Effect of mutations of murine lens αB crystallin on transfected neural cell viability and cellular translocation in response to stress

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Abstract We examined the influence of over-expressed native and mutant murine lens αB crystallin on the response of a murine neural cell line to heat and ionic strength shock. Native and mutant (F27R and KK174/175LL) murine αB crystallin amplicons were subcloned into a Lac-Switch IPTG-inducible RSV promoter eukaryotic vector, and transfected into N1E-115 cells using lipofectin. Expression was induced maximally 8 h after addition of IPTG (optimal final concentration 1 mM) to the medium. Cells grew normally after transfection with native and mutant murine αB crystallin. We demonstrated expression of the protein using specific anti-α crystallin antibodies. Viability under severe heat and ionic strength stress increased when cells had been transfected with native αB crystallin, but not with mutants F27R or KK174/175LL. Heat shock caused translocation of both native and mutant αB crystallins into the central region of the cells. These results show that mutations in αB crystallin that effect its chaperone-like activity may also influence viability of N1E-115 neural cells under stress, while not influencing the distribution of the protein within the cell.

Key words: Chaperone; Neurological disorder; Cataract; Neuroblastoma; Homeobox

1. Introduction

The mammalian small heat shock protein α crystallin is emerging as a key protein in a remarkable variety of cellular processes including: oxidative stress responses in heart and respiratory tissue [1], development of receptivity in the secretory phase endometrium [2], cellular differentiation in the eye, and in a variety of neurodegenerative disorders. It is the major antigen in the autoimmune response observed in multiple sclerosis [3], and is expressed by both oligodendrocytes and astrocytes in MS lesions [4].

α Crystallin is composed of two subunits, αA and αB, of approximately 20 kDa in mass each but the active protein exists as a large aggregate of varying mass between 300 and 1000 kDa [5,6]. The mechanism(s) involved in α crystallin activity remain unclear. It has been shown to confer increased stress resistance, enhanced adherence to surfaces and increased cytoskeleton on mammalian cell cultures [7] and promotion of proliferation, and reorganisation of the cytoskeleton appears a probable mechanism in α crystallin activity. Observations of stretch responses in human trabecular meshwork cells have provided further evidence of the association of α crystallin with actin filaments and of specific proteolysis of α crystallin during reorganisation of actin filaments [8]. It has also been shown to exhibit temperature dependant interaction with tubulin [9] and with intermediate filaments vimentin and peripherin during stress in vivo [10,11].

Observation of α crystallin behaviour in vitro and in vivo suggests that other pathways may also be involved. A number of researchers have proposed that α crystallin is a molecular chaperone. It is certainly able to prevent protein aggregation in vitro by sequestering denatured proteins [12,13]. Under certain conditions, we and others have found that α crystallin can assist in the refolding of citrate synthase, α-glucosidase [14] and prochymosin [15]. Recent evidence appears to suggest that α crystallin can function as a true molecular chaperone in vitro in the presence of ATP [16].

Peptide binding by molecular chaperones is thought to be largely mediated by hydrophobic interactions, particularly involving region(s) (e.g. the conserved RLFDQFF) near the N-terminus [17]. Using site-directed mutagenesis and heterologous expression in *Escherichia coli*, we showed that this region was indeed important in α crystallin chaperone-like activity, both in vitro and in vivo [13]. Recently published data [16] has confirmed our previous findings that recombinant α crystallin also confers increased viability on *E. coli* cells expressing the protein.

αB Crystallin is normally expressed in neurons, and may interact with intermediate filaments in NIH 3T3 cells [11]. We therefore investigated the influence of mutations of αB crystallin which we have shown to influence chaperone-like activity [13] on the viability of a murine neural cell line to heat and ionic strength shock. We also studied the cellular distribution of native and mutant αB crystallins in these cells during heat shock using confocal microscopy.

2. Materials and methods

2.1. Expression constructs

Native and mutant αB crystallins were expressed using the Lac-Switch expression system (Stratagene). This system involved the coupled use of an expression plasmid derived from the chloramphenicol acetyltransferase (CAT)-expressing vector pORSVICAT and the repressor plasmid p3′ss. The p3′ss plasmid produces high levels of the lac repressor protein, thereby preventing transcription from the strong RSV-LTR promoter of the pORSVICAT-derived expression plasmid. Induction using 1 mM IPTG inhibits this repression and protein expression is observed. Three pORSVICAT-derived expression constructs were made, one contained native αB-crystallin, the second

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and third contained the F27R and KK174/175LL mutations, which result in loss of chaperone-like activity [13].

