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Sense and Antisense Modification of Glial αB-Crystallin Production Results in Alterations of Stress Fiber Formation and Thermoresistance

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Abstract. The phenotypic effects of selectively altering the levels of αB-crystallin in cultured glial cells were analyzed using sense and antisense approaches. Rat C6 glioma cells and human U-373MG glioma cells were transfected with a rat αB-crystallin sense cDNA or an antisense cDNA regulated by a Rous sarcoma virus promoter to alter cellular levels of αB-crystallin. The antisense strategy resulted in decreased αB-crystallin levels, as revealed by Western blot and immunocytochemical analyses. The reduced αB-crystallin expression was accompanied by alterations in cellular phenotype: (*a*) a reduction of cell size and/or a slender cell morphology; (*b*) a disorganized microfilament network; and (*c*) a reduction of cell adhesiveness. Like HSP27, the presence of additional αB-crystallin protein confers a thermoresistant phenotype to stable transfectants. Thus, αB-crystallin in glioma cells plays a role in their thermal resistance and may contribute to the stability of cytoskeletal organization.

α-CRYSTALLIN is a major component of the vertebrate eye lens and consists of two genes, αA and αB. It has become clear in recent years that both subunits of α-crystallin are present in nonlenticular tissues. αB-crystallin is found in such extraocular tissues as heart, skeletal muscle, skin, brain and kidney, and in cultured rat astrocytes and neoplastic astrocytes (6, 15, 25, 27, 28, 34). αA-crystallin is also present in nonlens tissues, especially in spleen and thymus (33). Although α-crystallin under physiologic conditions is always isolated from lens as heterogeneous high molecular weight aggregates of αA-crystallin and αB-crystallin, the distribution of these two proteins in nonlenticular tissue is different.

Not only do the α-crystallins structurally resemble a class of small MW heat shock proteins (42, 50, 51) and αB-crystallin forms a complex with HSP27, one of the small heat shock proteins, in adenovirus-transformed rat kidney cells (52) and in human skeletal muscle (32), but also recent evidence indicates that αB-crystallin is itself a heat shock, or "stress" protein. Thus, αB-crystallin mRNA is increased in NIH 3T3 mouse fibroblasts (35), in cultured glioma cells (26), in normal cultured astrocytes (20), and in lens, kidney, and retinal epithelial cells (13, 39) in response to such diverse stimuli as heat shock, heavy metal exposure, hypoxia, hypertonicity, and hypoglycemia. αB-crystallin accumulates in reactive and neoplastic glial cells and in some neurons in a variety of pathologic situations (25, 29, 30) presumably reflecting some as yet unidentified stresses. In a remarkable example of stress protein expression, both αB-crystallin and hsp27 accumulate in massive amounts in the brains of patients with Alexander disease and is a major component of Rosenthal fibers, intracytoplasmic inclusions of reactive astrocytes (28). In addition, αB-crystallin plays a protective role against thermal stress, as postulated for small heat shock proteins (1). It is important to examine possible functions of αB-crystallin in glial cells to clarify how its expression might affect glial cell properties in a variety of neuropathologic conditions.

Several studies have indicated an association of the small HSPs with the cytoskeleton (12, 37, 38). αB-crystallin has also been thought to be associated with stress fibers (14, 19). Here we report that altering levels of αB-crystallin is associated with alterations in cellular morphology and actin organization, and that increased expression of αB-crystallin in glioma cells augments thermoresistance.

Materials and Methods

Plasmids

The entire coding region of the rat αB-crystallin cDNA was excised from the pRABX3 plasmid (23) at the *EcoRI* restriction sites. The ends were filled with the large Klenow fragment of *Escherichia coli* DNA polymerase I, and ligated into expression vector pRSVi-HindIII (17), which had been cut at a unique *Hind III* restriction site and treated with the Klenow DNA polymerase. The orientation of the pRSVi-derived plasmid in the transformants was determined by restriction enzyme analysis. The sense and antisense orientation plasmids were designated as pRSV-SSE and pRSV-SAS, respectively.

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Transfection

Rat C6 glioma cells (C6) (4) and human U373MG glioma cells (U373) (46) were obtained from the Amer. Type Culture Collection (Rockville, MD) and maintained as previously described (25). Glioma cells were transfected with 16 μg of pRSV-SSE or pRSV-SAS, together with 4 μg of pSVneo DNA containing the neomycin-resistance gene with the SV40 early promoter (49), using a modification of the calcium phosphate method (45). After screening with 400 μg/ml geneticin (G-418 sulfate, GIBCO BRL, Gaithersburg, MD) for 2 wk, visible colonies of transformed cells were isolated and then single cell cloning was performed by limited dilution. Each clone was characterized by immunoblotting, Northern blotting, Southern blotting, and immunocytochemistry as described below.

Preparation of Antibodies

Two distinct anti-αB-crystallin antisera were prepared for this study, a rabbit antiserum against αB-crystallin purified from rat heart (27, 28), and a rabbit antiserum against the carboxyl-terminal decapeptide (KPAVTAAPKK) of αB-crystallin, a sequence identical in rat and human αB-crystallin. The anti-peptide antiserum was produced in Japanese white rabbits by repeated injection of 0.2 mg of the octavalent peptide-antigen matrix (47) together with Freund's adjuvant. For comparison, anti-rat HSP27 antiserum (obtained from Dr. Yutaka Inaguma, Aichi Prefectural Colony, Japan) (22) and anti-human HSP27 monoclonal antibody (clone G3.1, StressGen, Victoria, Canada) were used for Western blotting, and anti-bovine-S-100 protein antiserum (Dakopatts, Copenhagen, Denmark) was used for immunocytochemistry.

SDS-PAGE and Immunoblotting

Cultured cells were homogenized in 2% SDS, 2 mM EDTA, 2 mM PMSF, 50 mM Tris-HCl, pH 6.8. Protein concentrations were determined by the modified Lowry's procedure using BSA as the protein standard (41). Protein samples were diluted with sample buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 15% 2-mercaptoethanol, 10% glycerin, 0.05% bromophenol blue) and denatured at 95°C for 4 min. Uniform quantities of the protein samples were electrophoresed on 13% polyacrylamide gels in the presence of SDS. Immunoblotting was carried out by transferring the proteins to polyvinylidene difluoride microporous membrane, blocking with 5% non-fat milk in TBS-Tween (50 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 0.05% NaN_3, and 0.05% Tween 20) and incubating overnight in primary antibodies diluted in 5% non-fat milk in TBS-Tween. The next day the blots were washed in TBS-Tween and incubated with alkaline-phosphatase conjugated secondary antibody (Promega Corp., Madison, WI) diluted 1:7,500 in TBS-Tween containing 5% non-fat milk for 30 min. The color reaction was developed in AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing 0.66% nitroblue tetrazolium and 0.33% 5-bromo-4-chloro-indolyl phosphate.

Immunoassay for Rat αB-Crystallin and HSP27

Concentrations of rat αB-crystallin and HSP27 in the C6 transformants were estimated by the sandwich-type enzyme immunoassay described previously (22, 34). The assay system consisted of polystylene balls with immobilized $F(ab')_2$ fragments of antibody and the same antibody Fab′ fragments labeled with β-D-galactosidase from *Escherichia coli*.

