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αB-Crystallin Expression in Mouse NIH 3T3 Fibroblasts: Glucocorticoid Responsiveness and Involvement in Thermal Protection

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αB-crystallin, a major soluble protein of vertebrate eye lenses, is a small heat shock protein which transiently accumulates in response to heat shock and other kinds of stress in mouse NIH 3T3 fibroblasts. Ectopic expression of an αB-crystallin cDNA clone renders NIH 3T3 cells thermoresistant. αB-crystallin accumulates in response to the synthetic glucocorticoid hormone dexamethasone. Dexamethasone-treated NIH 3T3 cells become thermoresistant to the same extent as they accumulate αB-crystallin. A cell clone in which αB-crystallin is superinduced upon heat shock acquires augmented thermotolerance. Expression of the *ras* oncogene causes a rapid but transient accumulation of αB-crystallin within 1 day. Later, sustained *ras* oncogene expression suppresses the dexamethasone-mediated αB-crystallin accumulation. Thus, oncogenic transformation triggered by the *ras* oncogene interferes with hormone-mediated accumulation of αB-crystallin and concomitant acquisition of thermoresistance. Other known heat shock proteins do not accumulate in response to ectopic αB-crystallin expression or to dexamethasone treatment. These results indicate that αB-crystallin can protect NIH 3T3 fibroblasts from thermal shock.

Studies on oncogene-induced alterations in cellular gene expression and their association with the transformation phenotype are essential for understanding the mechanisms by which oncogenes mediate the malignant phenotype (for a review, see reference 3). In an attempt to define specific cellular responses toward accumulation of oncoproteins, we have previously studied the effects of the *ras* and *mos* oncoproteins on cellular gene expression in mouse NIH 3T3 fibroblasts (34). We found that high amounts of αB-crystallin rapidly accumulate in NIH 3T3 cells in response to the expression of the c-Ha-*ras* and v-*mos* oncogenes controlled by the glucocorticoid-inducible promoter of mouse mammary tumor virus (MMTV) (32).

αB-crystallin is one of the major soluble proteins of vertebrate eye lenses (for reviews, see references 51, 52, and 67). The protein shares 60% amino acid sequence homology with αA-crystallin (62). αB-crystallin is also expressed in nonocular tissues such as heart muscle, skeletal muscle, kidney, lung, brain, spermatocyte, and placenta (5, 8, 12, 23–25, 31, 40). Moreover, evidence has been accumulating that elevated levels of αB-crystallin are associated with various pathological conditions in the brain and other tissues (13, 24, 26, 27, 43, 44, 48). The α-crystallins share amino acid sequence similarity with small heat shock proteins (hsps) of diverse organisms, including mycobacteria, fruit flies, plants, and mammals (for reviews, see references 39, 51, 52, 65, 66, and 67). The homology between mouse αB-crystallin (32) and hsp25 (unpublished data; 17) reaches approximately 40%. These observations suggest a possible functional relationship between α-crystallins and small hsps (11). Recently we have shown that αB-crystallin is indeed a bona fide small hsp (33). The characteristic features shared by αB-crystallin and small hsps are inducibility by heat and other kinds of stress, the formation of specific supramolecular structures, and the intracellular redistribution upon heat shock. We have also shown that the promoter of the αB-crystallin gene contains a perfect and a truncated heat shock element, both of which are functional under stress conditions (33).

hsps are synthesized in response to various kinds of stress by all analyzed organisms from bacteria to humans (for reviews, see references 46 and 63). This finding indicates that the stress response is a fundamental and universal reaction by which cells cope with adverse environmental circumstances. One interesting physiological aspect of the stress response is the phenomenon of thermotolerance (for a review, see reference 18). Cells exposed to a sublethal temperature transiently exhibit higher survival rates when exposed to a second, lethal heat shock treatment. The close correlation between the levels of the hsps and the degree of thermotolerance led to the hypothesis that hsps are involved in the establishment of thermotolerance. In fact, there are many data demonstrating an involvement of hsps in the ability of cells to survive a hyperthermic challenge. For example, microinjection of anti-hsp70 antibodies into rat cells (53) or interference with the transcriptional induction of the *HSP70* gene in Chinese hamster ovary (CHO) cells (29) increased their thermosensitivity. Transfection of Rat-1 fibroblasts with the human *HSP70* gene driven by a constitutive promoter has been shown to confer thermal resistance (38). In yeast cells, hsp104 has been shown to be indispensable for the development of thermotolerance by genetic knock-out experiments (50, 54). Similarly, a requirement for small hsps in the acquisition of thermotolerance has been presented. *Dictyostelium* mutants that were unable to synthesize small hsps failed to develop thermotolerance, despite the accumulation of other hsps (42). Selective induction of the small *HSP* genes by the steroid hormone ecdysone in a hormone-sensitive *Drosophila* cell line induced thermoresistance without heat shock (7). Enhanced constitutive expression of hsp27 was found in heat-resistant variants from Chinese hamster cells (9). Moreover, the forced constitutive

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expression of the human *hsp27* gene in CHO and mouse cell lines conferred thermoresistance (36).

The ability of hsp27 to protect cells from thermal insults suggests that αB-crystallin may have the same potential and that the oncogene-mediated alterations of αB-crystallin expression are reflected by the thermoresistance of cells. This observation prompted us to investigate the regulation and function of αB-crystallin. Here we provide evidence that αB-crystallin can protect NIH 3T3 cells from thermal shock. We show that glucocorticoid hormone causes a large accumulation of αB-crystallin and concomitant acquisition of thermoresistance. Previously we have shown that the expression of the Ha-*ras* (EJ) or v-*mos* oncogene causes the rapid accumulation of αB-crystallin within 1 day (32). However, this effect is of transient nature. Sustained expression of these oncogenes for 1 to 2 weeks prevented dexamethasone-mediated αB-crystallin expression and consequently the acquisition of the hormone-mediated thermoresistance.

MATERIALS AND METHODS

Cells and vectors. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (standard medium) unless otherwise indicated. Cells which were kept in culture for extended periods of time were split before they reached confluence. The cell lines HLE and HLM are NIH 3T3 derivatives which contain the Ha-*ras* (EJ) and v-*mos* oncogenes under the control of the MMTV long terminal repeat (LTR) promoter, respectively (28). NFR-2 is an NIH 3T3-derived cell line which contains the Ha-*ras* (EJ) oncogene. They were obtained by cotransfection with the Ha-*ras* (EJ) oncogene-containing plasmid pcH*ras*(A) (55) and pSV2neo. Expression of the Ha-*ras* oncogene was verified by Western immunoblot analysis using an antiserum directed against $p21^{ras}$. CMcry12 is a derivative of NIH 3T3 harboring the αB-crystallin expression vector pCMcry6. pSVcry6 and pCMcry6 are expression vectors which contain the αB-crystallin cDNA sequence under the control of the simian virus 40 early promoter and the cytomegalovirus (CMV) promoter, respectively. The cloning of αB-crystallin cDNA has previously been described (32). The *Xba*I-*Kpn*I fragment which contains the complete coding region of αB-crystallin was subcloned into the *Xba*I-*Kpn*I sites of the expression vectors pSVK3 (Pharmacia LKB Biotechnology) and pRc/CMV (Invitrogen).