Expression plasmids were constructed as shown in Fig. 1. The CAT gene was removed from the pORSVICAT vector by *Not*I digestion and replaced by native and mutant αB crystallins engineered by PCR to carry the *Not*I restriction site at each end.

2.2. Cell culture

N1E-115 cells, a mouse neuroblastoma cell line were obtained from ECACC (ECACC No. 88112303) and grown from frozen stock by the following protocol: 5 ml of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum, 2 mM glutamine, 200 U/ml penicillin and 100 μg/ml streptomycin (referred to later as 10% DMEM) was placed in a culture flask and cell stock solution was added. The flask was mixed gently and placed horizontally for 30–60 min to allow viable cells to adhere. The medium was replaced with fresh medium and the flask incubated at 37°C, 5% CO_2.

For routine passaging, the medium was removed from a 25 ml flask and the cell sheet washed with 5 ml phosphate buffered saline (PBS), 1 mM EDTA. The cell sheet was covered with 1 ml PBS/EDTA/Trypsin (the latter at 0.5 g/l) and incubated for 10–15 min at 37°C. To 1 ml of cell suspension, 4 ml of the medium described above was added and this suspension was further diluted into a flask containing fresh medium for onward growth.

2.3. Transfection

Transfections of the cells was carried out using the PerFect lipids from Invitrogen. The most effective lipid for N1E-115 cells was found to be Pfx-3, a 1:1 mix of a cationic lipid and L-dioleolyl phosphatidylethanolamine, with a transfection efficiency of 16%. This plasmid expresses a β-galactosidase and transfected cells can be detected by the blue coloration they acquire in the presence of the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Transfection was carried out as follows.

N1E-115 cells were trypsinised with 3 ml PBS/EDTA/Trypsin and seeded at 1:10 dilution in 2 ml 10% DMEM per well in 12 well culture plates. All transfections were carried out at least in duplicate. Cells were grown for 24–48 h, until 50–60% confluency had been obtained. On the day of transfection, the cells in each well were prepared by washing twice with 1 ml PBS, and were then overlaid with 1 ml of transfection solution. This solution was made by preparing a 24 μg/ml solution of the lipid in serum-free medium in a Sterilin bottle, and a 4 μg/ml concentration of plasmid DNA in 1 ml serum-free medium in a separate Sterilin bottle. The two solutions were combined and incubated at room temperature for 15 min to constitute the transfection solution used to overlay the cell sheet. Incubation of cells with the transfection solution was carried out at 37°C, 5% CO_2 for 24 h. The medium was then removed and replaced by 2 ml 10% DMEM supplemented with the selection antibiotics at 400 μg/ml. This medium was replaced every 3–4 days until colonies could be seen forming on the base of the well. On average 5–6 colonies formed per well.

Colonies were picked by removing the medium and replacing it with 150 μl PBS/EDTA/Trypsin. After 1 min at 37°C, cells were sucked up from each colony into a sterile pipette tip. The cells were transferred to a well of a fresh 12 well plate and grown in the presence of 2 ml medium. Two 75 ml flasks of cells were grown until the cells had reached confluency. Cells were trypsinised with 3 ml PBS/EDTA/Trypsin. The supernatant was decanted and the cell pellet resuspended in 4 ml 10% DMEM supplemented with 100 μg/ml selection antibiotics hygromycin and geneticin and 10% DMSO. The suspension was frozen in 1 ml aliquots, first in an isopropanol bath at −70°C overnight and then in liquid nitrogen.

2.4. αB crystallin expression

After preliminary studies on IPTG concentrations (0.5–5 mM) and time course of expression, expression of native and mutant proteins was induced maximally 8 h after addition of 1 mM IPTG to the media.

2.5. Gel electrophoresis

SDS polyacrylamide gel electrophoresis and Western blotting was performed as described previously [13,18].

