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Rat Hsp20 Confers Thermoresistance in a Clonal Survival Assay, but Fails to Protect Coexpressed Luciferase in Chinese Hamster Ovary Cells

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Hsp20 is a mammalian small heat shock protein with some deviating *in vitro* characteristics. We now compare the *in vivo* cellular thermoprotective abilities of Hsp20 with those of its direct relative, αB-crystallin. In a clonal survival assay Chinese hamster ovary (CHO) cells stably overexpressing Hsp20 survive equally well as αB-crystallin-expressing cells, after a heat shock. In a transient assay, however, overexpression of Hsp20 did not result in an enhanced recovery of coexpressed firefly luciferase after heat shock, in contrast to αB-crystallin. This might indicate that these highly homologous stress proteins are involved in at least partially distinct protective activities in cultured cells.

Key Words: **small heat shock protein; clonal survival assay; luciferase.**

Upon heat shock or other forms of stress a whole array of so-called heat shock proteins is expressed. A specific subset is formed by the small heat shock proteins (sHsps), comprising a ubiquitous superfamily of proteins that are characterized by a conserved sequence known as the α-crystallin domain (1). Some members of this superfamily are inducible upon stress, while others are expressed constitutively. In mammals, the oldest known representatives are αA- and αB-crystallin, which are highly expressed in the eye lens. Outside the lens αB-crystallin is also abundant in heart and some skeletal muscle tissues (2). Another familiar relative is Hsp27, also called Hsp25 in rodents. The α-crystallins and Hsp27 occur as 600- to 800-kDa multimeric structures of which the most conspicuous property is their *in vitro* chaperone-like capacity (3). Cryo-EM studies show that αB-crystallin forms hollow spherical complexes (4), and a similar structure with 14 "windows" is revealed by X-ray analysis of an archaebacterial sHsp (5).

During the last few years, new mammalian sHsps have been reported, of which Hsp20 is the best characterized (6, 7). For the other two representatives, HspB3 (8) and HspB2 (9), mainly sequence and expression data are as yet available. Like αB-crystallin, Hsp20 is highly expressed in heart and muscle tissue, but considerable amounts of Hsp20 are also found in smooth muscle of bladder and rectum, and in vascular smooth muscle (6). In rat hindlimb muscle, the expression of Hsp20 is developmentally regulated and related to muscle contraction (10). In vascular smooth muscle tissue, phosphorylation of Hsp20 seems to play a role in the contraction process (11). Furthermore, it has been reported that Hsp20 has an inhibitory function in the regulation of platelet activation (12).

Overexpression of various sHsps has been shown to enhance the survival of cells subjected to different forms of stress, including heat shock (13). The mechanisms involved in this cellular protection remain largely unknown. We recently found that recombinant rat Hsp20 deviates from α-crystallins and Hsp27 in being structurally a less stable protein, a poorer chaperone and having a tendency to dissociate into dimers (7). Wondering about the implications of these *in vitro* properties for the *in vivo* protective capacities, we here compare the abilities of Hsp20 and αB-crystallin to protect CHO cells against the deleterious effects of heat shock. To that end we used both a transient assay, in which the protection of cotransfected luciferase is assessed and a clonal survival assay of cells stably expressing Hsp20 or αB-crystallin. Hsp20 and αB-crystallin performed differently in these assays, indicating different physiological functions for these two sHsps.

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MATERIALS AND METHODS

Cell culture and transient transfection. CHO cells were cultured in DMEM (Gibco, Paisley, Scotland), supplemented with 10% fetal bovine serum (Gibco). One day before transfection with Lipofectamin (Gibco), CHO cells were plated at a density of 0.5×10^6 cells per 30-mm culture dish. Transfection mixtures for each dish contained 0.5 μg of a luciferase expression plasmid with an SV40 promoter (pGL2 control, Promega, Madison WI, USA) in combination with either the αB-crystallin or the Hsp20 expression vector or a negative control plasmid. In these vectors the hamster αB-crystallin gene (14) is controlled by an RSV promoter and the rat Hsp20 cDNA (7) is cloned downstream of a mouse Moloney virus LTR. As a negative control we chose an expression vector encoding the RSV promoter and the unrelated non-heat shock protein βB2-crystallin (15). The total amount of DNA in each mixture was 2 μg. One day after transfection, cells from each 30-mm dish were subcultured into 6-10 new 30-mm dishes, each containing 0.15×10^6 cells, representing parallel sister cultures. Approximately 48 h after transfection these parallel cultures were either subjected to a heat shock (for 40, 50, 60, or 70 min at 44.5°C) in a precision water bath, or kept at 37°C. At the same time, cells from parallel culture dishes were harvested to be used for Western blot analysis with anti-αB-crystallin (16) and Hsp20 (7) antisera. Purified recombinant proteins (7) were used to roughly estimate the expression levels of the overexpressed proteins. In a separate control experiment, transfection efficiencies of the different DNA mixtures were determined by co-transfection of a β-galactosidase expression plasmid (Promega, Madison WI) and staining for β-galactosidase activity according to the manufacturer's instructions.