Transient transfection assay. Cells were plated at a density of 10^4 cells per cm^2 in 15-cm-diameter tissue culture dishes and grown at 37°C for 24 h in the standard medium unless otherwise indicated. Transfection was performed with 40 to 50 μg of plasmid DNA by the Ca^{2+} phosphate precipitation method (64). Eighteen hours after the addition of the DNA precipitate, the cells were washed twice with phosphate-buffered saline (PBS), trypsinized, and seeded at a density of 10^4 cells per cm^2 into 25- or 75-cm^2 tissue culture flasks for heat treatment and 10- or 15-cm-diameter tissue culture dishes for protein analysis. The cells were grown for 2 days before being subjected to the heat treatment or protein analysis. Transfection efficiency was determined by cotransfecting cells with the β-galactosidase expression plasmid pRSV-β-gal. The β-galactosidase activity in the cell lysates was determined according to An et al. (1) and Miller (45). One unit of β-galactosidase is defined as A_{420}/(incubation time [hours] × milligram of protein).

Heat killing experiments. Cells were plated at a density of 10^4 cells per cm^2 in 25- or 75-cm^2 tissue culture flasks and grown at 37°C for 24 h in the standard medium unless indicated otherwise. The cells were subjected to the heat treatment by immersion of the tissue culture flasks in a water bath kept at either 45.0 or 45.3°C for an appropriate time period. In parallel, untreated cells were analyzed as controls. After heat treatment, the cells were washed with PBS, trypsinized, counted, and seeded at serial dilutions into six-well tissue culture plates or 6-cm-diameter tissue culture dishes. The plates were incubated at 37°C for 10 to 14 days. Colonies emerging from surviving cells were stained and counted. The fraction of cells which had survived the heat treatment was determined by dividing the number of colonies emerging from heat-treated cells by the number of colonies emerging from untreated cells and corrected on the basis of the plating efficiencies of the cell lines. Plating efficiencies were determined by dividing the number of colonies emerged by the number of cells seeded. Proteins were extracted for analysis from the cells grown on 10- or 15-cm-diameter dishes just before heat treatment.

Gel electrophoresis. Cells grown on tissue culture dishes or in plastic flasks were washed twice with PBS, collected, and lysed either in sodium dodecyl sulfate (SDS) sample buffer for one-dimensional (1D) gel analysis (35) or in buffer for two-dimensional (2D) gel analysis (49). The samples for 1D gel analysis were boiled for 5 min immediately after lysis. Protein concentrations were determined by spotting aliquots of the protein extracts onto a nitrocellulose filter and staining the filter with amido black (56). 1D gel analysis was performed in SDS–12.5% polyacrylamide gels; 2D gel electrophoresis was accomplished by the method of O'Farrell (49). For the first-dimension isoelectric focusing, the pH gradient was produced with a mixture of ampholines (LKB) of pH 3.5 to 10, pH 4 to 6, pH 6 to 8, and pH 9 to 11 at the ratio of 10:1:1:1. For the second dimension, SDS–12.5% polyacrylamide gels were used. Silver staining of gels was performed according to Morrissey (47). For Western blot analysis, proteins were transferred electrophoretically onto nitrocellulose membranes. The protein blots were incubated overnight either with an affinity-purified anti-αB-crystallin monospecific antibody or a mixture of anti-hsp72, anti-hsp25, and anti-αB-crystallin antisera at room temperature. The secondary antibody was anti-rabbit immunoglobulin G coupled to either alkaline phosphatase (Promega) or horseradish peroxidase (Amersham). The immunocomplexes were visualized by either enzymatic staining or chemiluminescence, respectively. Mouse α-crystallin and human αB-crystallin were purified from mouse and human eye lenses, respectively. Recombinant mouse hsp25 was produced in *Escherichia coli* transformed with the expression vector pAKHSP25 (17) and purified on SDS-polyacrylamide gels. The αB-crystallin- and hsp25-specific antisera were raised in rabbits, using purified human αB-crystallin and mouse hsp25, respectively, as the antigens. The αB-crystallin and hsp25 antisera were affinity purified as described by Iwaki et al. (24).

RESULTS

Dexamethasone-induced accumulation of αB-crystallin and its suppression by oncoproteins. Previously we found that high amounts of αB-crystallin rapidly accumulate in the NIH 3T3-derived cell lines HLE (NIH[LTR-*ras*]) and HLM (NIH[LTR-*mos*]) in response to the expression of the Ha-*ras* and v-*mos* oncogenes (32, 34). These cell lines were constructed by introducing c-Ha-*ras* (EJ) and v-*mos* oncogenes, respectively, controlled by the glucocorticoid hormone-inducible promoter of MMTV (28). Treatment of these cells with the synthetic hormone dexamethasone resulted in on-

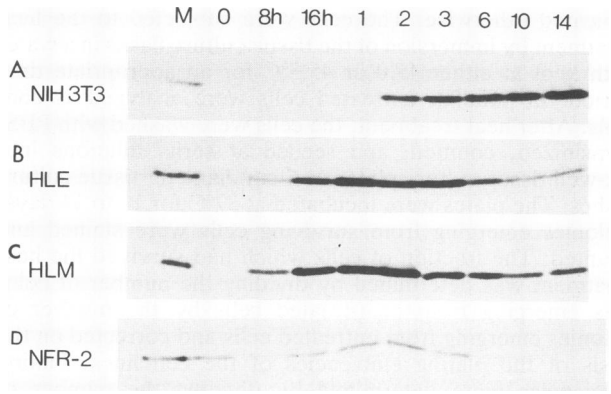

FIG. 1. Differential effects of dexamethasone treatment on αB-crystallin expression in untransformed and *ras*- or *mos*-transformed NIH 3T3 cells. The parental NIH 3T3 cells as well as NIH 3T3 cells transfected with the MMTV LTR–Ha-*ras* (EJ), MMTV LTR–v-*mos*, and pcH*ras* (A)–Ha-*ras* (EJ) constructs HLE, HLM, and NFR-2, respectively, were grown with 1 μM dexamethasone for the time periods (in days, unless specified otherwise) indicated above the lanes. Thirty micrograms of total cellular protein extracts was separated on SDS-polyacrylamide gels and subjected to Western blot analysis using an αB-crystallin monospecific antibody. Lane M, mouse eye lens α-crystallin.

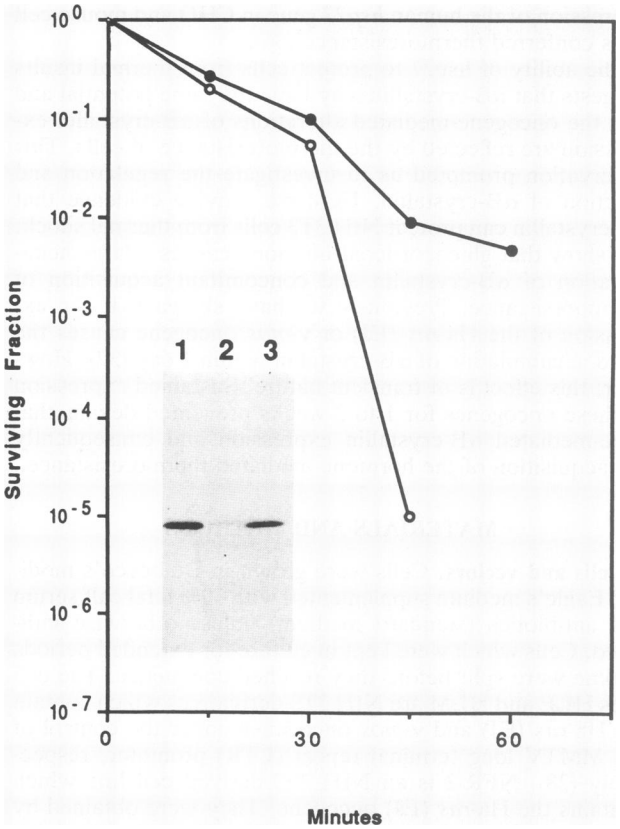

FIG. 2. Acquisition of thermoresistance mediated by expression of αB-crystallin. NIH 3T3 cells (○) and NIH 3T3 cells transfected with 40 μg of pSVcry6 (●) were heat treated at 45.3°C for the indicated time periods, and the fraction of surviving cells was determined. (Insert) Thirty micrograms of total cellular protein extracts was subjected to Western blot analysis using an anti-αB-crystallin monospecific antibody. Lanes: 1, mouse eye lens α-crystallin; 2, NIH 3T3 cells; 3, NIH 3T3 cells transfected with pSVcry6.

