Overexpression of the gene encoding the 70-kDa heat shock protein (hsp70) has previously been shown to protect neuronal cells against subsequent thermal or ischemic stress. It has no protective effect, however, against stimuli that induce apoptosis, although a mild heat shock (sufficient to induce hsp synthesis) does have a protective effect against apoptosis. We have prepared herpes simplex virus-based vectors that are able to produce high level expression of individual hsps in infected neuronal cells without damaging effects. We have used these vectors to show that hsp27 and hsp56 (which have never previously been overexpressed in neuronal cells) as well as hsp70 can protect dorsal root ganglion neurons from thermal or ischemic stress. In contrast, only hsp27 can protect dorsal root ganglion neurons from apoptosis induced by nerve growth factor withdrawal, and hsp27 also protects the ND7 neuronal cell line from retinoic acid-induced apoptosis. However, hsp70 showed no protective effect against apoptosis in contrast to its anti-apoptotic effect in non-neuronal cell types. These results thus identify hsp27 as a novel neuroprotective factor and show that it can mediate this effect when delivered via a high efficiency viral vector.

The heat shock proteins (hsps) were originally identified on the basis of their increased synthesis following exposure to elevated temperature. Subsequently, however, they were shown to be induced by a wide variety of other stresses in many different cell types (for review see Refs. 1–3) including neuronal cells exposed to ischemia (4), amphetamine treatment (5), or to sodium arsenite (6). Such induction of the hsps has been shown to have a protective effect against exposure to a subsequent stress in a variety of cell types. Thus, for example, exposure of primary neuronal cultures to a mild heat stress or ischemic stress sufficient to induce the hsps has been shown to be protective against subsequent exposure to the excitotoxin glutamate or to severe heat or ischemic stress (6–8), whereas similar exposure in vivo can protect against damaging effects caused by subsequent exposure to light (9) or ischemia (10, 11).

In a number of cases the protective effect of a mild hsps-inducing stress can be reproduced by the artificial overexpression of a single hsp. Thus, for example, dorsal root ganglion (DRG) neurons can be protected against thermal or ischemic stress by overexpression of either the 70-kDa hsp (hsp70) or the 90-kDa hsp (hsp90) (12–14), and a similar protective effect of hsp70 and hsp90 has also been observed in the ND7 immortalized cell line derived from sensory neurons (15). Interestingly, Fink et al. (16) were able to protect cultured hippocampal neurons against subsequent heat shock using a herpes simplex virus (HSV)-derived amplicon vector expressing hsp70 indicating that this effect applies to neurons derived from both the central and peripheral nervous systems. Moreover, the use of an HSV-based vector opens up the possibility of testing the protective effect of the hsps in vivo and of their ultimate therapeutic use in man. Such vectors can effectively deliver genes to neuronal cells following in vivo injection and may be useful for gene therapy procedures (for review see Refs. 17 and 18).

Such a possibility is of particular interest in view of the finding that a prior mild heat shock can protect neonatal DRG neurons against apoptosis induced by withdrawal of nerve growth factor (15) and similarly protects ND7 cells against apoptosis induced by serum withdrawal and addition of retinoic acid (19). Thus the ability to manipulate the rate of apoptosis would be of use in neurological diseases such as muscular dysgenesis (20) or spinal muscular atrophy (21) which involve changes in the normal level of neuronal cell apoptosis during development as well as in later onset diseases such as Alzheimer’s or Parkinson’s diseases where the excessive neuronal cell death may be apoptotic in nature (20).

Unfortunately, however, it has not proved possible thus far to identify a single hsp whose overexpression in neuronal cells can protect them from apoptosis. Thus, overexpression of hsp70 can protect several different non-neuronal cell types from apoptosis including fibrosarcoma cells (22), normal fibroblasts (23), and T cell leukemia cell lines (24, 25). In contrast, however, overexpression of hsp70 or hsp90 in ND7 cells (15) or DRG neurons (14) does not reproduce the protective effect of mild heat shock against subsequent apoptotic stimuli. Similarly, in the experiments of Fink et al. (16) overexpression of hsp70 with an HSV vector did not protect hippocampal neurons against glutamate toxicity which may act by inducing apoptosis (26).