After a further 24 h incubation under standard culture conditions, heat-treated and control cells were harvested and a luciferase activity assay was performed (Boehringer, Mannheim, Germany). In this particular transient assay the measured luciferase activities are derived exclusively from the transfected cells. Therefore, protective effects of the different transfected sHsps are directly reflected by the luciferase activity. The luciferase activities of the stressed cells were related to the activity of the non-stressed transfected control cells. The calculations were independent of transfection efficiencies since we used parallel sister cultures within the same experiment.

Transfection of virus helper cells, infection of CHO cells and clonal survival assay. The virus packaging cells ιCRE derived from NIH 3T3 helper cells (17) were cultured in DMEM (Gibco), supplemented with 10% fetal bovine serum (Gibco). Transfection was performed with a viral vector, pMV6 (18) containing the mouse Moloney virus LTR and either the rat αB-crystallin cDNA or the rat Hsp20 cDNA. After a ten days selection with G418/geneticin at a concentration of 400 μg/ml (Gibco) the resulting virus-producing colonies were pooled, and overnight medium from the pooled clones was used for infection of CHO cells. Again after selection with G418 for 5–6 days, surviving CHO cells were replated 24 h before the heat stress in medium without G418, in a concentration of 0.5×10^6 cells in each 30-mm dish, representing a cell population stably overexpressing either Hsp20 or αB-crystallin. The resulting cell population is not monoclonal and represents probably different viral integration sites. Heat shocks were applied in a precision water bath for various periods of time (40, 50, 60, 70 min) at 44.5°C. To perform a clonal survival assay, cells were trypsinized and replated directly after the heat shock in appropriate dilutions in 30-mm dishes. After 8–10 days incubation at 37°C in a humidified CO_2 incubator, colonies were stained with 1% crystal violet. Colonies containing more than 50 cells were counted. To determine the amounts of αB-crystallin and Hsp20 at the moment of the heat shock, parallel cultures of the virus-infected cells were harvested for Western blot analysis with polyclonal antisera directed against αB-crystallin (16) and Hsp20 (7).

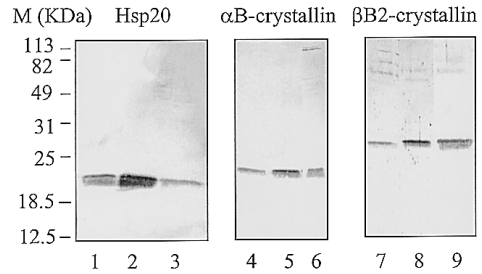

FIG. 1. Expression of Hsp20, αB-crystallin and βB2-crystallin in CHO cells 48 h after transient transfection. Western blot analysis was performed with polyclonal antisera directed against Hsp20, αB-crystallin and βB2-crystallin. Purified proteins were loaded as markers in lanes 1 and 2 (10 and 30 ng of Hsp20), 4 and 5 (2 and 5 ng of αB-crystallin) and 7 and 8 (5 and 30 ng of βB2-crystallin). In lanes 3, 6, and 9 extracts were applied from the respective transiently transfected CHO cells, equivalent to 0.3, 0.08, and 0.3×10^6 cells. Densitometric scanning revealed that expression levels for αB-crystallin and Hsp20 are within the same range, varying from 15 to 25 ng/10^6 cells.

RESULTS AND DISCUSSION

Hsp20 does not protect cotransfected luciferase. There are several methods to study the effect of a particular heat shock protein on cellular survival after stress. Conventional cytotoxicity assays are based on differences in metabolic activities of protected and non-protected cells, or on determining the colony-forming ability of such cells. Both methods rely upon the homogeneous overexpression of the potentially protective proteins of interest in a stably transfected cell population. However, overexpression of heat shock proteins sometimes results in decreased growth rates of cells (19, 20). In search of different approaches, involving transient overexpression of sHsps, we adapted a protocol developed by Williams *et al.* (21). In our assay, transient transfection is used to overexpress a heat shock protein together with luciferase as a reporter protein in CHO cells which are devoid of Hsp20 as well as αB-crystallin. Such cotransfection of heterologous plasmids results in simultaneous uptake of both plasmids in the majority of the cells that are successfully transfected (21). Since the expression of the protein of interest and the reporter protein are generally most pronounced after 48 h, this time point has been taken to subject the cells to heat shock. After a subsequent recovery period of 24 h, the activity of the reporter protein can be measured (21), reflecting exclusively the viability of the transfected cells.