cogene expression and the subsequent accumulation of αB-crystallin. However, it remained unknown whether αB-crystallin accumulation is a transformation-associated property. Since the morphological transformation of HLE and HLM cells requires 3 to 5 days of dexamethasone treatment, we examined whether αB-crystallin expression persists during long-term exposure to dexamethasone in these cells. αB-crystallin was detected in HLE and HLM cells 8 h after dexamethasone addition and reached the maximal level after 1 day (Fig. 1B and C). During the same period, dexamethasone did not cause the accumulation of substantial amounts of αB-crystallin in untransformed NIH 3T3 cells (Fig. 1A). However, we found that αB-crystallin accumulated in untransformed NIH 3T3 cells to large amounts (up to 1% of the total cellular proteins) upon prolonged dexamethasone treatment, while it disappeared slowly in HLE and HLM cells during the same period (Fig. 1A to C). These results demonstrate that the accumulation of αB-crystallin in untransformed NIH 3T3 cells is glucocorticoid responsive. The delayed accumulation of αB-crystallin in untransformed NIH 3T3 cells might result from a delayed transcriptional induction or from hormone-mediated posttranscriptional effects such as increased mRNA and protein stability. The effects of the oncoproteins on αB-crystallin expression are twofold. First, they cause αB-crystallin accumulation in a rapid but transient manner. Second, they suppress dexamethasone-mediated delayed accumulation of αB-crystallin. To study the effect of sustained oncogene expression on hormone-dependent and -independent αB-crystallin accumulation, we established NIH 3T3 cell lines which constitutively express the Ha-*ras* (EJ) oncogene. The analysis of one representative cell clone, NFR-2, indicates that *ras* transformation per se did not cause the constitutive expression of high amounts of αB-crystallin (Fig. 1D). Moreover, dexamethasone treatment of NFR-2 cells did not result in the accumulation of αB-crystallin (Fig. 1D). The same observation was made with other *ras*-transformed mouse and rat fibroblast cell lines (data not shown). These results indicate that the suppression of the dexamethasone-dependent αB-crystallin accumulation is a transformation-specific event associated with the sustained expression of the activated *ras* oncogene in NIH 3T3 fibroblasts.

Acquisition of thermoresistance by αB-crystallin. We have previously shown that αB-crystallin is a small hsp (33). This finding prompted us to investigate whether αB-crystallin can protect cells from a thermal shock. An αB-crystallin expression plasmid, pSVcry6, was constructed by cloning αB-crystallin cDNA behind the simian virus 40 promoter in the vector pSVK3 and was introduced into NIH 3T3 cells by transient calcium phosphate-DNA transfection. Two days after transfection, the cells were subjected to the lethal temperature of 45.3°C for various time periods. Immediately following the heat treatment, cells were trypsinized, replated at various dilutions, and incubated at 37°C for 10 to 14 days. After that time, the colonies emerging from the surviving cells could be counted. The fraction of NIH 3T3 cells surviving the lethal heat shock dropped rapidly during heat treatment (Fig. 2). However, the transfected cells expressing αB-crystallin were relatively resistant to a thermal shock (Fig. 2 and inset). To test whether the extent of thermal protection correlates with the amount of accumulated αB-crystallin, we transfected NIH 3T3 cells with increasing concentrations of pSVcry6. The ability of the transfected

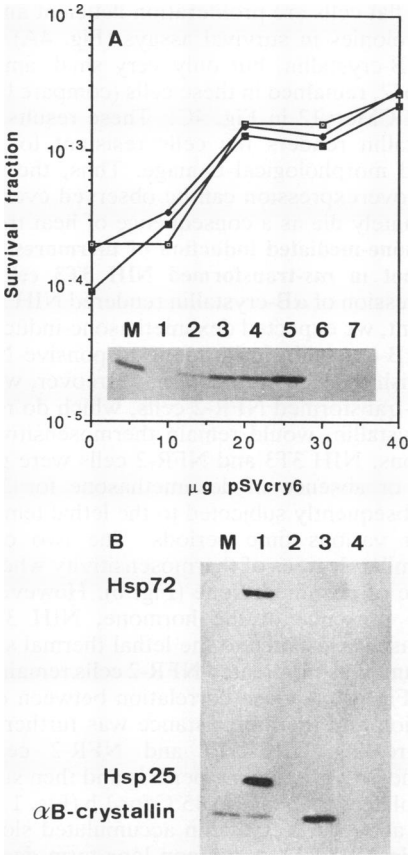

FIG. 3. Association of αB-crystallin expression and thermal protection. (A) NIH 3T3 cells were cotransfected with the indicated amounts of pSVcry6 DNA and 10 μg of β-galactosidase expression vector. DNA of the empty vector pSVK3 was added to a constant plasmid DNA amount of 50 μg per transfection. The transfected cells were divided into three sets and treated independently at 45°C for 1 h. The fractions of surviving cells from these three sets of experiments are shown. Transfection efficiencies were quantitated by measuring β-galactosidase activity. Cells transfected with 0, 10, 20, 30, and 40 μg of pSVcry6 contained 1.5, 1.4, 2.2, 1.4, and 2.4 U of β-galactosidase, respectively. Average survival fractions for the mock-transfected and untransfected cells from three experiments were 2.8×10^{-4} and 0.7×10^{-4}, respectively. (Insert) Forty micrograms of proteins from transfected and untransfected cells was subjected to Western blot analysis using an anti-αB-crystallin monospecific antibody. Lanes: M, mouse eye lens α-crystallin; 1 to 5, cells transfected with 0, 10, 20, 30, and 40 μg of pSVcry6 DNA, respectively; 6, mock-transfected cells; 7, untransfected cells. (B) Forty micrograms of total protein extracts was subjected to Western blot analysis using a mixture of anti-hsp72, anti-hsp25, and anti-αB-crystallin antisera. Lanes: M, mouse eye lens α-crystallin; 1, heat-shocked NIH 3T3 cells (the proteins were extracted 10 h after heat treatment at 42.5°C for 1 h); 2, cells transfected with 40 μg of pSVK3; 3, cells transfected with 40 μg of pSVcry6; 4, untransfected cells. The intensities of the signals of the three hsps are not a measure of their relative abundance, as the antisera recognize their antigens with greatly different efficiencies.

cells to survive on lethal heat shock at 45°C for 1 h did indeed correlate with the amounts of transfected pSVcry6 and accumulated αB-crystallin (Fig. 3A). Neither introduction of the empty vector nor the transfection procedure per se resulted in heat protection (see the legend to Fig. 3A). A general stress response may be triggered by introduction of foreign or denatured proteins into cells (2). Although pSVcry6 does not encode a foreign protein but encodes the normal mouse αB-crystallin, the forced overexpression of this protein could result in the formation of unusual macromolecular aggregates with aberrant protein structures. Such abnormal protein structures could induce the stress response and subsequently render the cells thermotolerant. Therefore, we investigated whether αB-crystallin overexpression causes the synthesis of other hsps. Western blot analysis using anti-hsp25 and anti-hsp72 antisera showed that neither hsp25 (the mouse homolog of human hsp27) nor hsp72 (a member of the heat-inducible hsp70 family in the mouse) accumulated in the cells transfected with the αB-crystallin expression vector (Fig. 3B). These results support the notion that αB-crystallin by itself rendered NIH 3T3 cells thermoresistant.