Southern Blotting

Genomic DNA was prepared from cultured cells as described (23). DNA samples (20 μg) were digested with various restriction enzymes, separated on a 0.8% agarose gel, and transferred to Gene Screen nylon filters (New England Nuclear, Boston, MA) in 20 × SSC. The probe used was a 3′ part of the human αB-crystallin cDNA, RFI, including exon 3 of the αB-crystallin gene (28) and was ^{32}P-labeled by random primer synthesis (Amersham, Buckinghamshire, England). Hybridizations were performed with 2.5×10^6 cpm/ml of ^{32}P-labeled probe in 5 × SSC, 5 × Denhardt's, 50 mM Na_2PO_4 (pH 6.8), 0.1% SDS and 100 μg/ml salmon sperm DNA overnight at 65°C. Filters were washed three times in 2 × SSC, 0.1% SDS; once in 0.2 × SSC, 0.1% SDS, for 15 min per wash at 65°C. Autoradiography was carried out at −70°C using Fuji X-ray film (Fuji, Kanagawa, Japan) with an intensifying screen.

Northern Blot Analysis

Total RNAs from culture cells were isolated by the guanidine isothiocyanate lysis method, followed by centrifugation through a 5.7-M cesium chloride cushion. RNA samples (20 μg) were electrophoresed in a 1.0% agarose-formaldehyde gel and were transferred to Gene Screen nylon filters in 10 × SSC. An ethidium bromide stain of the gel showed that uniform quantities of RNA were loaded in each gel lane for the Northern blot analysis. The probe used was a rat αB-crystallin cDNA, pRABX3, which includes the entire coding region (23). The washing conditions were the same as described above for Southern blotting.

Immunocytochemistry and Immunofluorescence

Cells (10^5) were plated into 25-sq cm culture wells containing poly-L-lysine-coated coverslips, and were cultivated for 2 d. The cells on the coverslips were washed in PBS, fixed with 2% paraformaldehyde in PBS for 30 min, rinsed in PBS and treated with 1% NP-40 in PBS for 10 min. After

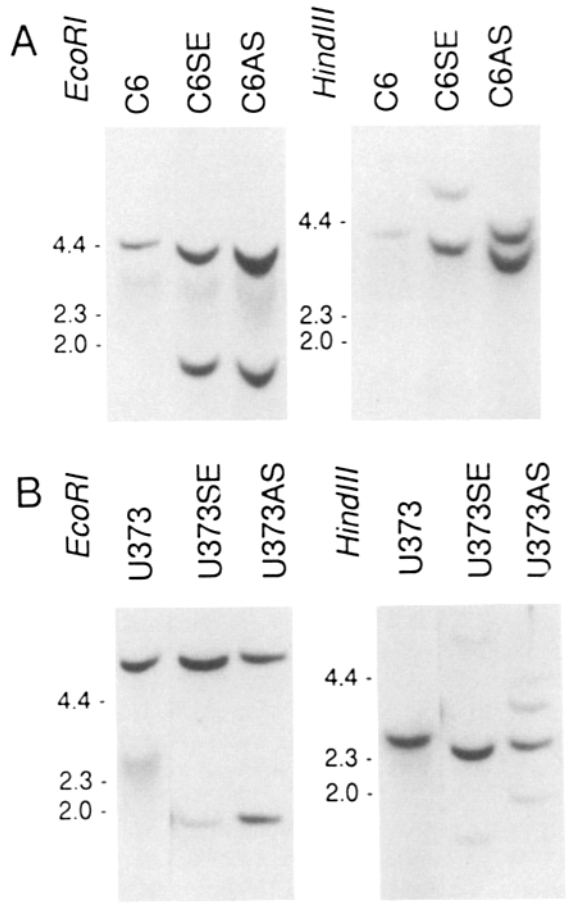

Figure 1. Southern blot analysis of αB-crystallin in the C6 transformants (*A*) and U373 transformants (*B*). A cDNA fragment, 0.35 kb in length, containing exon III of the human αB-crystallin gene was used as a probe. A single (extra) copy of αB-crystallin cDNA appears in the C6 transformants (*C6SE* and *C6AS*). The U373 transformants (*U373SE* and *U373AS*) are still heterogeneous with respect to the transfected cDNA even after the selection by limited dilution. *C6*, parental C6; *C6SE*, the sense C6 transformant; *C6AS*, the antisense C6 transformant; *U373*, parental U373MG; *U373SE*, the sense U373 transformant; and *U373AS*, the antisense U373 transformant. The smaller band in the C6 appears to be nonspecific. Under the hybridization conditions used, the αB-crystallin probe does not bind to hsp27 transcripts or genomic DNA. Size markers are given in kb.

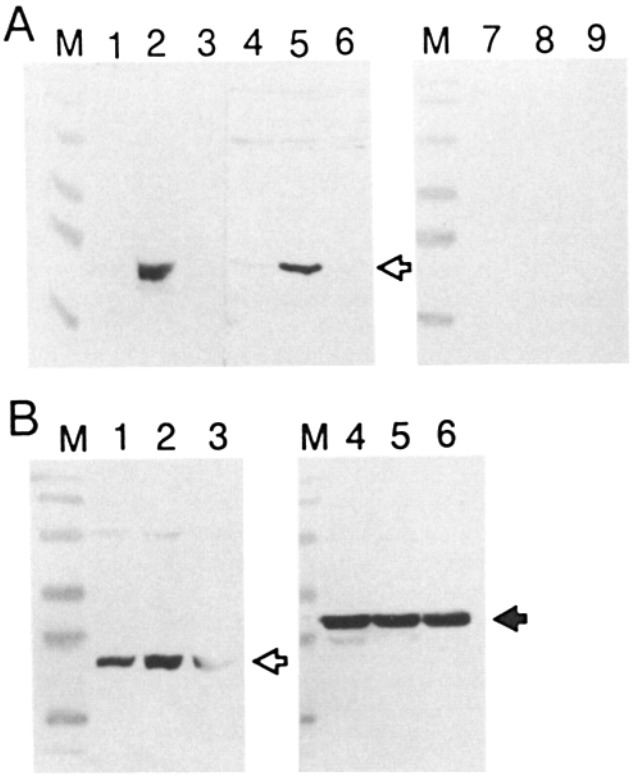

Figure 2. Western blots of total proteins (30 μg per lane) from the glioma cells probed with an affinity-purified antiserum against rat αB-crystallin (*A*: lanes *1–3*) and with antiserum against synthetic carboxyl decapeptide of αB-crystallin (*A*: lanes *4–6* and *B*: lanes *1–3*) and with affinity-purified antiserum against rat HSP27 (*A*: lanes *7–9*), and with a monoclonal antibody against human HSP27 (*B*: lanes *4–6*). The positions of αB-crystallin and HSP27 are indicated by the open arrow and the closed arrow, respectively. In *A* (lanes *1*, *4*, and *7*) C6; (lanes *2*, *5*, and *8*) C6SE; (lanes *3*, *6*, and *9*) C6AS. In *B*, (lanes *1* and *4*) U373; (lanes *2* and *5*) U373SE; (lanes *3* and *6*) U373AS. The prestained molecular weight markers (Bio-Rad Labs.) are shown in lanes *M* (from the *top*: 106, 80, 49.5, 32.5, 27.5, 18.5 kD).

washing with PBS, cells were then incubated sequentially in a diluent (10% goat serum in PBS) for 15 min; primary antibody at 4°C overnight; PBS for 3 min, three times. For immunoperoxidase staining cells were treated with horseradish peroxidase-labeled goat anti-rabbit IgG (Vector Labs, Burlingame, CA) for 1 h (diluted 1:200 in PBS) and the reaction was visualized with 0.03% DAB containing 0.01% H_2O_2. Alternatively, cells were treated with FITC-labeled donkey anti-rabbit IgG for 1 h (diluted 1:50 in PBS) for immunofluorescence.