2.6. Ionic strength shock

Ionic strength shock experiments were carried out in 25 ml flasks or 8 well chamber slides (Life Technologies) as appropriate. Cells were seeded in the wells in 5 ml or 0.45 ml respectively of 10% DMEM supplemented with selection antibiotics as required and allowed to grow to 50–60% confluency. Cells were induced with 1 mM IPTG for 8 h and then supplemented with 0, 25, 50, 100, 150 or 200 mM KCl respectively as appropriate. Cells were incubated at 37°C, 5% CO_2 overnight.

2.7. Heat shock

Heat shock experiments were carried out in 25 ml flasks or slide flasks (Life Technologies), as appropriate. Cells were seeded in the flasks in 5 ml or 3 ml respectively of 10% DMEM supplemented with selection antibiotics as required and allowed to grow to 50–60% confluency. Cells were induced with 1 mM IPTG for 8 h and then shocked by incubation of the flasks at 43°C, 5% CO_2 for 0, 3, 6 and 24 h respectively.

2.8. Cell viability

Floating cells were decanted into a Falcon tube. The cell sheet remaining was washed with 5 ml PBS/EDTA and the wash decanted into the same tube. The sheet was then trypsinised with 1 ml PBS/EDTA/Trypsin and after 15 min at room temperature, cells were dislodged and again decanted into the tube. Remaining cells were collected by washing the flask with a final 5 ml PBS/EDTA. Cells were collected by centrifugation and washed with 5 ml PBS, centrifuged again and finally resuspended in 20–100 μl PBS. To an aliquot of this was added an equal volume of 0.4% trypan blue dye, previously diluted 1:1 with PBS and filtered through a Millipore 0.2 μm filter to remove any precipitates. After 15 min incubation blue cells (dead) and colourless cells (viable) were counted using a haemocytometer and light microscope. The average of at least two counts was taken and converted into a concentration of cells/ml.

2.9. Confocal microscopy

Cell sheets on the slide surface were washed with PBS and then fixed with freshly prepared 4% paraformaldehyde for 10 min at room temperature. Blocking was done with 1% foetal calf serum in PBS, 0.05% azide for 1 h at room temperature. Following four washes of the cells with PBS, cells were permeabilised by incubation with 50% methanol, 50% acetone for 10–15 min at room temperature. Cells were washed four times with PBS prior to use of the primary antibody. This rabbit anti-mouse α crystallin polyclonal antibody serum [13] was used at a 1:1000 dilution in PBS and incubation was for 1 h at room temperature. Quantitatively, ≥ 0.1 ng of bovine α crystallin under the conditions used gave a colour reaction with the antibody, while 0.01 ng failed to react. The fluorescence antibody (goat anti-rabbit IgG-FITC conjugate, Sigma) was added at 1:500 dilution in PBS and incubation followed for 1 h at room temperature in the dark. After a final four washes with PBS, the cells were mounted in Vectralabs H-1300 mounting medium containing propidium iodide, covered by a coverslip (0.17 μm ± 0.1 μm) and stored in the dark until examined by confocal microscopy, using a Leica DMRBE microscope with TCSNT software.

3. Results

Stable N1E-115 cell lines transfected with the pORSVI vector containing native αB crystallin, the F27R αB crystallin mutant and the KK174/175LL mutant αB crystallin were successfully established.

Untransfected N1E-115 cells grown under normal conditions had a very low level of α crystallin expression, as shown by Western blotting (Fig. 2) and confocal microscopy (Fig. 3). Cells transfected with native or mutant αB crystallin cDNA all produced αB crystallin upon induction with IPTG; Fig. 2 shows the results from cells transfected with native and mutant F27R αB crystallin cDNA. The background level of αB crystallin increased slightly upon shock (see Fig. 3a,d), but shock did not induce the expression of any other proteins that cross-reacted with the anti-αB crystallin antibody.