Augmented thermotolerance in a cell line which superinduces αB-crystallin. Attempts to isolate NIH 3T3 cell clones which constitutively overexpress αB-crystallin after transfection with pSVcry6 have failed. The expression plasmid pCMcry6, in which the CMV promoter should direct constitutive αB-crystallin expression, was constructed and stably transfected into NIH 3T3 cells. No constitutively overexpressing clones were obtained. However, we have isolated cell clones of pCMcry6-transfected NIH 3T3 cells which accumulate large quantities of αB-crystallin (several percent of the total cellular proteins) in response to a sublethal heat shock. We have not investigated the mechanism of heat-induced expression of the CMV promoter-driven αB-crystallin cDNA. Since the primary αB-crystallin transcript from this expression vector is without an intron, we speculate that it is properly processed only under stress conditions. We examined whether the superinduction of αB-crystallin in one of these cell lines (CMcry12) increases the extent and duration of thermotolerance. CMcry12 and NIH 3T3 cells were preheated at the severe but sublethal temperature of 44°C for 1 h and were subsequently maintained at 37°C. The cells were then subjected to the lethal temperature of 45°C for 1 h at various times after the preheat treatment. Both cell lines developed thermotolerance with similar kinetics. However, CMcry12 cells became more thermotolerant than NIH 3T3 cells (Fig. 4A). Moreover, the thermotolerant state of CMcry12 cells persisted for a longer period than did that of NIH 3T3 cells. The amount of accumulated αB-crystallin was much higher and longer lasting in CMcry12 cells, as shown by Western blot analysis (Fig. 4C). In contrast, the induced levels of hsp25 and hsp72 were similar in NIH 3T3 and CMcry12 cells (Fig. 4C). The two cell lines had similar growth curves, with a lag period immediately following the preheat treatment (Fig. 4B). Thus, the accumulation of an extraordinarily large amount of αB-crystallin apparently did not affect the growth properties of the cells. A similarly augmented thermotolerance in CMcry12 cells was observed when the cells were preheated at 43°C for 1 h (data not shown). These results indicate that the extent of thermotolerance is dependent on the amount of αB-crystallin present in cells.

Morphological protection from thermal shock in the αB-crystallin-superinducing cell line. Alteration of cell morphology is another parameter by which the effects of heat shock can be examined. NIH 3T3 and CMcry12 cells have a similar flat morphology under normal growth conditions (Fig. 5A). Both cells became rounded immediately after the lethal heat treatment at 45°C for 1 h (Fig. 5B, 0h). Practically all of these cells died. They either detached from the tissue culture flask or remained attached but lost most of the cytoplasmic

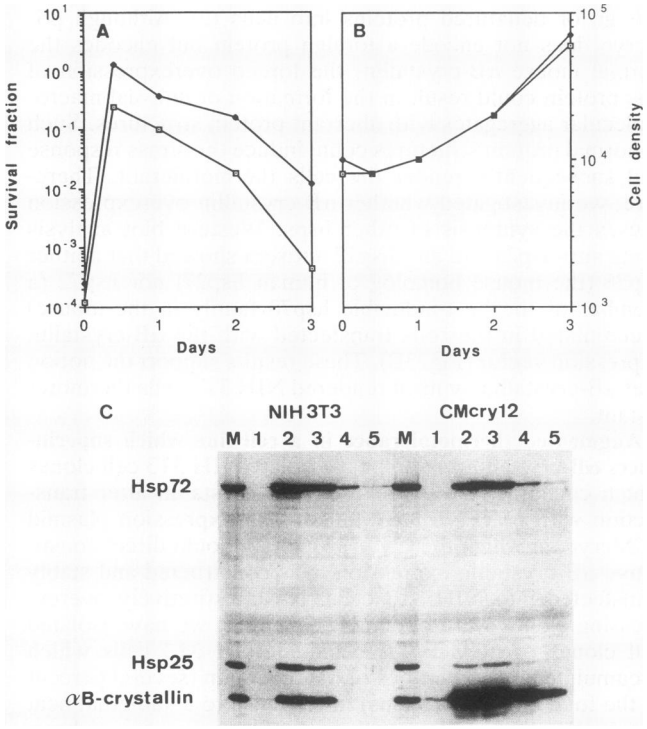

FIG. 4. Augmented thermotolerance in αB-crystallin-superinducing cells. (A) NIH 3T3 (⊡) and CMcry12 (■) cells were plated at a density of 0.5×10^4 cells per cm^2 in 25-cm^2 tissue culture flasks and grown at 37°C for 24 h. The cells were then heated at 44°C for 1 h and subjected to lethal heat treatment at 45°C for 1 h after the indicated time periods. (B) The cell densities (cell number per square centimeter) were determined immediately before the lethal heat treatment. The zero time point was taken immediately after the preheat treatment at 44°C. ⊡, NIH 3T3 cells; ■, CMcry12 cells. (C) Forty micrograms of proteins from cells extracted at 0 h (lane 1), 10 h (lane 2), 1 day (lane 3), 2 days (lane 4), and 3 days (lane 5) after preheat treatment was subjected to Western blot analysis using a mixture of anti-hsp72, anti-hsp25, and anti-αB-crystallin antisera. Lane M, a mixture of mouse eye lens α-crystallin and the protein extracts from heat-shocked (42.5°C) NIH 3T3 cells.

contents within 1 day (Fig. 5B, 1d). To examine the effects of thermotolerance on cell morphology, the cells were subjected to the lethal heat shock, 1 day after the preheat treatment at a sublethal temperature, as described above. NIH 3T3 cells became rounded immediately after the lethal heat shock, as did the cells which were not preheated at the sublethal temperature (Fig. 5C, 0h). However, a substantial portion of the cells regained a flat morphology after 1 day (Fig. 5C, 1d). In contrast, a large portion of the preheated CMcry12 cells remained flat even immediately after the lethal heat shock (Fig. 5C, 0h) and exhibited the original flat morphology after 1 day (Fig. 5C, 1d). When the cells were subjected to the lethal heat shock, 3 days after the preheat treatment, NIH 3T3 cells became rounded immediately after the lethal heat shock (Fig. 5D, 0h). Most of these cells died and detached from the tissue culture flask within 1 day (Fig. 5D, 1d). The preheated CMcry12 cells also became rounded immediately after the lethal heat shock (Fig. 5D, 0h). However, a large portion of the cells regained a flat morphology after 1 day (Fig. 5D, 1d). Thus, the preheated CMcry12 cells are highly resistant to the heat shock-induced morphological changes even 3 days after the preheat treatment. However, most of these flat cells are proliferation deficient and do not give rise to colonies in survival assays (Fig. 4A). A large quantity of αB-crystallin, but only very small amounts of hsp25 and hsp72, remained in these cells (compare lanes 5 of NIH 3T3 and CMcry12 in Fig. 4C). These results indicate that αB-crystallin renders the cells resistant to the heat shock-induced morphological damage. Thus, the effect of αB-crystallin overexpression can be observed even in cells that will ultimately die as a consequence of heat treatment.