It is clear therefore that the protective effect of heat shock against apoptosis in neuronal cells cannot be reproduced by hsp70 alone despite its protective effect in other cell types. The protective effect could involve another hsp such as hsp27 which has also been shown to protect non-neuronal cell types against apoptosis (22, 27) but has not been artificially overexpressed in neuronal cells. Alternatively, it could require a combination of several hsps or some other effect of heat shock not involving...
hsp synthesis. Such protective effects not involving hsp synthesis are likely to account for cases in non-neuronal cells where tolerance to stress can be enhanced in the absence of hsp synthesis (28, 29) or where cells with the same hsp levels exhibit different levels of thermotolerance (30). In this case it would evidently not be possible to produce an effective anti-apoptotic effect using gene therapy procedures involving the overexpression of one or more hsp genes.

To resolve these possibilities and attempt to identify the hsp involved in the protective effect against apoptosis, we have constructed HSV-based vectors expressing individual hsps and used these to investigate their protective effects in vitro. These viruses offer a high efficiency means of delivering specific genes to primary neuronal cells and could be used, for example, to overexpress several different hsps in the same neuronal cell. Moreover, they could also ultimately be used to direct similar overexpression of hsps in vivo, in gene therapy procedures.

MATERIALS AND METHODS

Virus Construction—Hsp cDNAs or a control β-galactosidase gene (from pCH110; Pharmacia) under the control of the cytomegalovirus immediate early promoter (from pJ7; Ref. 54) were inserted into a plasmid containing the region of the HSV-1 genome encoding the latency-associated transcript promoter region (HSV-1 strain 17+; GenBank™ accession number HE11C0). Hsp cDNAs were Chinese hamster hsp27 (from Jacques Landry (31)), rabbit hsp56 (from Marie-Claire Lebeau), inducible human hsp70 (32), and human hsp90 (33, 41). Hsps were introduced into the latency-associated transcript region of an HSV vector deleted for ICP27 (34, 49) directly replacing the lacZ gene in the control virus described above by standard homologous recombination. Here a blue/white selection for recombinant plaques was performed after 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside staining, while Hsp-expressing plaques were being picked and plaque-purified five times followed by Western blotting to confirm expression of the appropriate Hsp from each virus. ICP27 deleted viruses were grown in B130/2 BHK cells that have been stably transfected to express ICP27 (49) allowing lytic growth of the virus. There is no overlap between sequences inserted into the cell line to complement ICP27 and the ICP27-deleted virus in this virus cell line combination, and thus homologous recombination and repair of the ICP27 deletion is undetectable (reversion frequency <1 in 10^7 plaque-forming units (49)).

Viruses Infections—B130/2 cells, parental BHK cells (35), ND7 cells (36), and neonatal rat primary dorsal root ganglion cells were infected in each case at a multiplicity of infection (m.o.i.) of 10 in serum-free media for 1 h. Virus containing media was then replaced with full growth media and incubation continued for a further 16 h prior to the induction of cell stress or harvesting of the cells for Western blotting.