Patterns of actin filaments in the cells were examined with a rhodamine-coupled phalloidin (Molecular Probes, Eugene, OR). The cells were fixed and permeabilized as described above, and then incubated with rhodamine-phalloidin (1:10 in PBS) for 1 h at room temperature. Double staining with phalloidin and appropriate antibodies were also performed.

Protease Detachment Assay

Control and αB-crystallin-transfected glioma cells were grown in 100-mm plastic dishes. Tumor cells just reaching confluency were rinsed twice with Dulbecco's PBS and 10 ml of trypsin preparation (10 USP U per ml, Mochida Pharmaceutics, Tokyo, Japan) in Dulbecco's PBS was added. The dishes were then placed on a rotary shaker at 10 rpm at room temperature and 1-ml samples of the culture medium were removed every 5 min from each dish. At the end of the incubation, the detached cells in each sample were counted after Trypan blue staining.

Table I. Concentrations of αB-Crystallin and HSP27 in C6 Transformants

Cell line	Phase	αB-crystallin (ng/mg protein)	HSP27 (ng/mg protein)
C6	growth	381 ± 23.8	ND (<1.40)
C6SE	growth	2550 ± 134	ND (<0.952)
C6AS	growth	16.1 ± 2.18	ND (<1.44)
C6	confluent	16.1 ± 1.01	0.349 ± 0.042
C6SE	confluent	1330 ± 95.8	0.432 ± 0.058
C6AS	confluent	5.55 ± 1.26	0.648 ± 0.066

ND, not detected; Average ± Standard error (n = 5)

Heat Shock Treatment and Survival Assay

For heat treatment, each dish of the cells was floated on a water bath at 44.0°C for 15, 30, or 60 min. Immediately after heat treatment, the cells were trypsinized and replated into six plastic dishes at a minimum of two cell dilutions for each dose point. After 10 d of incubation, the tumor cells were rinsed with PBS briefly and fixed with methanol for 10 min. After drying, the cells were stained with 3% Giemsa solution in PBS for 20 min. Only colonies containing more than 50 cells were scored.

Results

Establishment of αB-Crystallin Sense DNA and Antisense DNA-transfected Cell Lines

C6 glioma cells and U373 glioma cells constitutively express αB-crystallin (23). To produce cell lines with different levels of αB-crystallin, glioma cells were cotransfected with two expression vectors, the first of which produced sense or antisense αB-crystallin mRNA under the control of a Rous sarcoma virus promoter and an SV40 polyadenylation signal,

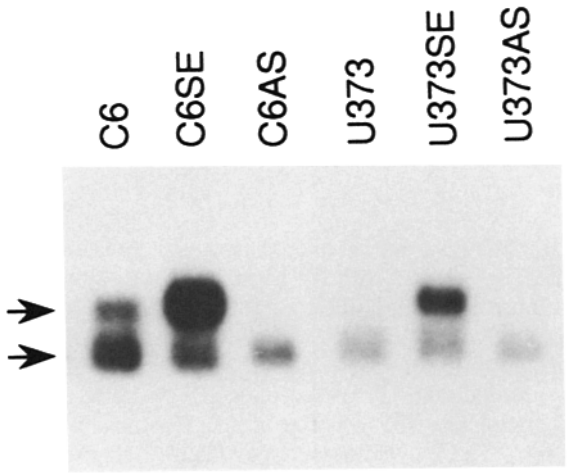

Figure 3. Northern blot of RNA (20 μg) from the parental cells and their transformants probed with a rat αB-crystallin cDNA. Two species of the transcripts in C6 (~0.9 and 1.2 kb) generated by alternative initiation (23) are denoted by arrows. The αB-crystallin sense cDNA transformants (*C6SE* and *U373SE*) express an additional longer mRNA species (~1.3 kb). In the antisense transformants (*C6AS* and *U373AS*), the levels of αB-crystallin mRNAs are slightly decreased, and the endogenous, longer mRNA is not detected in C6AS.

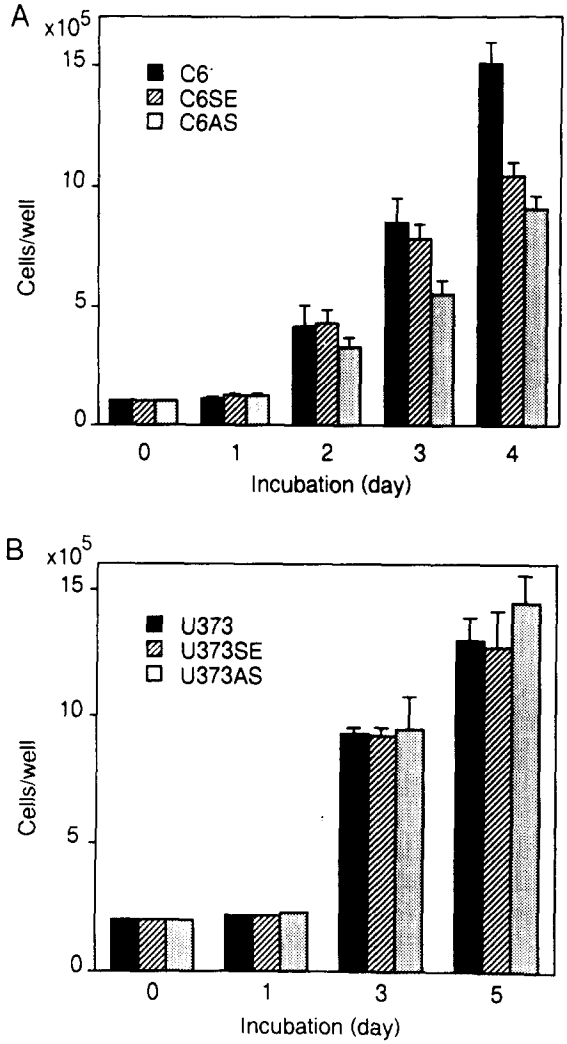

Figure 4. Growth of glioma cells in vitro (*A*: C6 and the C6 transformants, *B*: U373 and the U373 transformants). Cells (*A*: 10^5, *B*: 2×10^5) were plated into 25-sq cm culture wells, and then at the indicated times cells were removed and counted. The culture medium was not changed during the incubation. Values and vertical bars indicate the mean ± SD for triplicate wells.

and the second of which contained the neo gene (pSV40neo). After selecting colonies with G-418 sulfate, each colony was further cloned by limited dilution. It was easy to clone the C6 transformants, but the U373 transformants tended to die or grow slowly at very low cell density. Southern blotting (Fig. 1) revealed that a single copy of αB-crystallin cDNA was integrated into the genome of the C6 transformants (C6SE and C6AS). However, the U373 transformants (U373SE and U373AS) were still heterogeneous judging from the pattern of the introduced cDNA.

