αA-crystallin confers cellular thermoresistance

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Abstract   The bovine eye lens protein αA-crystallin has been overexpressed both by stable transfection of HeLa cells and by transient transfection of NIH 3T3 cells. In both experimental systems αA-crystallin overexpression results in an increased cellular thermoresistance as judged by different clonal survival assays. In contrast, similar overexpression of another stable lens protein, βB2-crystallin, does not confer thermoresistance. These results indicate that the structural relationship of αA-crystallin to the small heat shock proteins HSP25/27 and to αB-crystallin is sufficient for the shared thermoprotective function of all of these molecules and strongly suggests that the chaperone-like properties that they have in common are responsible for the conferred cellular thermoresistance.

Key words: α-Crystallin; β-Crystallin; Small heat shock protein; Thermoresistance

1. Introduction

Heat shock proteins can protect cells from damage caused by heat or other forms of stress. Accordingly, for several members of different HSP families it has been demonstrated that their artificial overexpression confers increased thermoresistance in various cellular systems (for recent reviews see Refs. [1–4]). For the small heat shock proteins (sHSPs), which are ubiquitous and range in size from 15–30 kDa [5], cellular thermoresistance provided by their overexpression has been reported [6–8]. A striking sequence similarity exists between the C-terminal parts of proteins belonging to this group of HSPs and the αA- and αB-crystallins [5,9]. The latter show a sequence identity of 55% [10]. Together, the 20 kDa αA- and αB-crystallins are the subunits that form soluble complexes of up to 800 kDa, constituting one of the most abundant protein components in the vertebrate eye lens [11].

The discovery that expression of αB-crystallin is not restricted to the lens [12–14], was a major breakthrough in crystallin research. Expression of αB-crystallin was found in many tissues, reaching levels of up to 2% in soleus muscle [15], and is increased in neuro-degenerative diseases [16,17], in brain tumors [18] or during oncogene expression [19]. In addition, expression of αB-crystallin can be induced by heat, osmotic and arsenite stress [20,21]. Finally, the recent finding that αB-crystallin confers cellular thermoresistance [22] left no doubt as to its being not only evolutionarily, but also functionally a member of the sHSP family. The extra-lenticular expression of αA-crystallin has also been reported, but only at very low levels [23,24], and, in contrast to the αB-crystallin gene [25], no heat shock element has been identified in its promoter region. It was thus conceivable that between the two subunits, αB-crystallin had retained its function as a stress protein, whereas αA-crystallin had become adapted to function as a lens structural protein.

Recently, a major piece of the puzzle concerning the functional similarities between members of the sHSP family was solved by the discovery that αA- and αB-crystallin as well as HSP25 showed molecular chaperone-like activity in vitro [26–28]. Still little is known about the other piece of the puzzle, the respective functions of αA- and αB-crystallin in vivo. Whereas αB-crystallin seems to behave as a genuine sHSP [21,22], this is not clear for αA-crystallin. However, because of the structural and functional relationships between αA-crystallin, αB-crystallin and HSP25/27, we wondered whether αA-crystallin is also able to provide cells with increased thermoresistance.

In this paper, we present evidence that, in contrast to overexpression of an unrelated lens protein, βB2-crystallin, overexpression of αA-crystallin indeed confers cellular thermoresistance to different cellular systems. These data provide new insight into the mechanism and the minimal structural requirements for the thermoprotective function of sHSPs.

2. Materials and methods

2.1. Plasmid constructions

For the construction of αA-crystallin eukaryotic expression vectors, the 524 bp *NcoI–Bam*HI fragment, containing the coding sequence of the bovine αA-crystallin cDNA, was taken from the vector pET8CαA [29]. For the expression of βB2-crystallin, the 707 bp *NcoI–Eco*RI fragment of the rat βB2-cDNA [30] was used. Both fragments were independently ligated into the *Sma*I-site of the pSVK3 vector (Pharmacia) leading to pSVK3αA and pSVK3βB2, respectively. The αA-fragment was also ligated into the *Hin*dIII-site of the vector pECV5 (obtained from A. Zantema, Leiden), which contains an RSV-promoter and a hygromycine-resistance cassette.

2.2. Transfections, heat shock treatments and clonal survival assays

HeLa cells were grown in DMEM (GIBCO) supplemented with 10% fetal calf serum (GIBCO) and antibiotics, and transfected according to Wigler et al. [31]. Stably transfected cell lines were obtained using hygromycine (ICN) as selecting agent, after which the hygromycine was left out of the medium. One day prior to heat treatment, cells were seeded into 25 cm^2 flasks at a density of 2×10^4 cells per cm^2. In each experiment cells from one αA-expressing and one control cell line were subjected to a heat shock of 60 min at 45.0 ± 0.1°C. For the heat treatment the flasks were immersed into a waterbath. Immediately after the heat treatment, cells were trypsinized, counted and seeded into 9 cm petri dishes at appropriate dilutions and allowed to form colonies for 9–11 days, after which clonal survival was determined.