As shown in Fig. 1, both Hsp20 and αB-crystallin are highly expressed, at comparable levels, in transiently transfected CHO cells. This is also true for the unrelated non-heat shock protein βB2-crystallin, which is used as a negative control in this assay. The transfection efficiencies of the different DNA mixtures were comparable, and estimated at approximately 10%, as

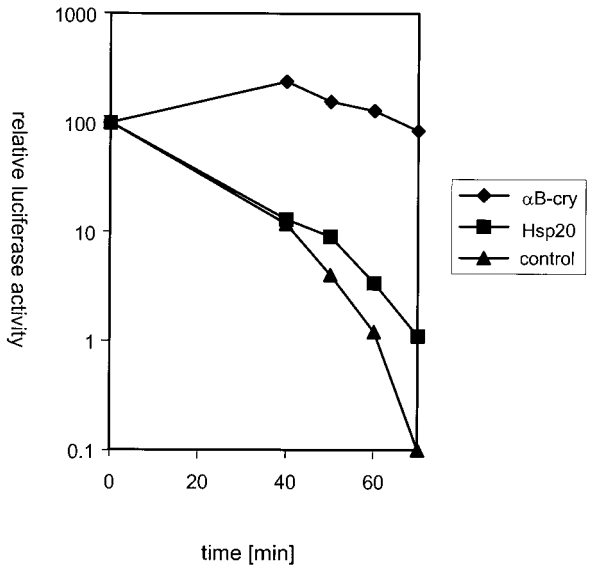

FIG. 2. Comparison of protection of luciferase activity by overexpressed Hsp20 and αB-crystallin in CHO cells. Luciferase activity was determined 24 h after a 40- to 70-min heat shock at 44.5°C, in CHO cells cotransfected with Hsp20, αB-crystallin (αB-cry) or βB2-crystallin (control) expression vectors in combination with a luciferase plasmid. The measured luciferase activity is represented as a percentage of the activity of non-heat-shocked cells. Experiments were repeated four times and representative results are shown.

determined in a control transfection experiment by coexpression and staining for β-galactosidase (results not shown).

In Fig. 2 the luciferase activity detected 24 h after heat treatment of the transiently transfected cells is shown relative to the activity detected in non-stressed cells. It is immediately clear that far more luciferase activity can be measured in heat-shocked cells overexpressing αB-crystallin, than in cells overexpressing Hsp20 or a control protein such as βB2-crystallin. In fact, the luciferase activity detected in cells overexpressing Hsp20 is almost as low as that in cells transfected with the negative control. Thus, in comparison to αB-crystallin, Hsp20 is far less adequate in providing cellular protection in such a way that it results in retaining the luciferase activity. Interestingly, in cells transfected with αB-crystallin the luciferase activity detected after a heat shock of 40, 50, or 60 min is even higher than in non-treated cells. This is a common observation, presumably caused by overactivation of different cellular repair mechanisms after the heat shock (G.J.J.S., unpublished).

Hsp20 performs well in a clonal survival assay. Since both αB-crystallin and Hsp25 perform well in the luciferase assay (22), the poor performance of Hsp20 in the luciferase protection assay was rather unexpected. Therefore, we also performed a more conventional clonal survival assay with CHO cells stably overexpressing either αB-crystallin or Hsp20. The expression levels of both heat shock proteins in the stably transfected cells are presented in Fig. 3. Although we did not try to determine the exact protein levels, it is clear that both proteins are expressed at comparable, though rather low, levels. Surprisingly, a clonal survival assay reveals that Hsp20 displays a similar protective capacity as does αB-crystallin (Fig. 4). The protective effect is most pronounced after a heat shock of 50 min, where Hsp20- and αB-crystallin-overexpressing cells both provide approximately 100 times more colonies than control cells.

Overexpression of αB-crystallin and Hsp20 did not result in decreased growth rates. Similar numbers of cells of both lines result in equal numbers of colonies in comparison with non-infected control cells. Also the plating efficiency for all three lines was close to 100%. The number of colonies arising ten days after the heat shock in our experimental setting is somewhat lower than previously reported (20, 23). This may be due to the fact that we used a pooled population, whereas usually the cell line with the highest expression is used. In a control experiment, using cells infected with a virus only expressing the geneticin selection marker, it was excluded that the enhanced cellular survival was somehow induced by the viral infection. It can be concluded that Hsp20, like all other sHsps tested so far (13) exhibits the ability to confer thermoresistance in a clonal survival assay.