The levels of αB-crystallin and HSP27 were analyzed by Western blotting (Fig. 2). The parental C6 cells constitutively expressed only a small amount of αB-crystallin, and did not express detectable HSP27 (below 1 ng per mg protein). In contrast, the parental U373 cells constantly expressed both proteins at high levels. Transfection resulted in a marked increase in αB-crystallin in the sense C6 transformant (C6SE) and a decrease in the protein level in the antisense C6 transformant (C6AS). HSP27 levels in these C6 transformants were not altered by the transfection procedures, remaining at an undetectable level. αB-Crystallin was reduced in U373 by antisense transfection (U373AS), but the high level of HSP27 expression was not altered. αB-Crystallin appeared to be slightly increased in the sense transfectant U373SE, but the parent line already produces substantial amounts of the protein, and transfection did not substantially increase endogenous levels. Since αB-crystallin mRNA levels in C6 decreased with increasing cell density (24), we determined the αB-crystallin and HSP27 protein levels in C6 cells and transformants grown at low and high densities (Table I). In parental and transformants, αB-crystallin levels decreased at high density. The Western blotting described above (Fig. 2) was performed with samples of high density, at which differences in protein levels are the greatest. The immunofluorescence studies were performed at a lower density to visualize cell morphology and stress fibers more clearly. Thus, the results of immunofluorescence of the parental C6 cells reflect the properties of cells containing the highest amounts of αB-crystallin. Even at low density, however, there is a large difference in αB-crystallin content between parental and transformant lines.

Next, the alterations in mRNA levels were examined by Northern blotting (Fig. 3). The parental C6 glioma cells contain two species of transcripts (about 0.9 and 1.2 kb) generated by alternative initiation and the parental U373 cells contain a single 0.9-kb transcript (23, 25). The exogenous αB-crystallin gene in the sense transformants (C6SE and U373SE) express additional mRNA in the larger size range, ~1.3 kb. The larger size is probably due to a longer 3′-untranslated region from the SV40 polyA signal. In the antisense transformants (C6AS and U373AS), the levels of αB-crystallin mRNAs were only slightly decreased (Fig. 3), but the protein levels were remarkably reduced (Figs. 2, 5, and 6). The mechanism of action of antisense constructs is either to block processes that require physical access to the sense sequence or to promote cleavage of the sense strand by stimulating intracellular degrading enzymes. The Northern results suggest that the presence of antisense mRNA does not accelerate αB-crystallin mRNA turnover but rather may result in relatively stable, double-stranded RNA hybrids and may inhibit translation of the endogenous mRNA.

Growth of the Transformed Glioma Cells

The growth of the transformants, compared to the parent lines, is shown in Fig. 4. Proliferation of the C6 transformants appeared to be reduced compared to that of the parental C6. Growth rates of the U373 transformants was the same as that of the parental cells.

Morphology of the Transfected Glioma Cells

Increased αB-crystallin expression in C6 cells did not change cell morphology substantially, although a flattened appearance was more pronounced (Fig. 5). There appeared to be more microfilament bundles, visualized with rhodamine-conjugated phalloidin in the sense transfectant line (Fig. 7). Since the parental U373 already expresses αB-crystallin at high levels (2 μg per mg soluble protein), the

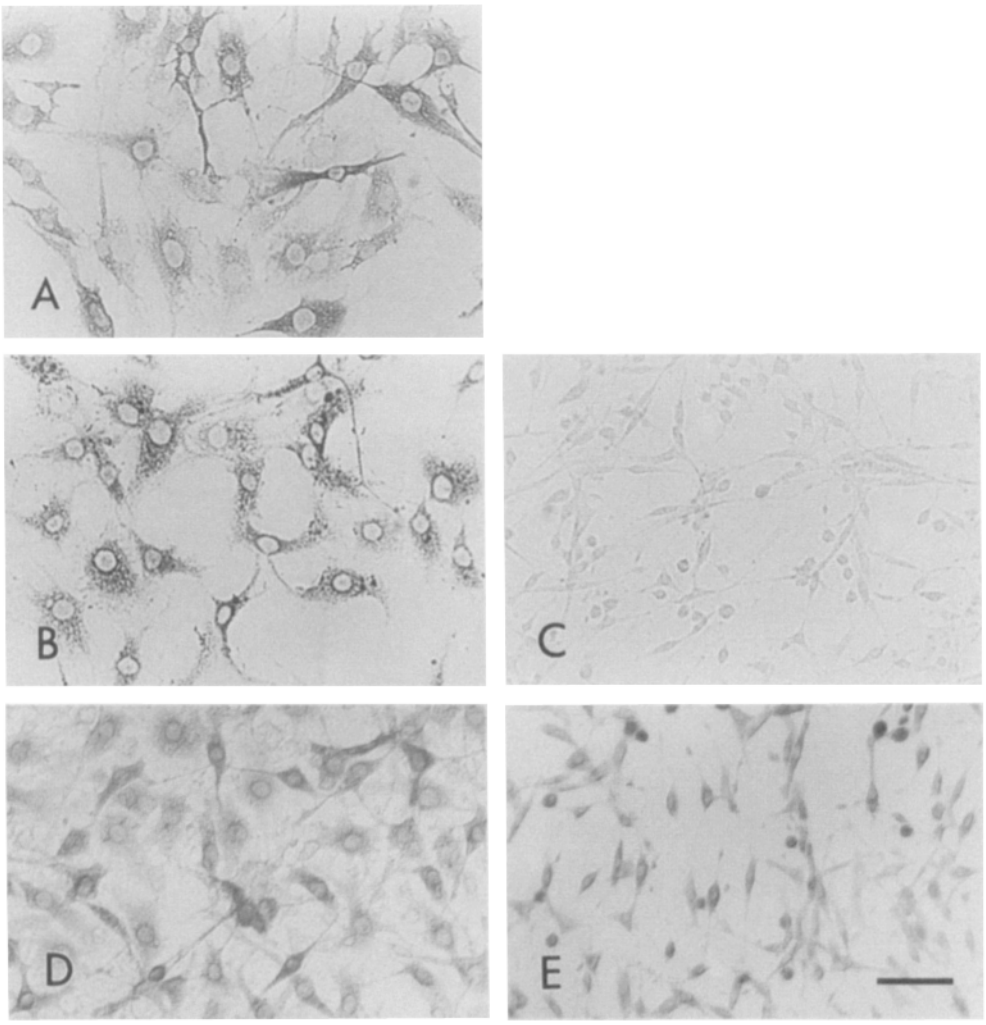

Figure 5. Immunoperoxidase staining of the parental C6 (*A*), the αB-crystallin sense cDNA transformant (C6SE) (*B* and *D*), and the αB-crystallin antisense cDNA transformant (C6AS (*C* and *E*) with a polyclonal antiserum against αB-crystallin (*A*, *B*, and *C*) and a polyclonal anti-S-100 protein antiserum (*D* and *E*). These results demonstrate that the C6AS has become αB-crystallin negative and the C6SE accumulates αB-crystallin, but the expression of S-100 protein, a marker for glial cells, is not altered in those transformants. ×200 after reduction, bar, 50 μm.

additional expression of the rat αB-crystallin in the U373SE caused little change in their morphology (Fig. 6). In contrast, the inhibition of αB-crystallin production in both C6 and U373 antisense transformants (C6AS and U373AS) correlated with remarkable alterations in cellular phenotype. The antisense-transfected cells assumed a bipolar, compact shape (C6AS) or a slender, multipolar shape (U373AS). These differences were accompanied by a loss of stress fibers in these antisense transformants (Figs. 7 and 8). Since the selective inhibition of S-100β protein expression in C6 glioma cells by antisense techniques resulted in a flattened morphology and a more organized microfilament network that is similar to the morphology of C6SE (48), we analyzed S-100 protein in those transfectants by immunocytochemistry. Both C6SE and C6AS were immunopositive to S-100 protein at similar level to the parental C6 cells (Fig. 5, *D* and *E*).