There were no significant differences in viability (86% ± 2%;

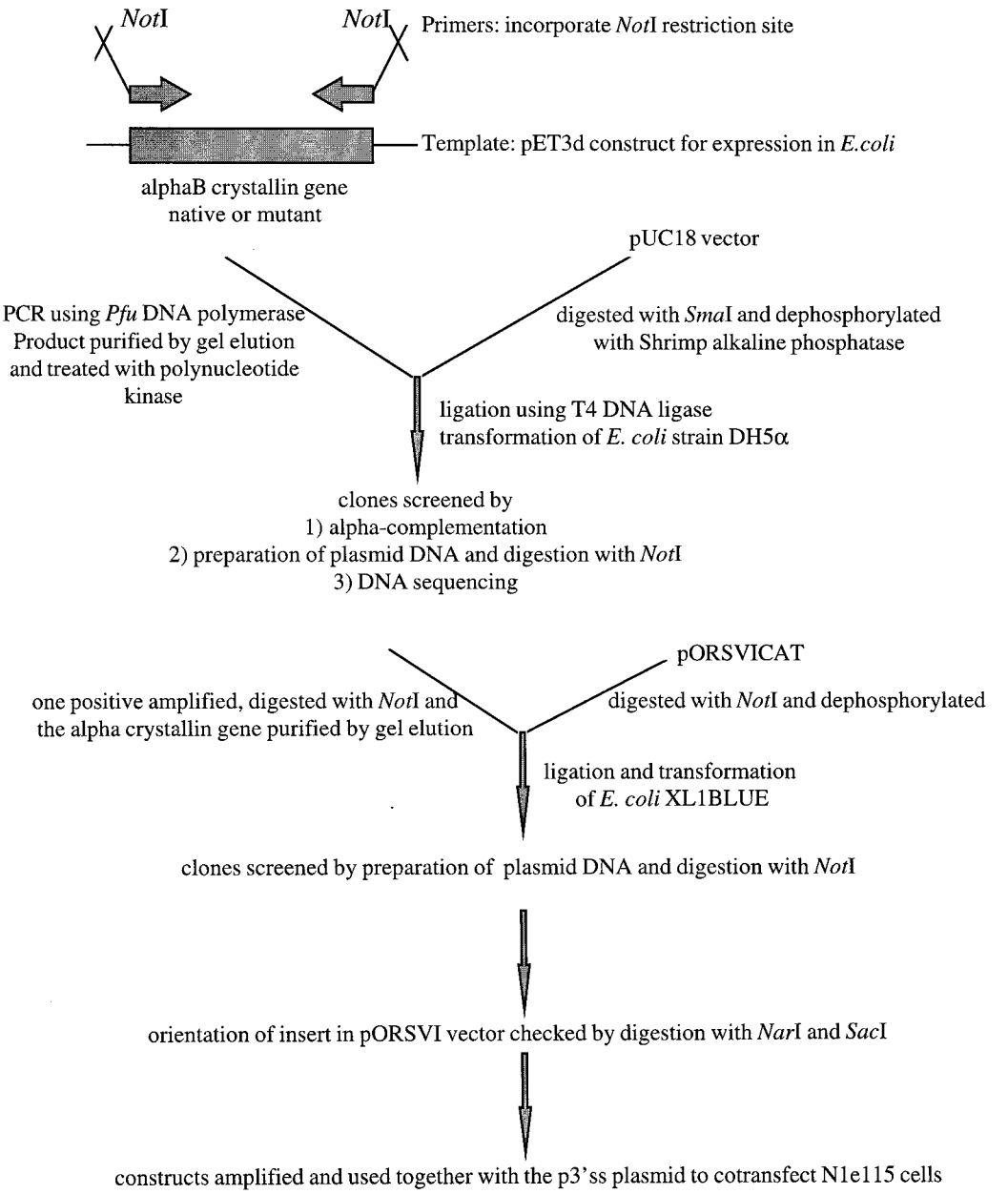

Fig. 1. Method of construction of the pORSVICAT-derived expression vectors for native and mutant αB crystallins.

$P > 0.05$) between cells that were transfected but not induced, and cells that were transfected with either native αB crystallin, mutant F27R, or mutant KK174/175LL. There were no significant differences ($P > 0.05$) in cell viability between cells that had been transformed with vector only and were not subjected to induction conditions, and cells that had been transformed with vector only and were subjected to induction conditions. Both sets served as controls. Cells were not viable at concentrations of KCl ≥ 100 mM, whether untransfected or transfected with native or mutant αB crystallin. Cells subjected to shock using KCl (25 or 50 mM), which caused a significant drop in viability ($56 \pm 4\%$; $P = 0.00092$) showed increased viability when transfected with native αB crystallin ($75 \pm 4\%$; $P = 0.003$), but not with mutants F27R or KK174/175LL ($58 \pm 4\%$; $P > 0.05$).