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Abbreviations: sHSP, small heat shock protein; HSP25/27, mouse/human small heat shock protein.

NIH 3T3-cells were from clone EC0 [32]. Transfection, heat shock treatment and the clonal survival assay were performed as described by Knauf et al. [33].

2.3. SDS-polyacrylamide gel electrophoresis and immunoblotting

Protein samples of the transfected cells were prepared by lysing 10^4 cells in 10 μl SDS-sample buffer at 100°C for 5 min. Proteins were separated by one-dimensional SDS-polyacrylamide gel electrophoresis according to Laemmli [34] and subsequently transferred to PVDF-membrane (Millipore) by the method of Towbin et al. [35]. Immuno-analysis of the blots was performed using specific polyclonal antisera and a chemiluminescence Western Blot kit from Boehringer. The anti-serum against αA-crystallin was raised in rabbits against bovine lens αA-crystallin. The antiserum against bovine βB2-crystallin was obtained from J. Horwitz (UCLA).

3. Results and discussion

In a first attempt to investigate whether expression of αA-crystallin confers cellular thermoresistance, we stably trans-fected HeLa cells with the eukaryotic expression vector for αA-crystallin pECV5αA and, as a control, with the expression vector without insert (pECV5). Two independent αA-crystallin overexpressing cell lines as well as two independent control cell lines were isolated. Expression of αA-crystallin was monitored by immunoblotting as shown in the insert of Fig. 1. The West-ern blot clearly shows expression of αA-crystallin in the αA-transfected cell lines whereas in the control cell lines no expres-sion of αA-crystallin could be detected. The thermoresistance of the different stably transfected cell lines was determined by subjecting the cells to a single heat shock treatment and subse-quently analysing clonal survival. The results of 4 independ-ently performed experiments are presented in Fig. 1. These results show a substantial variability, probably due to minor differences in the condition of the cells between the different experiments. This variability is, however, overcome by the ob-servation that within each experiment the increase in ther-moresistance by αA-crystallin expression is significant. Fur-thermore, the ratio between clonal survival of αA-crystallin overexpressing cells and control cells always ranks between
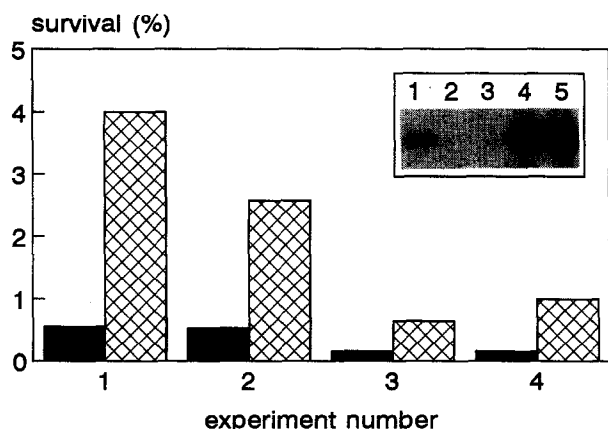
Fig. 1. Analysis of αA-crystallin expression and thermoresistance of stably transfected HeLa cells. Columns represent the clonal survival of cells expressing αA-crystallin (hatched boxes) versus control cells (black boxes) after the heat treatment as a percentage of non heat-treated cells. The results of 4 independent experiments are shown. Insert: Western blot analysis of the expression of αA-crystallin using 2.5 ng of bovine lens αA-crystallin as a marker (lane 1) and protein samples of 10^4 cells of the clones transfected with the control vector (lanes 2,3) as well as the two αA-crystallin transfected clones (lanes 4,5).
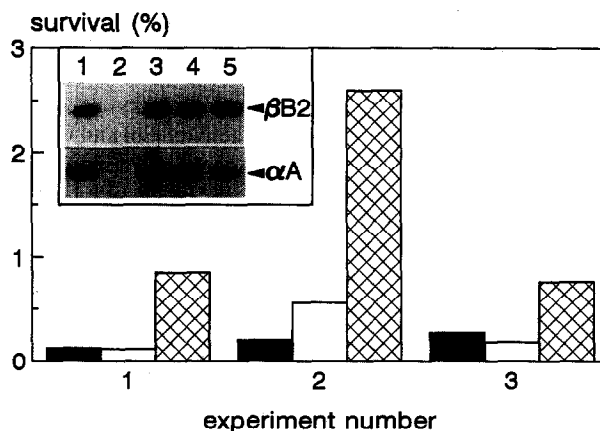
Fig. 2. Analysis of αA- and βB2-crystallin expression and thermoresis-tance of transiently transfected NIH 3T3 cells. Columns represent the clonal survival of NIH 3T3 cells transfected with the expression vector for αA-crystallin (hatched boxes) or βB2-crystallin (blank boxes) or with the control vector (black boxes) after the heat treatment as a percentage of non heat-treated cells. The results of 3 independent exper-iments are shown. Insert: Western blot analysis of the expression of αA-and βB2-crystallin. For comparison of the respective expression levels 2.5 ng of bovine lens αA- and βB2-crystallin were used as markers (lane 1). Protein samples of 10^4 cells transfected with the control vector (lane 2) as well as those of the αA- or βB2-crystallin transfected cells of the 3 experiments (lanes 3–5) were analyzed.