We performed double-label immunofluorescence to ask if any of the αB-crystallin was localized to actin filaments. Most of the αB-crystallin signal appeared diffusely throughout the cytoplasm, as expected, since most of the protein is soluble, but in some cells, particularly near the edges of well-spread cells, a signal that corresponded to stress fibers could be observed (Fig. 8, *C* and *E*).

Adhesive Properties of the Transfected Glioma Cells

We measured the rate of detachment of both the parental controls and their transformants after exposure to trypsin (Fig. 9). The sense-transfected C6 cell line was more resistant to protease detachment than the parent line, and the antisense-transfected C6 and U373 cells detached earlier than the control cells. No difference was seen between the U373 parent and the sense transfectant; αB-crystallin levels were similar in these two lines (see Fig. 2). Thus, the expression of αB-crystallin in the glioma cells correlated in part with their adhesive properties.

Relationship of αB-Crystallin to Thermal Resistance in the Transfected Glioma Cells

Increased αB-crystallin confers a thermoresistant phenotype to the stable transformant (C6SE) (Fig. 10). Similarly a decrease in αB-crystallin made the C6 transformant (C6AS) more thermosensitive than the parental C6 cells. Since neither C6 nor the transformants expressed HSP27 at the time of the heat shock treatment, the result indicates that levels of endogenous αB-crystallin play a role in determining thermal resistance.

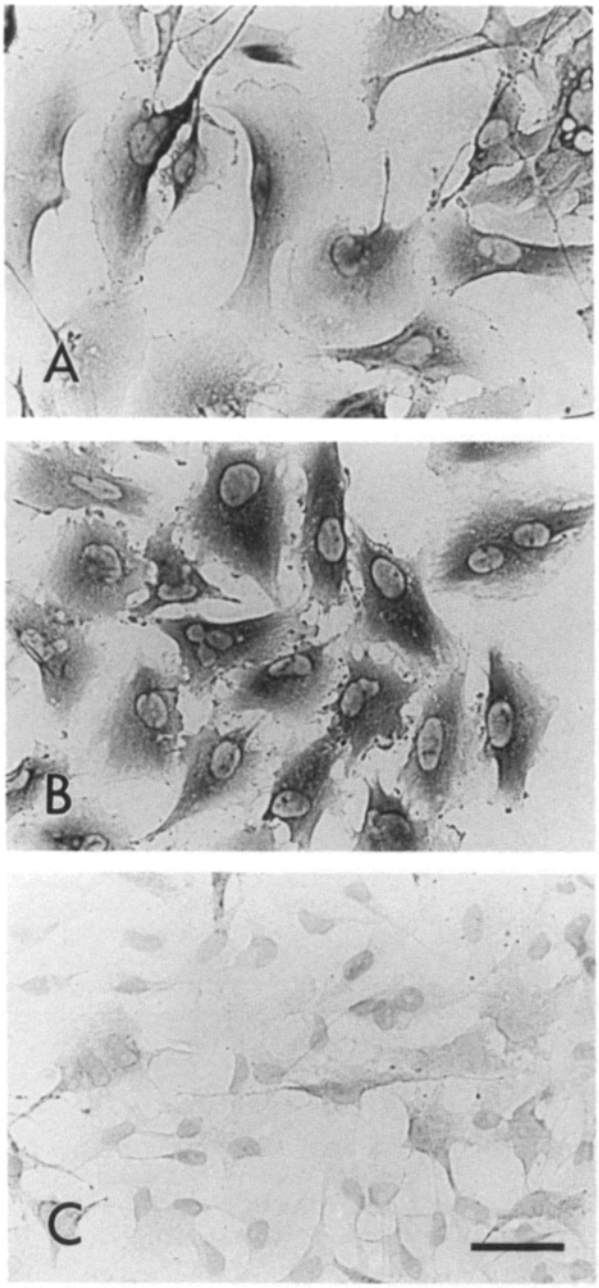

Figure 6. Immunoperoxidase staining of (*A*) the parental U373, (*B*) the αB-crystallin sense cDNA transformant (U373SE), and (*C*) the αB-crystallin antisense cDNA transformant (U373AS) with a polyclonal antiserum against αB-crystallin, counterstained with hematoxylin. These results demonstrate that the U373AS cells have become largely negative to αB-crystallin. ×250, bar, 50 μm.

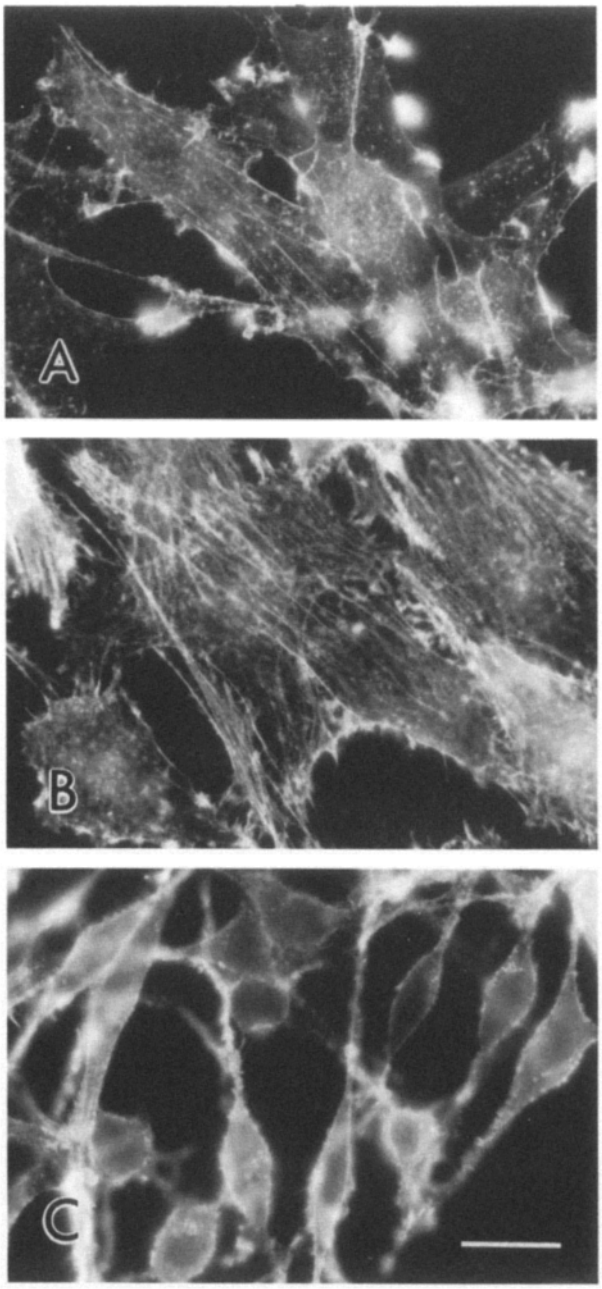

Figure 7. Stress fibers in the parental C6 (*A*) and the transformants (*B*, C6SE; *C*, C6AS) are revealed with rhodamine-conjugated phalloidin. The stress fibers in C6AS cells disappear, whereas those in C6SE are well developed. ×650, bar, 20 μm.