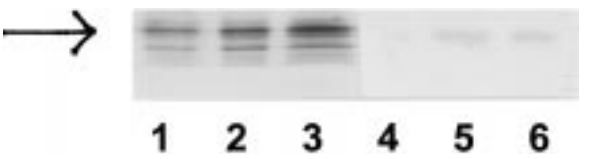

Fig. 2. α Crystallin identification in N1E-115 mouse neuroblastoma cells. The arrow shows the migration position of αB crystallin. SDS polyacrylamide gel electrophoresis lanes are 1, 2 and 3; Western blot lanes are 4, 5, and 6. Lanes 1, 4: cells transfected with native αB crystallin cDNA but not induced with IPTG; 2, 5: cells transfected with native αB crystallin cDNA and induced with IPTG; 3, 6: cells transfected with αB crystallin mutant F27R cDNA and induced with IPTG.

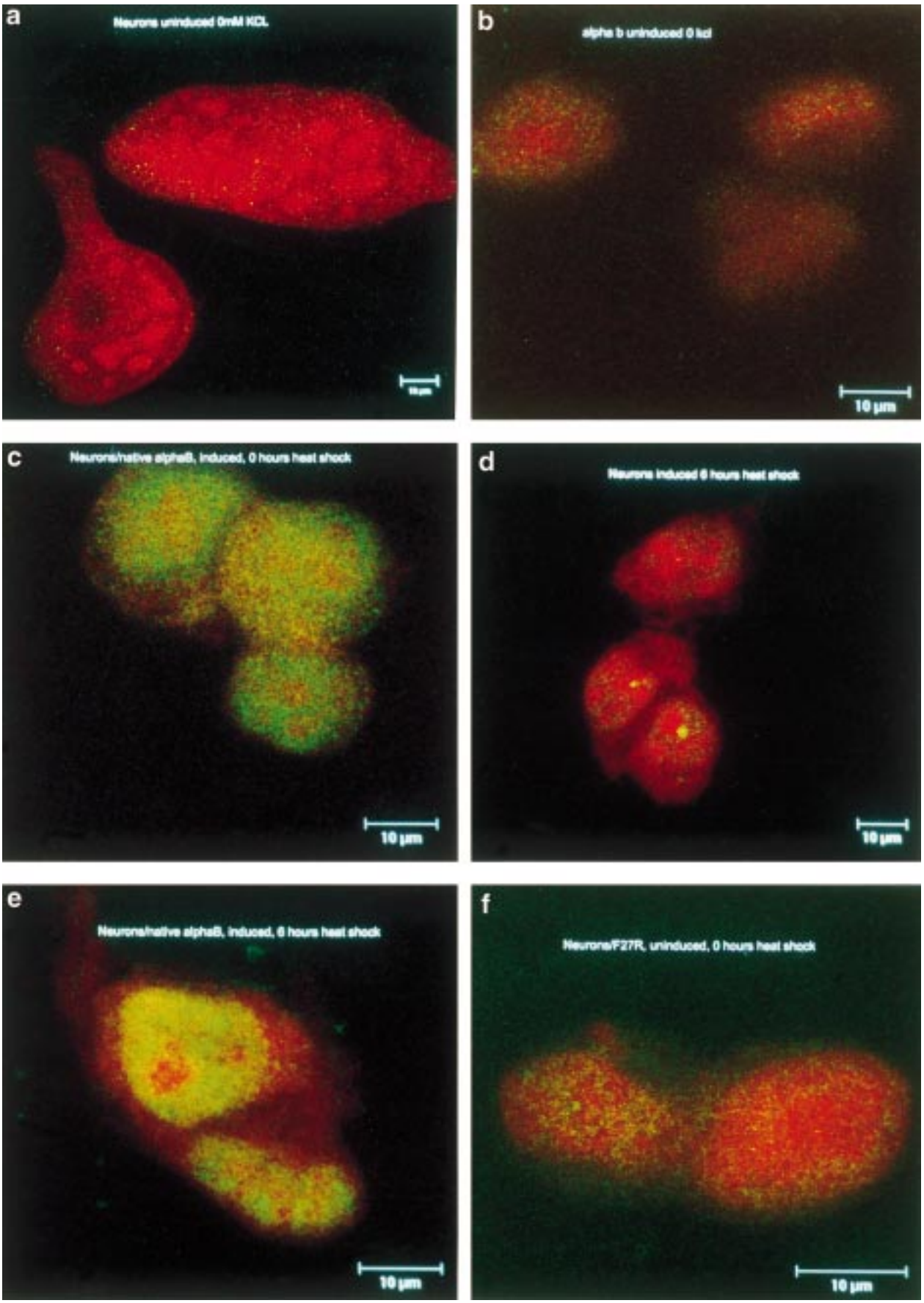
a
Neurons uninduced 0mM KCL
b
alpha b uninduced 0 kcl
10 µm
c
Neurons/native alphaB, induced, 0 hours heat shock
10 µm
d
Neurons induced 6 hours heat shock
10 µm
e
Neurons/native alphaB, induced, 6 hours heat shock
10 µm
f
Neurons/F27R, uninduced, 0 hours heat shock
10 µm