4 to 8 fold. Therefore, these results strongly indicate that ex-pression of αA-crystallin is responsible for an increase in cellu-lar thermoresistance. However, it is generally recognized that stably transfected cells are genetically altered and thus could have a changed phenotype. Although not likely, the observed increase in thermoresistance could also be due to effects other than the expression of αA-crystallin per se.

To avoid this problem of stable transfectants and to general-ize the observation of increased thermoresistance by using a different cellular system, we transiently transfected NIH 3T3 EC0 cells with an αA-crystallin expression vector. For these experiments we used the vector pSVK3αA and the same vector without insert as a control. Two days after transfection expres-sion of αA-crystallin was examined by immunoblotting as shown in the insert of Fig. 2. In parallel, another batch of the transfected cells was subjected to a heat shock at $44.5 \pm 0.1°C$ for 60 min. The ability of the cells to survive the heat treatment was determined by examining the number of colonies that had grown 6–8 days after seeding the cells into soft agar. The results of three independent experiments are depicted in Fig. 2. These results indicate that overexpression of αA-crystallin can also provide NIH 3T3 cells with thermoresistance, thus confirming and extending the results of the experiments with HeLa cells.

It is possible that introduction of denatured or mutated pro-teins into eukaryotic cells as well as transient expression of exogenous proteins can trigger a general stress response [36]. It has been previously reported [33] that overexpression of HSP25 in these NIH 3T3 EC0 cells does not change the intra-cellular levels of HSP70, HSP90 and αB-crystallin, which are all known to be heat inducible proteins [21,37]. Because of the structural relationship between αA-crystallin and HSP25 [27] we reasoned that this would also be the case for expression of αA-crystallin. Nevertheless, to eliminate the possible effect of expression of an exogenous protein on the cellular thermoresis-tance, we determined the effect of expression of the unrelated

βB2-crystallin. This protein was chosen because, like αA-crystallin [38], it is a very stable protein [39,40] and its expression is mainly restricted to the lens and has not been reported for any eukaryotic cell line. In addition, unlike αA-crystallin, βB2-crystallin has no molecular chaperone activity [26] and is therefore not expected to have heat shock protein properties [2,3]. Since it has been reported that the increase in cellular thermoresistance caused by overexpression of sHSPs in mammalian cells correlates with the expression level [22,33], it is important that those of βB2- and αA-crystallin be comparable. For that reason, we determined these expression levels by semi-quantitative immunoblotting (see the insert of Fig. 2) and found that the level of βB2-crystallin overexpression was comparable with that of αA-crystallin and estimated to be 2.5 ng protein/10^4 transfected cells. Analysis of the clonal survival of the cells overexpressing βB2-crystallin was carried out in parallel with those of the αA-crystallin transfected cells and shows convincingly that there is no significant increase in thermoresistance when an unrelated protein such as βB2-crystallin is overexpressed (Fig. 2).

The data presented allow the conclusion that expression of αA-crystallin in mammalian cells provides these cells with thermoresistance. Apparently, despite its almost exclusive function as a lens structural protein and its evolutionary divergence, αA-crystallin has retained the thermoprotective capacity shared by αB-crystallin and HSP25/27 [6,22,33]. The present data and those in the literature [6,22,33] do not yet allow a good comparison of the relative thermoprotective capacities of these proteins. It will be interesting to assess this matter in further detailed comparative studies. Our present findings further support the functional relationship between these members of the small HSP family. In addition, our results support the notion that there is a direct relationship between the in vitro activity of a protein as a molecular chaperone and its capability to confer cellular thermoresistance [2,3,33] by showing that of two proteins, αA- and βB2-crystallin, the one with molecular chaperone activity is able to confer cellular thermoresistance, whereas the other is not. Because no stress related function of αA-crystallin has been reported until now, the physiological significance of the thermoresistance conferring activity of αA-crystallin remains to be elucidated.

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