Discussion

We have established glioma cell lines in which the levels of αB-crystallin have been selectively altered by genetic manipulation, and have shown a correlation between the levels of αB-crystallin and stress fibers, adhesive properties, and thermal resistance. Two different glioma cell lines, C6 and U373, were used, because the parental U373 cells constitutively expressed a large amount of HSP27, whereas the parental C6 cells did not express HSP27. In addition, the levels of HSP27 in the sense and antisense transformants derived from those glioma cell lines were not altered by the transfection procedures. Our direct examination of the organization of the microfilament system in the transfected cells was in line with the findings of HSP27 cDNA transfected cell lines (37). That is, microfilament bundles were lost in the αB-crystallin-depleted cells and appeared to have increased in cells with increased αB-crystallin levels. The results presented in this paper provide a direct indication for a potential

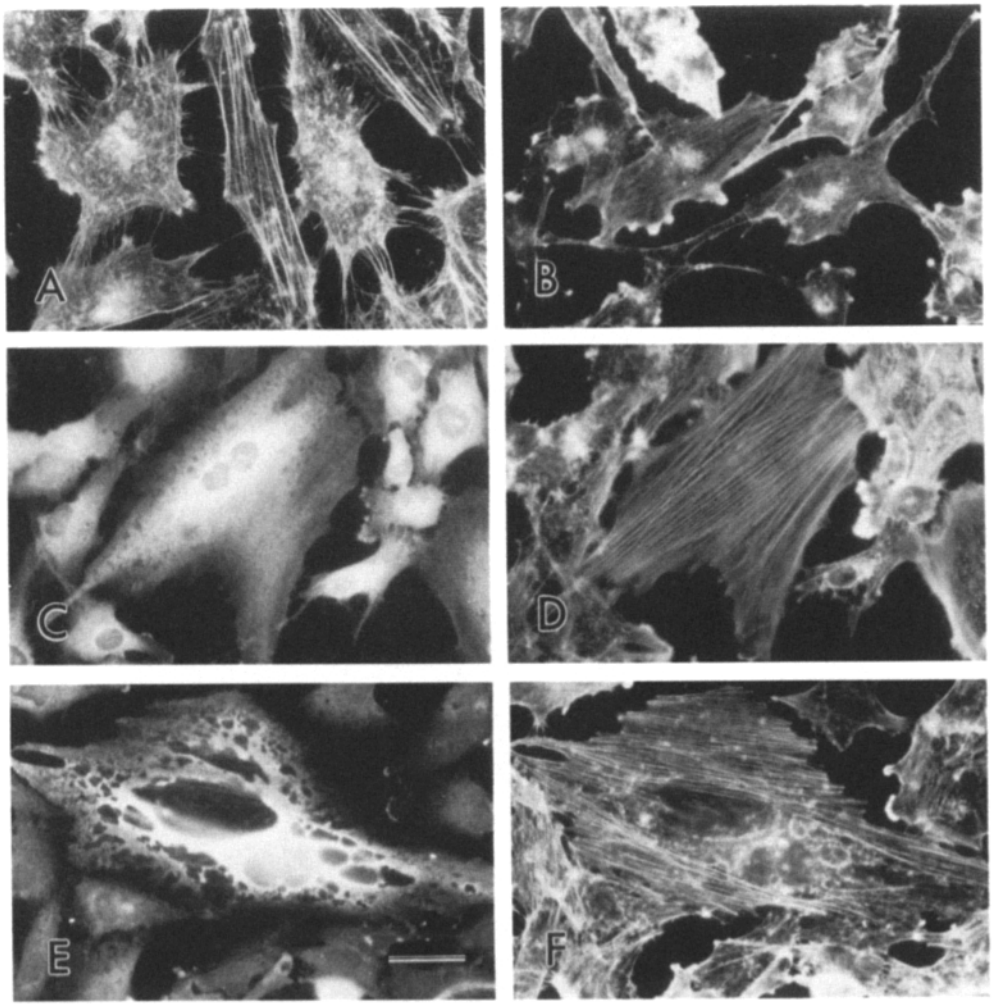

Figure 8. Stress fibers revealed by rhodamine-phalloidin staining combined with immunofluorescence probed with a polyclonal antiserum against αB-crystallin. (*A*) Parental U373 probed with phalloidin. (*B*) The αB-crystallin antisense cDNA transformant (U373AS) probed with phalloidin. Stress fibers in the antisense transformant are markedly reduced. (*C–F*) Double labeling of the αB-crystallin sense cDNA transformant (U373SE) with anti-αB-crystallin antiserum followed by FITC-labeled secondary antibody (*C* and *E*) and with phalloidin (*D* and *F*). U373SE cells show stress fibers as well as ruffling membranes, like the parental U373 cells. ×200 after reduction, bar, 50 μm.

link between the induction of αB-crystallin and the reorganization of the cytoplasmic matrix.

Although most of the αB-crystallin is readily solubilized in aqueous buffers, a small proportion of the protein is associated with cytoskeletal and membrane constituents. For example, lens α-crystallin is found in a membrane-cytoskeletal fraction and its relationship to the lens cytoskeleton, including actin filaments and intermediate filaments has been extensively studied (5, 7, 8, 9, 10, 14, 16, 19). By using a double-labeling immunofluorescence method, Del Vecchio et al. demonstrated that some of the α-crystallin in lens cells is associated with actin (14). In addition, the binding of actin to αA-crystallin and αB-crystallin was demonstrated using affinity column chromatography (19). In non-lens tissue, αB-crystallin has been localized in the Z lines of slow muscle and heart muscle (3, 40), and affinity chromatography has suggested that cardiac actin can bind to cardiac αB-crystallin (11). Moreover, the levels of αB-crystallin mRNA in muscle can be regulated by mechanical tension and denervation (2). These results suggest that αB-crystallin may regulate myofibril structures as a myofibril-stabilizing protein.

Recently it has been shown that αB-crystallin and small heat shock protein are chaperones in vitro, displaying a general capacity to interact with a variety of cellular proteins and possibly modulate their cellular distribution, stability, or reactivity (21, 31). Actin-containing microfilaments have been reported to become disorganized under heat stress (18). Perhaps αB-crystallin interacts with microfilaments as a molecular chaperone, which can recognize a structural change, rather than as a specific actin-binding protein such as gelsolin. This idea may be further supported by the recent finding that an αB-crystallin-related small heat shock protein behaves as a barbed-end capping protein devoid of nucleating activity (43, 44). However, stabilization of microfilaments by αB-crystallin may not be due to a direct interaction. One could conceive models in which crystallin interacts with actin-binding proteins to change actin equilibria or with membrane proteins to change cell shape and microfilament anchorage.

The thermotolerant state has recently been correlated with increased resistance of the cytoskeleton, translational machinery, and mRNA splicing mechanisms to heat damage. Several lines of evidence suggest that the small heat shock proteins have an important role in the development of thermoresistance in mammalian cells. Induction of additional HSP27 gene expression in rodent cells confers resistance to heat shock (36, 37). αB-Crystallin accumulates in response to the synthetic glucocorticoid hormone dexamethasone and