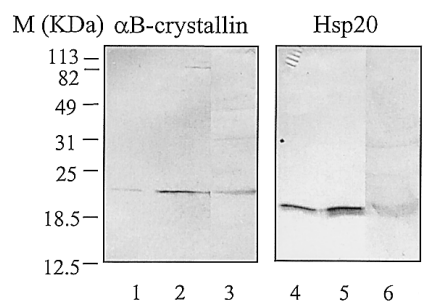

FIG. 3. Expression of Hsp20 and αB-crystallin in virus-infected CHO cells. Western blot analysis was performed as described in the legend to Fig. 1. Lanes 1, 2, 4, and 5 contain 1 and 5 ng of αB-crystallin, and 5 and 10 ng of Hsp20, respectively. In lanes 3 and 6 equal amounts of protein were loaded, corresponding with 10^6 CHO cells overexpressing either αB-crystallin or Hsp20. The expression levels in these stable cell lines are indeed much lower (approximately 1 ng αB-crystallin and less than 1 ng Hsp20/10^6 cells) than in the transient transfections depicted in Fig. 1, also considering the fact that in the transient transfections only about 10% of the cells overexpresses the transfected protein. The low expression upon stable transfection is due to the fact that overexpression of sHsps results from a single integrated copy of the expression virus per cell.

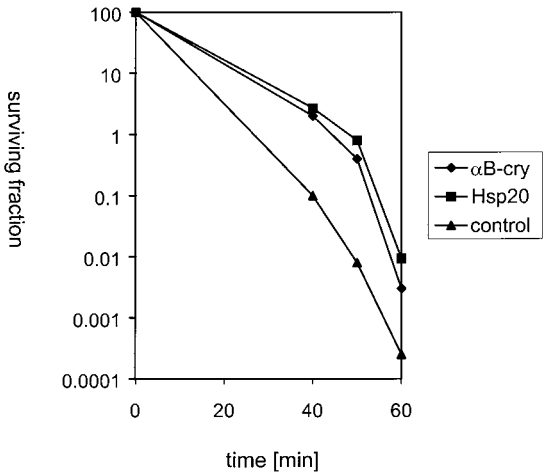

FIG. 4. Thermoresistance of CHO cells stably overexpressing Hsp20 or αB-crystallin, in comparison with non-infected control cells. Surviving colonies were counted 10 days after heat shock and trypsinization, and numbers are presented as percentages of the number of colonies arising from non-treated cells. Experiments were repeated four times with similar results and a representative graph is shown. Cells infected with an empty virus (not shown) behave similar to the noninfected control CHO cells.

Comparison of luciferase protection and clonal survival assays. The mechanisms involved in the protective effect of sHsps on cellular survival after a heat shock remain largely elusive. *In vitro,* the sHsps prevent protein aggregation by binding to hydrophobic regions in unfolding polypeptides. This chaperone-like behavior is likely to play a role in the processes occurring during heat shock. In previous experiments we found that luciferase activity in αB-crystallin- and Hsp27-transfected cells vanishes directly after a heat shock (22). Restoration of the enzyme activity could be blocked by cycloheximide, indicating that sHsps are involved in the protection of the protein synthesizing machinery allowing *de novo* synthesis of luciferase. Alternatively, or additionally, it is possible that during heat shock the rather heat-labile luciferase denatures irreversibly in the control cells, whereas it is kept in a folding-competent state (24, 25) in shsp-overexpressing cells. Directly after the heat shock, the sHsp-bound luciferase may be refolded, perhaps in conjunction with other Hsps, resulting in an increased luciferase activity in comparison to control cells. In the latter case newly synthesized proteins are involved because no luciferase activity could be measured in cycloheximide-treated cells.

Speculating that the chaperone-like properties of sHsps play a role in the restoration or protection of luciferase activity, it is quite conceivable that Hsp20, being a poorer chaperone (7) is less effective in facilitating the renewed synthesis or refolding of luciferase. Since no major differences were found between Hsp20 and αB-crystallin in the clonal survival assay, this might suggest that the chaperone-like activity does not play a similar role in this assay. A fundamental difference between the luciferase protection assay and the clonal survival assay is the fact that in the latter case cell division and attachment are essential. It is generally accepted that the cytoskeleton plays an important role in both these processes. One of the most conspicuous *in vivo* properties of αB-crystallin and Hsp27 is that they are not only able to colocalize with cytoskeletal structures, but may also be involved in maintaining the integrity of the cytoskeleton (16, 23, 26–28). Therefore, the cytoskeletal protective capacities of sHsps may be especially important in dividing cells. Our results with Hsp20 and αB-crystallin, indicate that the various thermoprotective properties of the sHsps reside in different mechanisms. The current data only allow speculations concerning the possibly lesser importance of the chaperone-like behavior of sHsps for the protection of cytoskeletal structures, and require future experimental corroboration.

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