Fig. 3.

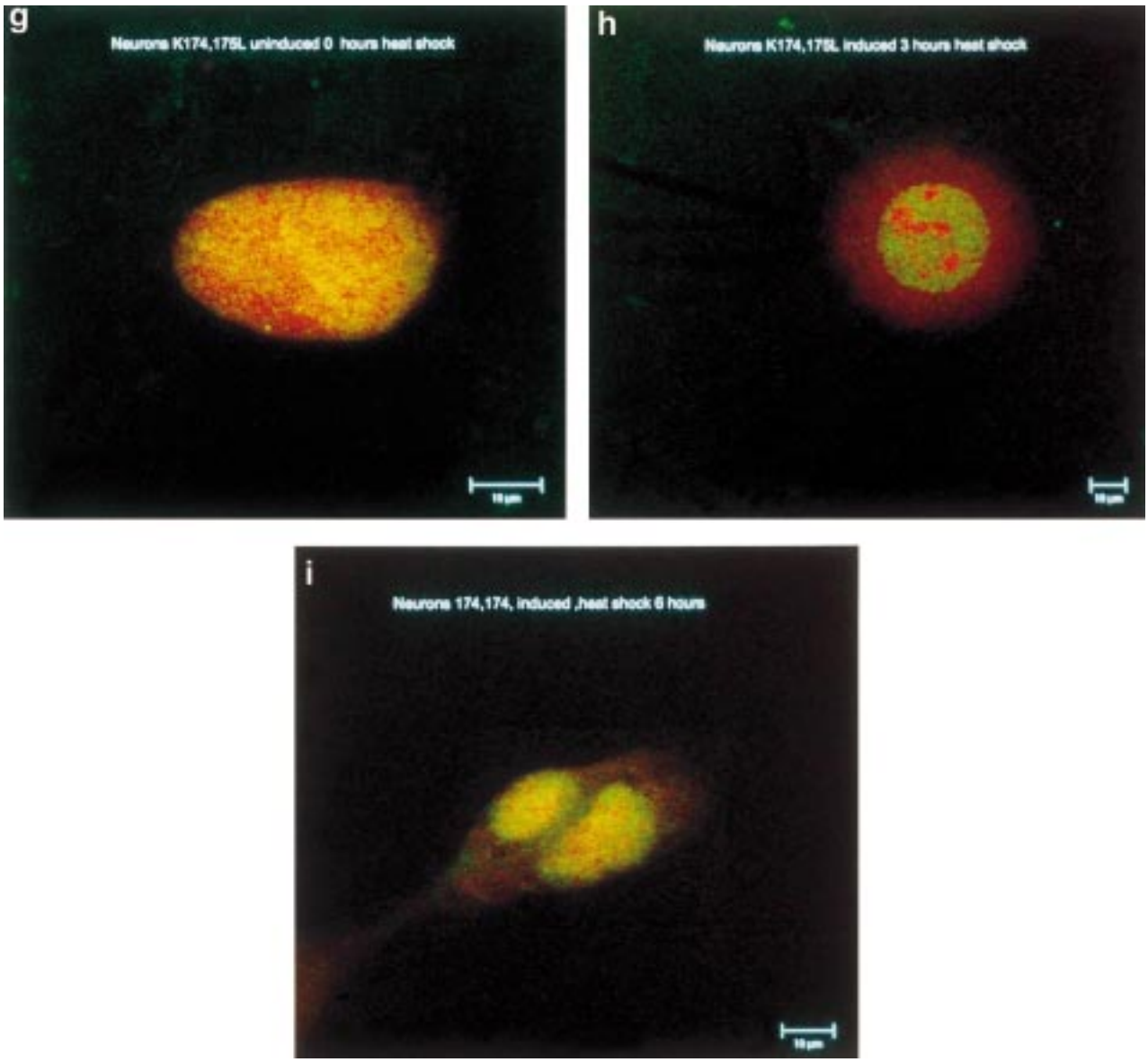

Fig. 3. Confocal microscopy of N1E-115 mouse neuroblastoma cells. a: Untransfected cells, without induction and without heat shock or KCl; b: cells transfected with native αB crystallin cDNA but not induced with IPTG and without heat shock or KCl; c: cells transfected with native αB crystallin cDNA and induced with IPTG, without heat shock or KCl; d: untransfected cells, induced with IPTG, 6 h heat shock at 43°C; e: cells transfected with native αB crystallin cDNA, induced with IPTG, 6 h heat shock at 43°C; f: cells transfected with mutant F27R αB crystallin cDNA and induced with IPTG, without heat shock or KCl; g: cells transfected with mutant KK174/175LL, not induced with IPTG, without heat shock or KCl; h: cells transfected with mutant KK174/175LL αB crystallin cDNA, induced with IPTG, 3 h heat shock at 43°C; i: cells transfected with mutant KK174/175LL αB crystallin cDNA, induced with IPTG, 6 h heat shock at 43°C.

Mild heat stress (3 h at 43°C) reduced overall cell viability relative to unstressed control cells ($53 \pm 5\%$; $P = 0.00055$), but there were no significant differences after 3 h between untransfected cells and those transfected with native αB crystallin, mutant F27R, or mutant KK174/175LL ($P > 0.05$). In contrast, severe heat stress (24 h at 43°C) significantly reduced control cell viability ($9 \pm 3\%$; $P = 0.00016$); viability was significantly improved under these conditions when cells had been transfected with native αB crystallin ($21 \pm 2\%$; $P = 0.013$), but not when transfected with mutants F27R or KK174/175LL ($10 \pm 2\%$; $P > 0.05$).