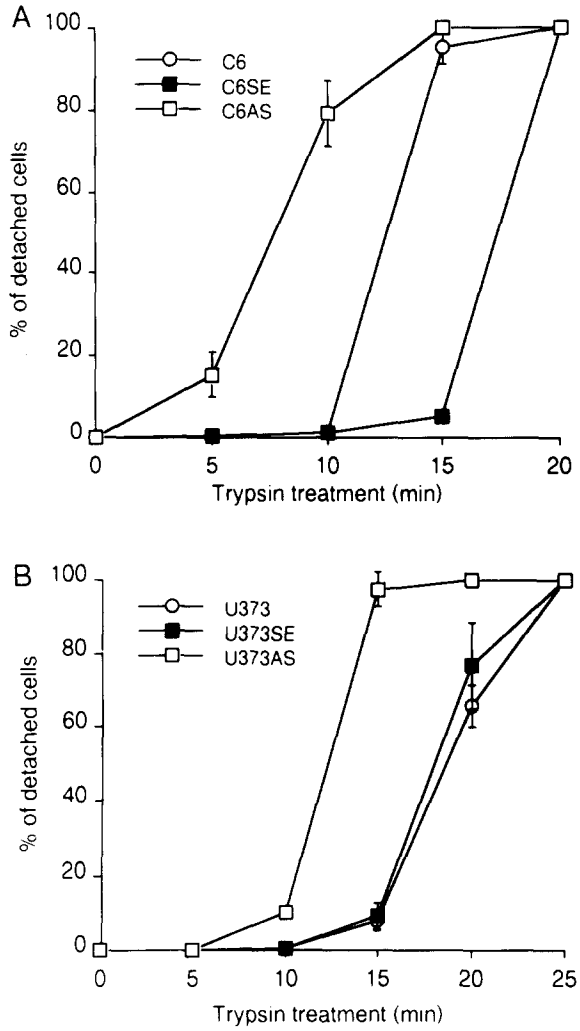

Figure 9. Trypsin-induced detachment of the parental controls (*C6* and *U373*) and the transfected glioma cell lines (sense: C6SE and U373SE, antisense: C6AS and U373AS). The cultivated cells were subject to trypsinization as described in Materials and Methods. Values, expressed as the percentage of total cells that have detached at the end of the incubation, represent the mean ± SD of triplicate determinations.

dexamethasone-treated NIH3T3 cells become thermoresistant in proportion to the accumulation of αB-crystallin (1). Acquired thermoresistance was further demonstrated in our study by using genetic manipulations. The overexpression of αB-crystallin in the C6SE, which still lack HSP27, provided increased thermoresistance. Because of the marked effect of αB-crystallin and HSP27 on the stabilization of actin filaments, the acquired thermoresistance of the genetically manipulated cells may result in part from the stabilization of the cytoplasmic matrix.

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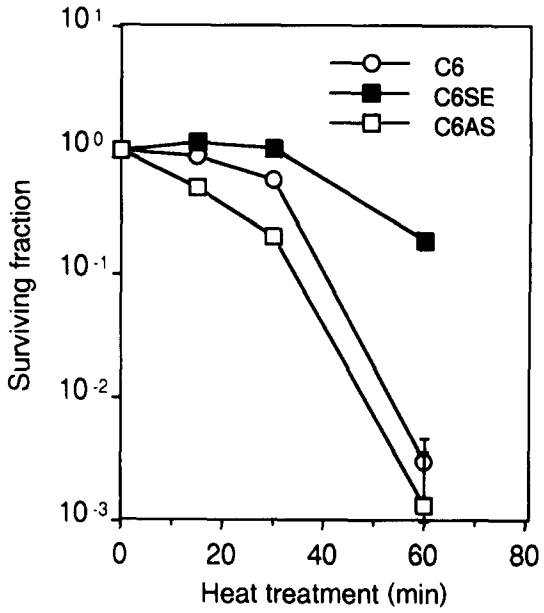

Figure 10. Survival of C6 and the C6 transformants (*C6SE, C6AS*) after heat shock treatments. Exponentially growing cells were treated at 44°C for various incubation times and were replated into plastic dishes as described in Materials and Methods. After 10 d of incubation, colonies were scored. Each point represents the mean ± SD of three independent determinations.

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References

1. Aoyama, A., E. Fröhli, R. Schäfer, and R. Klemenz. 1993. αB-crystallin expression in mouse NIH 3T3 fibroblasts: glucocorticoid responsiveness and involvement in thermal protection. *Mol. Cell. Biol.* 13:1824–1835.
2. Atomi, Y., S. Yamada, and T. Nishida. 1991. Early changes of αB-crystallin mRNA in rat skeletal muscle to mechanical tension and denervation. *Biochem. Biophys. Res. Commun.* 181:1323–1330.
3. Atomi, Y., S. Yamada, R. Strohman, and Y. Nomura. 1991. αB-crystallin in skeletal muscle: purification and localization. *J. Biochem.* 110: 812–822.
4. Benda, P., J. Lightbody, G. Sato, L. Levine, and W. Sweet. 1968. Differential rat glial cell strain in tissue culture. *Science (Wash. DC).* 161:370–371.
5. Benedetti, E. L., I. Dunia, F. C. S. Ramaekers, and M. A. Kibbelaar. 1981. Lenticular plasma membranes and cytoskeleton. Molecular and Cellular Biology of the Eye Lens. 137–184.
6. Bhat, S. P., and C. N. Nagineni. 1989. αB subunit of lens-specific protein α-crystallin is present in other ocular and non-ocular tissues. *Biochem. Biophys. Res. Commun.* 158:319–325.
7. Bloemendal, H. 1977. The vertebrate eye lens. A useful system for the study of fundamental biological processes on a molecular level. *Science (Wash. DC).* 197:127–138.
8. Bloemendal, H., E. L. Benedetti, F. C. S. Ramaekers, I. Dunia, M. A. Kibbelaar, and A. J. M. Vermorken. 1979. Is the cytoskeleton-plasma membrane complex involved in lens protein biosynthesis? *Mol. Biol. Rep.* 5:99–103.
9. Bloemendal, H., G. A. M. Berbers, W. W. de Jong, F. C. S. Ramaekers, A. J. M. Vermorken, I. Dunia, and E. L. Benedetti. 1984. Interaction of crystallins with the cytoskeletal-plasma membrane complex of the bovine lens. *Human Cataract Formation (Ciba Foundation symposium 106).* 177–190.
10. Bloemendal, H., T. Hermsen, I. Dunia, and E. L. Benedetti. 1982. Association of crystallins with the plasma membrane. *Exp. Eye Res.* 35:61–67.
11. Chiesi, M., S. Longoni, and U. Limbruno. 1990. Cardiac alpha-crystallin. III. Involvement during heart ischemia. *Mol. Cell. Biochem.* 97:129–136.
12. Collier, N. C., and M. J. Schlesinger. 1986. The dynamic state of heat

