phosphorylation of αB-crystallin to increase the level of apoptosis following treatments with anti-cancerous drugs. However, it is not possible to specifically manipulate the endogenous level of αB-crystallin phosphorylation, without modifying other functions such as p38 MAPK or MAPKAP kinases, which have pleiotropic effects (38). The most obvious possibility is to inhibit the level of endogenous αB-crystallin, which confirmed the protective effect of endogenous molecules in MCF7 cells subjected to anti-cancer treatment (Fig. 2).

αB-Crystallin is considered to be a soluble cytoplasmic protein but has also been described in association with subcellular organelles. It was shown to reside in the nucleus (39) and the perinuclear Golgi (40), and is known to associate with centrosomal structures (22), as well as to localize near to cellular membranes (41). In our study, we found a preferential association of the phosphorylated αB-crystallin with the mitochondria versus the “nonphosphorylated” protein with the mitochondria. Interestingly, it has been recently shown that localization of αB-crystallin at the mitochondria is regulated by oxidative stress and that normal or mutant (R120G) αB-crystallin interact with heart mitochondria in wild-type and CryABR120G mice (42). Mitochondrial permeability transition is clearly affected in CryABR120G-transfected cardiomyocytes and precedes the increased levels of apoptotic markers. This result raises the possibility that αB-crystallin and other sHSP may have a direct impact on either VDAC or mitochondrial proteins associated with the permeability transition pore (PTP). Our data and other results found in cardiomyocytes, although opposed in terms of cellular survival, suggest that phosphorylation could be important for specific mitochondrial localization and regulation of anti-apoptotic functions of αB-crystallin (43).

In summary, serine 59 phosphorylation of αB-crystallin plays a crucial role in vinblastine-induced apoptosis in MCF7 cells, by down-regulating the anti-apoptotic function of Bcl-2. All our data indicate that inducing specifically the phosphorylation of
**αB-Crystallin and Cancer**

Serine 59 on αB-crystallin in complement to other therapies should greatly improve the efficiency of treatments in breast cancer.

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