Confocal microscopy showed little immunofluorescence for αB crystallin in untransfected cells (Fig. 3a), in cells transfected with native αB crystallin cDNA but not induced with IPTG (Fig. 3b), or in cells transfected but not induced with mutant KK174/175LL αB crystallin cDNA (Fig. 3g), compared to cells transfected with native αB crystallin cDNA and induced with IPTG (Fig. 3c). Cells transfected and induced with mutant F27R αB crystallin cDNA (Fig. 3f), in common with cells transfected and induced with mutant KK174/175LL αB crystallin cDNA (not shown) showed similar immunofluorescence with the polyclonal αB crystallin antibody as with the native αB crystallin, also mostly throughout the cells (Fig. 3c). Increases in ionic strength of the medium produced effects indicating membrane depolarisation, where the immunofluorescence concentrated around the cell periphery, and were not analysed in detail by confocal microscopy.

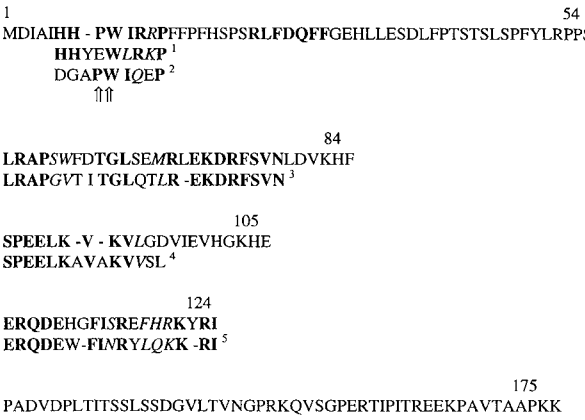

Fig. 4. Sequence comparisons between human αB crystallin (175 residues) and homeobox proteins. Residues in bold show homology; residues in italics show similarity. [1], hexapeptide region of xcad1. [2], hexapeptide region of m-msx-2. ⇑ ⇑, conserved PW in the hexapeptide of the majority of homeobox proteins (W in all). [3], residues encountered in helix 1 of homeodomain proteins (predicted as helix for αB crystallin). [4], residues encountered in helix 2 of homeodomain proteins (predicted as helix for αB crystallin). [5], residues encountered in helix 3/4 of homeodomain proteins (predicted as helix for αB crystallin).