Dexamethasone-mediated induction of thermoresistance in normal but not in *ras*-transformed NIH 3T3 cells. Since transient expression of αB-crystallin rendered NIH 3T3 cells thermoresistant, we expected dexamethasone-induced accumulation of αB-crystallin in hormone-responsive NIH 3T3 cells to establish the same protection. Moreover, we speculated that *ras*-transformed NFR-2 cells, which do not accumulate αB-crystallin, would remain thermosensitive under these conditions. NIH 3T3 and NFR-2 cells were grown in the presence or absence of dexamethasone for 2 weeks. They were subsequently subjected to the lethal temperature of 45.3°C for various time periods. The two cell lines manifested similar degrees of thermosensitivity when grown in the absence of dexamethasone (Fig. 6). However, when grown in the presence of the hormone, NIH 3T3 cells showed increased resistance to the lethal thermal shock. In contrast, dexamethasone-treated NFR-2 cells remained thermosensitive (Fig. 6). A close correlation between αB-crystallin expression and thermoresistance was further demonstrated by treating NIH 3T3 and NFR-2 cells with dexamethasone for various time periods and then subjecting them to a lethal heat challenge at 45°C for 1 h (Fig. 1 and 7A). As discussed above, αB-crystallin accumulated slowly and continuously in NIH 3T3 cells upon long-term dexamethasone treatment (Fig. 1 and 7A). The amount of αB-crystallin in NFR-2 cells stayed low during the hormone treatment (Fig. 7A). The kinetics of the acquisition of thermoresistance paralleled that of αB-crystallin expression in NIH 3T3 cells (Fig. 7A). No hormone-mediated establishment of thermoresistance was observed in NFR-2 cells (Fig. 7A). This result is consistent with the inability of NFR-2 cells to accumulate αB-crystallin in response to dexamethasone treatment. We could rule out the possibility that thermoresistance resulted from a hormone-mediated general stress response by demonstrating the absence of hsp25 and hsp72 accumulation in long-term dexamethasone-treated cells (Fig. 7B).

Dexamethasone-mediated morphological change of NIH 3T3 cells. The time course of the delayed accumulation of αB-crystallin in dexamethasone-treated NIH 3T3 cells and the concomitant development of thermoresistance paralleled those of morphological changes of the cells. Formation of patches of flat cells became apparent 2 days after dexamethasone treatment and persisted thereafter (Fig. 8A). The effects of the long-term exposure to dexamethasone on heat shock-mediated morphological alterations were examined. NIH 3T3 cells became rounded immediately after the lethal heat shock and detached rapidly from the tissue culture flask (Fig. 8B). In contrast, the dexamethasone-treated cells retained a flat morphology immediately after the heat shock and resisted the heat shock-induced morphological deformation for prolonged time periods (Fig. 8C). These results further support the notion that αB-crystallin renders the cells resistant to the heat shock-induced morphological damages.

αB-crystallin is the only hsp which is induced by dexamethasone or whose synthesis is augmented in CMcry12 cells. So far, we have excluded the possibility that dexamethasone treatment causes a general stress response in NIH 3T3 cells.

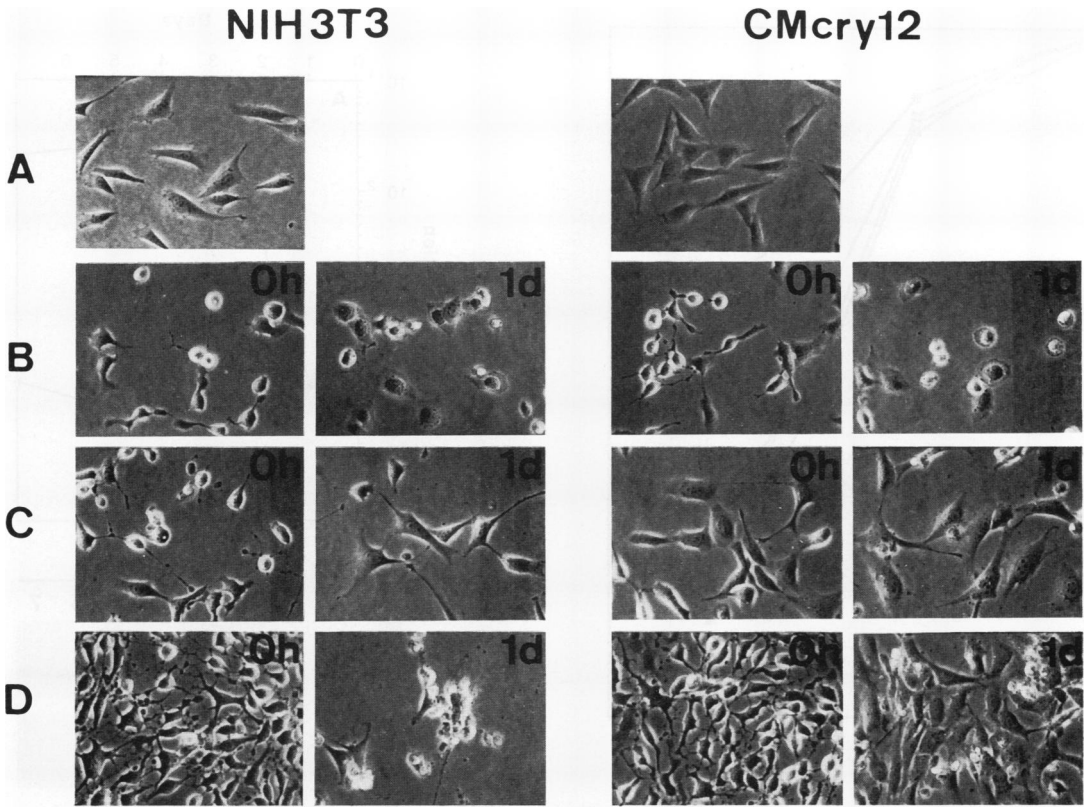

FIG. 5. Thermotolerance as judged by cell morphology. NIH 3T3 and CMcry12 cells were grown, preheated, and subjected to a lethal heat shock as described in the legend to Fig. 4. Photographs were taken immediately (0h) and 1 day (1d) after the lethal heat shock. (A) Untreated cells; (B) heat-shocked cells without preheat treatment; (C and D) heat-shocked cells 1 day (C) and 3 days (D) after preheat treatment.

The possibility remains that hsps other than hsp25 and hsp72 accumulate in response to dexamethasone. Likewise, hsps other than hsp25 and hsp72 might be superinduced in heat-stressed CMcry12 cells. We compared the protein patterns of long-term dexamethasone-treated, heat-shocked, and untreated NIH 3T3 cells as well as heat-shocked and untreated CMcry12 cells by 2D gel electrophoresis (Fig. 9). To identify hsps, NIH 3T3 cells were either heated at the sublethal temperature of 42.5°C for 1 h or left untreated, and proteins were subsequently labeled with [^{35}S]methionine for 1 h at 37°C (data not shown). Protein extracts were subjected to 2D gel electrophoresis, and fluorograms were prepared. Comparison of the patterns of labeled proteins from heated and unheated cells led to the identification of hsps with molecular masses of around 110, 100, 90, 70 (hsp72), and 20 (αB-crystallin) kDa. αB-crystallin was identified by 2D Western blot analyses (Fig. 9A to C, lower panels). The abundant hsps accumulated after a heat shock to amounts which are sufficient for their visualization on silver-stained gels (Fig. 9A and B, upper panels). αB-crystallin was the only hsp which accumulated in dexamethasone-treated cells (Fig. 9C, upper panel). The 2D gel patterns of the protein extracts from NIH 3T3 (Fig. 9A, upper panel) and CMcry12 (Fig. 9D) cells are essentially identical. At the sublethal temperature of 44°C, αB-crystallin was induced to extraordinarily high amounts in CMcry12 cells (Fig. 9E), whereas the other hsps accumulated to similar levels in the two cell lines (Fig. 9E and F). Thus, αB-crystallin is the only hsp whose synthesis is superinduced upon heat shock in CMcry12 cells.