shock proteins in chicken embryo fibroblasts. *J. Cell Biol.* 103:1495–1507.
13. Dasgupta, S., T. C. Hohman, and D. Carper. 1992. Hypertonic stress induces αB-crystalin expression. *Exp. Eye Res.* 54:461–470.
14. Del Vecchio, P., K. MacElroy, M. Rosser, and R. Church. 1984. Association of alpha-crystallin with actin in cultured lens cells. *Curr. Eye Res.* 3:1213–1219.
15. Dubin, R. A., E. F. Wawrousek, and J. Piatigorsky. 1989. Expression of the murine αB-crystallin gene is not restricted to the lens. *Mol. Cell. Biol.* 9:1083–1091.
16. FitzGerald, P., and D. Graham. 1991. Ultrastructural localization of alpha-A crystallin to the bovine lens fiber cell cytoskeleton. *Curr. Eye Res.* 10:417–436.
17. Forman, B. M., C. Yang, F. Stanley, J. Casanova, and H. H. Samuels. 1988. c-erbA protooncogenes mediate thyroid hormone-dependent and independent regulation of the rat growth hormone and prolactin genes. *Mol. Endocrinol.* 2:902–911.
18. Glass, J. R., R. G. DeWitt, and A. E. Cress. 1985. Rapid loss of stress fibers in Chinese hamster ovary cells after hyperthermia. *Cancer Res.* 45:258–262.
19. Gopalakrishnan, S., and L. Takemoto. 1992. Binding of actin to lens alpha crystallins. *Curr. Eye Res.* 11:929–933.
20. Head, M. W., E. Corbin, and J. E. Goldman. 1994. Coordinate and independent regulation of αB-crystallin and hsp27 expression in response to physiological stress. *J. Cell. Physiol.* In press.
21. Horwitz, J. 1992. α-crystallin can function as a molecular chaperone. *Proc. Natl. Acad. Sci. USA.* 89:10449–10453.
22. Inaguma, Y., S. Goto, H. Shinohara, K. Hasegawa, K. Ohshima, and K. Kato. 1993. Physiological and pathological changes in levels of the two small stress proteins, HSP27 and αB crystallin, in rat hindlimb muscles. *J. Biochem.* 114:378–384.
23. Iwaki, A., T. Iwaki, J. E. Goldman, and R. K. H. Liem. 1990. Multiple mRNAs of rat brain α-crystallin B chain result from alternative transcriptional initiation. *J. Biol. Chem.* 265:22197–22203.
24. Iwaki, A., T. Iwaki, Y. Sakaki, R. K. H. Liem, and J. E. Goldman. 1991. Opposite effects of cyclic AMP and cell density on expression of αB crystallin and glial fibrillary acidic protein in C-6 glioma cells. *In* Biological Aspects of Brain Tumors. K. Tabuchi, editor. Springer-Verlag, Tokyo. 396–401.
25. Iwaki, T., A. Iwaki, M. Miyazono, and J. E. Goldman. 1991. Preferential expression of αB-crystallin in astrocytic elements of neuroectodermal tumors. *Cancer.* 68:2230–2240.
26. Iwaki, T., A. Iwaki, J. Tateishi, Y. Sakaki, and J. E. Goldman. 1993. αB-crystallin and 27 kilodalton heat shock protein are regulated by stress conditions in the central nervous system and accumulate in Rosenthal fibers. *Am. J. Pathol.* 143:487–495.
27. Iwaki, T., A. Kume-Iwaki, and J. E. Goldman. 1990. Cellular distribution of αB-crystallin in non-lenticular tissues. *J. Histochem. Cytochem.* 38:31–39.
28. Iwaki, T., A. Kume-Iwaki, R. K. H. Liem, and J. E. Goldman. 1989. αB-Crystallin is expressed in non-lenticular tissues and accumulates in Alexander's disease brain. *Cell.* 57:71–78.
29. Iwaki, T., and J. Tateishi. 1991. Immunohistochemical demonstration of alphaB-crystallin in hamartomas of tuberous sclerosis. *Am. J. Pathol.* 139:1303–1308.
30. Iwaki, T., T. Wisniewski, A. Iwaki, E. Corbin, N. Tomokane, J. Tateishi, and J. E. Goldman. 1992. Accumulation of αB-crystallin in central nervous system glia and neurons in pathologic conditions. *Am. J. Pathol.* 140:345–356.
31. Jakob, U., M. Gaestel, K. Engel, and J. Buchner. 1993. Small heat shock proteins are molecular chaperones. *J. Biol. Chem.* 268:1517–1520.
32. Kato, K., H. Shinohara, S. Goto, Y. Inaguma, R. Morishita, and T. Asano. 1992. Copurification of small heat shock protein with αB-crystallin from human skeletal muscle. *J. Biol. Chem.* 267:7718–7725.
33. Kato, K., H. Shinohara, N. Kurobe, S. Goto, Y. Inaguma, and K. Ohshima. 1991. Immunoreactive αA crystallin in rat non-lenticular tissues detected with a sensitive immunoassay method. *Biochim. Biophys. Acta.* 1080:173–180.
34. Kato, K., H. Shinohara, N. Kurobe, Y. Inaguma, K. Shimizu, and K. Ohshima. 1991. Tissue distribution and developmental profiles of immunoreactive αB crystallin in the rat determined with a sensitive immunoassay. *Biochim. Biophys. Acta.* 1074:201–208.
35. Klemenz, R., E. Fröhli, R. H. Steiger, R. Schäfer, and A. Aoyama. 1991. αB-crystallin is a small heat shock protein. *Proc. Natl. Acad. Sci. USA.* 88:3652–3656.
36. Landry, J., P. Chrétien, H. Lambert, E. Hickey, and L. A. Weber. 1989. Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells. *J. Cell Biol.* 109:7–15.
37. Lavoie, J. N., G. Gingras-Breton, R. M. Tanguay, and J. Landry. 1993. Induction of Chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. HSP27 stabilization of the microfilament organization. *J. Biol. Chem.* 268:3420–3429.
38. Leicht, B. G., H. Biessmann, K. B. Palter, and J. J. Bonner. 1986. Small heat shock proteins of *Drosophila* associated with the cytoskeleton. *Proc. Natl. Acad. Sci. USA.* 83:90–94.
39. Lin, L.-R., D. Carper, T. Yokoyama, and V. N. Reddy. 1993. The effect of hypertonicity on aldose reductase, alphaB-crystallin and organic osmolytes in retinal pigmented epithelium. *Invest. Ophthalmol. Vis. Sci.* 34:2352–2359.
40. Longoni, S., S. Lattonen, G. Bullock, and M. Chiesi. 1990. Cardiac alpha-crystallin. II. Intracellular localization. *Mol. Cell. Biochem.* 97:121–128.
41. Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* 87:206–210.
42. Merck, K. B., P. J. T. A. Groenen, C. E. M. Voorter, W. A. de Haard-Hoekman, J. Horwitz, H. Bloemendal, and W. W. de Jong. 1993. Structural and functional similarities of bovine α-crystallin and mouse small heat shock protein. A family of chaperones. *J. Biol. Chem.* 268: 1046–1052.
43. Miron, T., K. Vancompernolle, J. Vandekerckhove, M. Wilchek, and B. Geiger. 1991. A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein. *J. Cell Biol.* 114:255–261.
44. Miron, T., M. Wilchek, and B. Geiger. 1988. Characterization of an inhibitor of actin polymerization in vinculin-rich fraction of turkey gizzard smooth muscle. *Eur. J. Biochem.* 178:543–553.
45. Parker, B. A., and G. R. Stark. 1979. Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infectins by virus or viral DNA. *Virology.* 31:360–369.
46. Pontén, J., and B. Westermark. 1978. Properties of human malignant glioma cells in vitro. *Med. Biol.* 56:184–193.
47. Posnett, D. N., and J. P. Tam. 1989. Multiple antigenic peptide method for producing antipeptide site-specific antibodies. *Method Enzymol.* 178:739–746.
48. Selinfreund, R. H., S. W. Barger, M. J. Welsh, and L. J. Van Eldik. 1990. Antisense inhibition of glial S100β production results in alterations in cell morphology, cytoskeletal organization, and cell proliferation. *J. Cell Biol.* 111:2021–2028.
49. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under the control of the SV40 early region promoter. *J. Mol. Appl. Genet.* 1:327–341.
50. Wistow, G. 1985. Domain structure and evolution in α-crystallins and small heat-shock proteins. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 181:1–6.
51. Wistow, G. 1993. Possible tetramer-based quaternary structures for α-crystallins and small heat shock proteins. *Exp. Eye Res.* 56:729–732.
52. Zantema, A., M. Verlaan-De Vries, D. Maasdam, S. Bol, and A. Van der Eb. 1992. Heat shock protein 27 and αB-crystallin can form a complex, which dissociates by heat shock. *J. Biol. Chem.* 1992:12936–12941.