Heat shock of untransfected cells, induced with IPTG with 6 h heat shock at 43°C, showed some increase in immunofluorescence, particularly in the central region of the cells (Fig. 3d), relative to the control (Fig. 3a), suggesting that αB crystallin is a stress-inducible protein in these cells, although immunofluorescence levels were much less than with transfected cells. Cells transfected and induced with native αB crystallin cDNA, with 6 h heat shock at 43°C showed translocation of immunofluorescence to the cental region of the cells, (Fig. 3e), as did cells transfected and induced with mutant KK174/175LL αB crystallin cDNA, with 3 h heat shock at 43°C (Fig. 3h), cells transfected and induced with mutant KK174/175LL αB crystallin cDNA, with 6 h heat shock at 43°C (Fig. 3i); both mutant crystallins behaved in the same way as the native αB crystallin.

4. Discussion

In the present study, while SDS-PAGE and Western blotting revealed that there was a constitutive level of αB crystallin expression in mouse N1E-115 neural cells, confocal microscopy showed that cells transfected with native and mutant αB crystallin cDNAs had significantly greater expression of αB crystallins.

Upon heat shock, αB crystallin tended to concentrate in the central region of the cells. The heat shock behaviour is very similar to that observed with NIH 3T3 cells and constitutively expressed αB crystallin [11]. Interestingly, in the N1E-115 cells, heat shock or cold shock results in translocation of β crystallin predominantly from the central or nuclear region to the outer or cytoplasmic region of the cells [18], possibly to stabilise other proteins and/or to ensure that storage levels of cytoplasmic Ca^{2+} are maintained.

N1E-115 cells transfected with native αB crystallin showed increased thermotolerance under severe heat shock and increased tolerance to high ionic strength. Cells transfected with mutant αB crystallins showed decreased tolerance to heat shock and high ionic strength, although the distribution of αB crystallin in the cells was unaffected by the mutations. The tolerance to heat and ionic shock was similar to that observed previously over a range of concentrations of native and mutant αB crystallins, in vitro, as well as in *E. coli* cells expressing native and mutant protein [13]. Our results had suggested that both charge-charge and hydrophobic interactions are important in protein binding by αB crystallin and that the N-terminal hydrophobic region(s) are important for chaperone-like activity; this is borne out by the recent crystal structure of a small heat shock protein which contains an α crystallin domain [6], although hydrophobic interactions could also take place at other exposed regions on the protein, as 49% of the solvent-accessible surfaces inside the sphere, and 22% of the surfaces on the outside, are composed of nonpolar residues [6]. The results presented in the present paper show that the mutations tested are not involved in the events responsible for any translocation of the protein under stress.

αB Crystallin can bind to DNA (e.g. [19]), and translocation of αB crystallin into the nucleus under heat shock may reflect the binding of αB crystallin to DNA or to DNA binding proteins under stress; a related protein, SEC-1, appears to be adapted to perform essential functions in early embryogenesis of the nematode *Caenorhabditis elegans* [20]. It is of some interest that there is some sequence homology between αB crystallin and the hexapeptide region of two homeobox gene products, xcad1 and m-msx-2, which are involved in cell development [21]. In particular, a tryptophan residue, which is common to all homeobox proteins, appears in this region (see Fig. 4). Furthermore, three other regions of αB crystallin, which are predicted from secondary structure analysis to be α-helical in nature, show sequence similarities to consensus sequences from helix 1, helix 2, and helix 3/4 in homeobox proteins (see Fig. 4), and which in those proteins are involved in DNA binding interactions [21]. It is too early to say whether there is any evolutionary relationship between the small heat shock proteins and the homeobox proteins, but it is possible that αB crystallin may have evolved from a protein important in development into a protein that can be recruited into the nucleus in times of cellular stress.

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