DISCUSSION

We have demonstrated in this report that one of the two known murine small hsps, αB-crystallin, can render NIH 3T3 cells thermoresistant. αB-crystallin accumulates in response to dexamethasone. Expression of the *ras* oncogene abrogates dexamethasone-mediated αB-crystallin accumulation and the establishment of thermoresistance.

Dexamethasone responsiveness of αB-crystallin. Hormone responsiveness is a common characteristics of small *HSP* genes. In *Drosophila melanogaster*, the expression of the small hsps was shown to be subject to regulation by the molting hormone ecdysone (21, 22). The human hsp27 gene is estrogen responsive and is expressed in several estrogen-sensitive human tissues and breast tumors (10, 16, 59, 60). Here we have shown that murine αB-crystallin accumulates in response to the glucocorticoid hormone dexamethasone. Preliminary experiments indicate that dexamethasone leads to the transcriptional induction of the αB-crystallin gene. There are two glucocorticoid-responsive element-like sequences within the known promoter region of the αB-crystallin gene. Footprint analyses have revealed that one of these glucocorticoid-responsive elements can indeed bind glucocorticoid receptor in vitro (unpublished results).

αB-crystallin-mediated thermal protection. Three different manipulations resulted in the accumulation of αB-crystallin in NIH 3T3 cells: sublethal heat treatment, dexamethasone treatment, and transfection with αB-crystallin expression vectors. Among them, only sublethal heat treatment led to

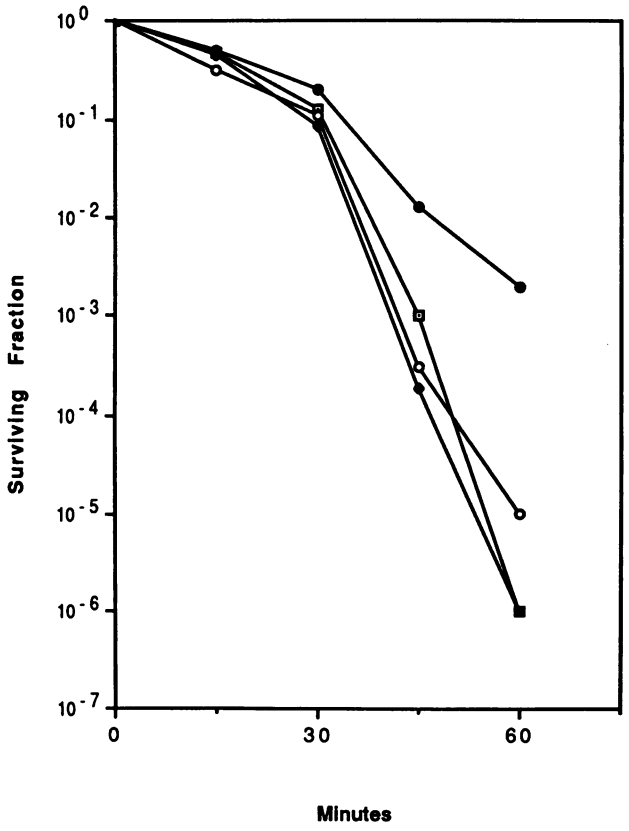

FIG. 6. Differential potential of dexamethasone to establish thermoresistance in NIH 3T3 and NFR-2 cells. NIH 3T3 and NFR-2 cells were grown with or without 1 μM dexamethasone for 2 weeks and were subjected to a heat treatment at 45.3°C for the indicated periods. ○, NIH 3T3 cells; ●, dexamethasone-treated NIH 3T3 cells; ⊡, NFR-2 cells; ■, dexamethasone-treated NFR-2 cells.

the induction of other known hsps in addition to αB-crystallin. In all cases, we observed the establishment of thermal protection. The extent of thermal protection always closely paralleled that of accumulated αB-crystallin. This is particularly evident in NFR-2 cells, in which the *ras* oncoprotein abrogates hormone-mediated αB-crystallin accumulation and the establishment of thermal protection. These results strongly support the notion that αB-crystallin can induce the thermoresistant state under conditions when other *HSP* genes are not induced. Phosphorylated and unphosphorylated forms of αB-crystallin are known. The αB-crystallin expressed in the experiments described in this report is not phosphorylated. It seems that phosphorylation is not required for the thermoprotective activity of αB-crystallin in the analyzed cell lines. Involvement of other hsps has been implicated in thermotolerance of mammalian cells. The forced expression of either human hsp27 or hsp70 alone rendered Rat-1 fibroblasts or CHO and mouse cells, respectively, thermoresistant (36, 38). Apparently, several hsps can develop the thermoresistant state to a certain extent on their own if drastically overexpressed. Under physiological conditions, they are required in lower amounts, indicating that their concerted action potentiates the extent of thermotolerance. An example of the collaboration of different hsps has indeed recently been demonstrated in *E. coli* (37).

Previously, short-term glucocorticoid hormone treatment

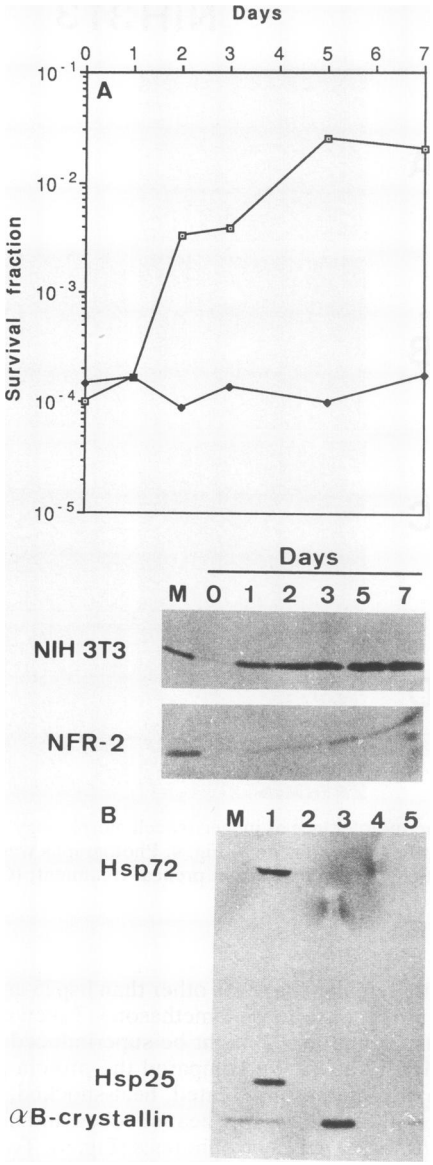

FIG. 7. Close correlation between αB-crystallin expression and thermoresistance during long-term dexamethasone treatment of cells. (A) NIH 3T3 (⊡) and NFR-2 (■) cells were grown with 1 μM dexamethasone at 37°C for the indicated periods and subjected to a heat treatment at 45°C for 1 h. Forty micrograms of proteins from cells treated with dexamethasone for the indicated periods was subjected to Western blot analysis using an anti-αB-crystallin monospecific antibody. M, mouse eye lens α-crystallin. (B) Forty micrograms of proteins was subjected to Western blot analysis using a mixture of anti-hsp72, anti-hsp25, and anti-αB-crystallin antisera. Lanes: M, mouse eye lens α-crystallin; 1, heat-shocked (42.5°C) NIH 3T3 cells; 2, untreated NIH 3T3 cells; 3, 1-week dexamethasone-treated NIH 3T3 cells; 4, untreated NFR-2 cells; 5, 1-week dexamethasone-treated NFR-2 cells.

was found to cause the development of partial thermoresistance in Chinese hamster cells despite the lack of apparent *hsp* gene induction (15). This observation was regarded as evidence for Hsp-independent acquisition of thermoresistance. In view of our present results, dexamethasone-mediated accumulation of αB-crystallin may have caused thermal protection of Chinese hamster cells.

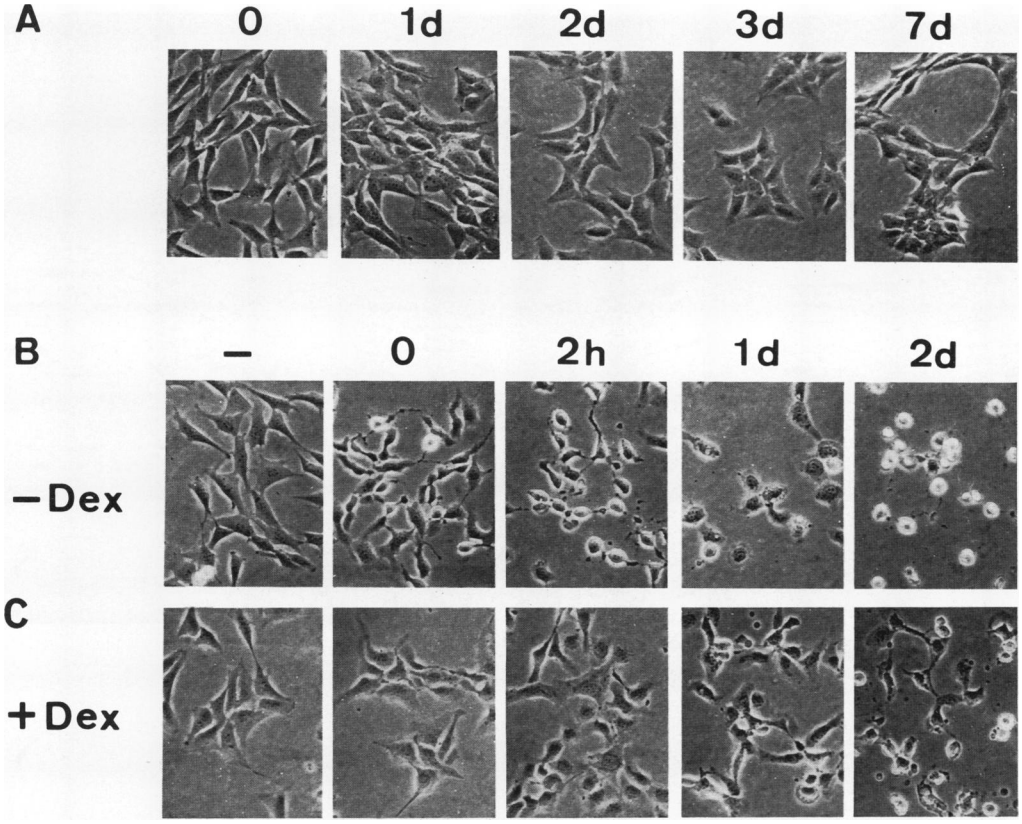

FIG. 8. Dexamethasone-mediated morphological changes in NIH 3T3 cells. (A) NIH 3T3 cells were grown with 1 μM dexamethasone for the indicated periods in days. (B and C) NIH 3T3 cells were grown with (C) or without (B) 1 μM dexamethasone (Dex) for 1 week. Photographs were taken before (–) or at the indicated times after a lethal heat shock at 45°C for 1 h.

Expression of αB-crystallin in normal and diseased tissues. To investigate the nonlenticular function of αB-crystallin, several groups have examined the expression patterns of αB-crystallin in adult organisms as well as during development. In adults, αB-crystallin is expressed in many tissues, including eye lens, heart muscle, skeletal muscle, kidney, lung, brain, spermatocyte, and placenta (5, 8, 12, 23–25, 31, 40). The expression patterns of αB-crystallin in many of these tissues seem to coincide with those of oxidative enzymes (23). αB-crystallin has been shown to be localized in the Z bands of slow muscle (5) and heart muscle (41). Disuse atrophy of the slow soleus muscle of rats leads to a specific loss of αB-crystallin (5). This response is accompanied by the disappearance of myofibrillar proteins such as slow myosin, troponins I and C, and tropomyosin. Moreover, it was shown that the amount of αB-crystallin mRNA in muscles was regulated by mechanical tension and denervation (4). These results suggest that αB-crystallin is a myofibril-stabilizing protein which may regulate myofibril structures depending on nerve innervation and mechanical stimuli in muscles (4, 5). During development, αB-crystallin seems to be associated with major morphological changes of tissues. In rat kidneys, αB-crystallin expression is closely associated with the prominent elongation of Henle's loop during the first 10 days of development and is temporally correlated with the acquisition of tubule function in early postnatal life (25). Scotting et al. (58) demonstrated by immunohistochemical analyses that αB-crystallin and ubiquitin-protein conjugates are coexpressed in those regions of early chicken embryos which undergo extensive morphological reorganization. They proposed that ubiquitin and αB-crystallin may have a coordinate role in the extensive architectural remodeling occurring in the developing tissues. Elevated levels of αB-crystallin mRNA or protein are associated with various pathological conditions in the brain and other tissues. These conditions include degenerative brain diseases such as scrapie infection (13). Alexander's disease, a rare inheritable degenerative brain disease of children (24), diffuse Lewy body disease, one of the major human neurodegenerative dementing diseases (43, 44), glial tumors (27), tuberous sclerosis, an autosomal dominant disease characterized by the growth of benign tumors (hamartomas) and malformations (hamartias) in the skin, central nervous system, heart, kidney, and other organs (26), and Werner syndrome, a rare inherited disorder involving premature aging (48). In degenerative brain diseases, αB-crystallin was found associated with ubiquitin-protein deposits (24, 43, 44).

Function of αB-crystallin. The model that αB-crystallin is a molecular chaperone-like protein seems to fit our results best. It is known that αB-crystallin multimerizes to form a large complex (33, 67) which can associate with architectural proteins such as myofibrils, actin, and desmin filaments (4–6, 41). Large protein complex formation is a characteristic of some of the molecular chaperons such as the prokaryotic chaperonin GroEL, eukaryotic cytoplasmic chaperonin, and mitochondrial chaperonin hsp60 (14). Likely targets for a molecular chaperone-like ability of αB-crystallin are components of the structures which determine the cellular architecture. It is notable that factors which are shown here to cause transient or permanent accumulation of large quanti-

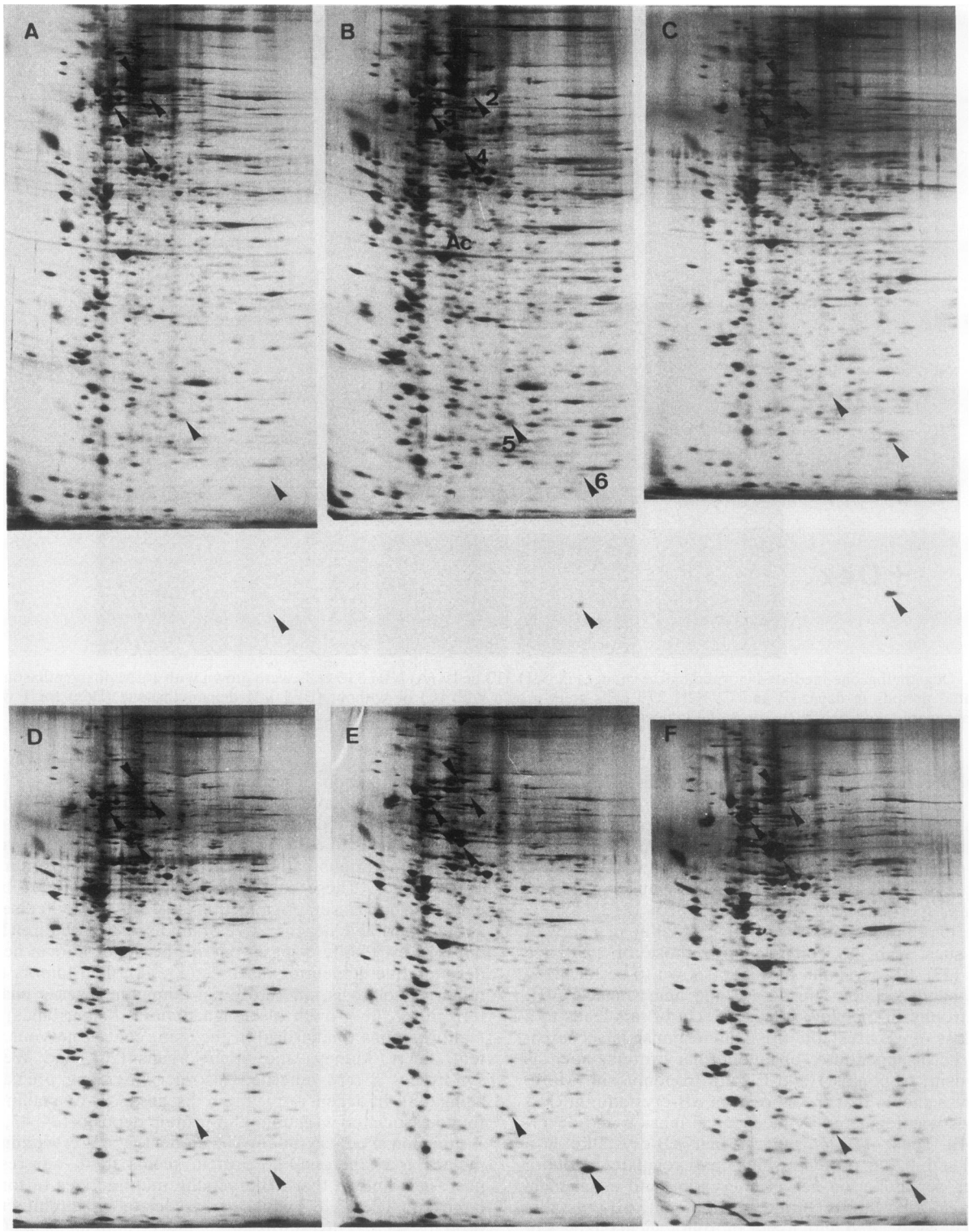

FIG. 9. Specific accumulation of αB-crystallin in dexamethasone-treated NIH 3T3 cells or heat-shocked CMcry12 cells. Fifty micrograms of proteins from lysates of NIH 3T3 or CMcry12 cells was analyzed by isoelectric focusing 2D gel electrophoresis. Proteins were visualized by silver staining. For Western blotting, the lower part of a 2D gel run in parallel was blotted onto a nitrocellulose filter. αB-crystallin was detected by anti-αB-crystallin monospecific antibody (A to C, lower panels). (A) Untreated NIH 3T3 cells. (B) Heat-shocked NIH 3T3 cells. Cells were incubated at 42.5°C for 1 h and collected 10 h thereafter. (C) Dexamethasone-treated NIH 3T3 cells (1 week). (D) Untreated CMcry12 cells. (E) Heat-shocked CMcry12 cells. Cells were incubated at 44°C for 1 h and collected 10 h thereafter. (F) Heat-shocked NIH 3T3 cells. Cells were incubated at 44°C for 1 h and collected 10 h thereafter. Molecular weights of proteins were determined by comparing mobilities of prestained marker proteins and ^{35}S-labeled proteins from heated and unheated cells in a 1D SDS-polyacrylamide gel. Arrows indicate hsps with apparent molecular masses of 110 (1), 100 (2), 90 (3), 70 (hsp72) (4), 25 (hsp25) (5), and 20 (αB-crystallin) (6) kDa. The position of actin (Ac) is also indicated.

ties of αB-crystallin, such as a severe but sublethal heat shock, oncogenic transformation, and dexamethasone treatment, also cause major morphological changes. We would like to postulate that αB-crystallin either directly interacts with structural proteins or recognizes specific structural features of proteins which are created during reorganization of the cellular architecture. Such structural features may be produced by denaturation, proteolysis, or ubiquitination. Binding of αB-crystallin to such proteins may protect, facilitate renaturation of, or promote degradation of proteins or architectural networks. We would further like to postulate that such a function of αB-crystallin is relatively nonspecific with respect to the cellular architecture affected. Thus, αB-crystallin may participate in such diverse biological processes as differentiation, stress response, degenerative diseases, neoplastic transformation, and aging. In the cells of eye lens, brain, kidney, and muscle, αB-crystallin may be required for the differentiation-associated morphological changes and later for maintenance of the cellular architecture. In addition, in view of our present results, the existence of αB-crystallin may help these cells to resist various kinds of stress such as the constant exposure to light in eye lenses, mechanical constraints in muscles, and osmotic stresses in kidneys. In fact, quite recently, Horwitz and coworkers have demonstrated that α-crystallin can protect various proteins, including eye lens proteins, from thermal denaturation in vitro (19, 20). It is quite likely that such a molecular chaperone-like activity of α-crystallin operates in vivo.

Effects of oncoproteins on αB-crystallin expression and their biological consequences. We have shown that the suppression of dexamethasone-mediated accumulation of αB-crystallin is a *ras* transformation-associated characteristic of NIH 3T3 cells. The mechanism by which *ras* transformation suppresses the dexamethasone-mediated accumulation of αB-crystallin is unknown. However, mutual inactivation of transcription factors such as AP-1 and glucocorticoid receptor through direct interactions has been shown elsewhere (30, 57, 61, 68). It is possible that elevation of AP-1 activity (augmented c-*fos* gene expression or activation of c-*jun* protein through phosphorylation [for a review, see reference 3]) in *ras*-transformed cells suppresses glucocorticoid receptor activity and thus the accumulation of αB-crystallin. Alternatively, *ras* transformation might affect posttranscriptional regulatory mechanisms such as mRNA or protein stability. Since αB-crystallin is induced by various kinds of stress (33), its accumulation may protect cells not only from thermal shock but also from other stresses. Thus, *ras*-transformed fibroblasts may be impaired in their ability to establish tolerance toward various forms of stress in response to glucocorticoid hormone treatment. Further investigations should evaluate whether this phenomenon can be clinically exploited. It remains to be analyzed whether αB-crystallin accumulation occurs in vivo, for instance in tissues of cancer patients treated by hyperthermia or glucocorticoids. The cellular response to these treatments and likewise the success of the therapeutic intervention might depend on the relative amounts of αB-crystallin in cancerous and noncancerous tissues.

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