Cell Culture, Neonatal Dorsal Root Ganglion Cultures— Cultures were prepared from 2-day-old Sprague-Dawley rats. Spinal ganglia were removed aseptically, digested with 0.3% collagenase (Boehringer Mannheim) for 90 min, and then mechanically dissociated through a 1-ml Gilson tip. Routinely cultures contain approximately 90% neurons and 10% glial cells as assayed by morphology and staining with appropriate antibodies. Cells were plated and grown overnight in defined media (37) on sterile glass coverslips and subsequently transferred to 24-well plates (Nunc, Roskilde, Denmark). Cells were infected/mock-infected over 1 h in defined media in the absence of bovine serum albumin. After overnight incubation the cells were either exposed to heat shock (see below), simulated ischemia (see below), or stimulated to undergo apoptosis by prolonged incubation in defined media in the absence of nerve growth factor (NGF). Viable neurons were assessed at 12 and 24 h recovery after insult (heat shock and ischemia) and at 24 and 48 h after prolonged NGF withdrawal by visualization at 400 × magnification under light microscopy. The parameters used to define viable neurons were phase bright bodies, agranular appearance, and non-ruffled membranes. Non-viable neurons were defined by phase dark bodies, lack of neurites, granular/vacuolated appearance, and ruffled membranes (14).ND7 Cells—ND7 cells were originally generated as a fusion of primary sensory DRG neurons and neuroblastoma cells and display many of the characteristics of sensory neurons (36). ND7 cells were grown in Leibovitz L15 medium with 10% fetal calf serum and infected as above before exposure to either lethal heat shock (see below) or simulated ischemia (see below) prior to assay of cell viability by trypan blue exclusion (see below). To induce the cells to cease dividing and undergo morphological differentiation or apoptosis, they were transferred to serum-free medium consisting of a 1:1 mix of Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) and nutrient mix Ham’s F12 (Life Technologies, Inc.) supplemented with human transferrin (5 μg/ml), bovine insulin (250 ng/ml), and sodium selenite (30 nM). All-trans-retinoic acid was added to a final concentration of 1 μM to increase the proportion of cells undergoing apoptosis. This procedure has previously been shown to induce cell death by apoptosis in these cells (43).

In Situ Detection of Apoptosis—ND7 cells were seeded into 8-well chamber slides and infected at an m.o.i. of 10 with each of the HSV vectors expressing lacZ (control) or each of the Hsps individually. After serum withdrawal/addition of retinoic acid, the cultures were incubated for 48 h at 37 °C, 5% CO2. Media were removed, and the cells were fixed with 1% paraformaldehyde for 30 min. Slides were then washed twice in phosphate-buffered saline at 37 °C and TUNEL reactions (Boehringer Mannheim) performed according to the manufacturer’s instructions. The samples were visualized using fluorescein optics, and the numbers of positive staining, apoptotic cells were counted in three confluent fields of view for each sample.

Heat Shock—Growth media was pre-warmed to 48 °C and 1 ml/well added to ND7 cells or primary rat DRG cells in vitro. The plates were wrapped in parafilm and incubated in a water bath at 48 °C (lethal heat shock) for 20 (ND7 cells) or 30 min (rat primary DRG cultures) (18) followed by transfer to a 37 °C, 5% CO2 incubator for recovery for a period of 1 (ND7 cells) or 24 h (primary neurons) as in our previous study (43).
Hsp27 and Apoptosis

FIG. 2. Hsp expression in recombinant HSV infected ND7 cells. Western blotting was carried out with antibody to hsp27 (a), hsp56 (b), or hsp70 (c) using extracts from uninfected cells (control, C), cells infected with virus expressing β-galactosidase (lac), or virus expressing the appropriate hsp as indicated. RM, molecular mass in kilodaltons.

TheND7 cells were gently harvested and gently resuspended in 100 μl of ice-cold phosphate-buffered saline before trypan blue exclusion assay for cell viability (see below). Primary DRG neurons were assessed in situ at 0, 12, and 24 h time points, similar to work reported previously (14).

Simulated Ischemia—Ischemia can be simulated in vitro by incubating the cells in a physiological buffer containing raised levels of lactic acid, high potassium, and decreased pH and inhibitors of electron transport and glycolysis (38). ND7 and rat DRG cells were cultured in a 24-well plate and mock-infected or infected with the viruses 24 h prior to ischemia. The cells were incubated for 4 h at 37 °C, 5% CO₂ in either control buffer (pH 7.4) or ischemic buffer (38). Viable ND7 cells were harvested as above and counted immediately after insult. Viable primary DRG neurons were visualized and counted after 12 and 24 h of ischemia as described above, and in each case the degree of hsp induction was determined in comparison to cells infected with lacZ expressing control virus. Equal loading of protein samples was confirmed by Coomassie staining of duplicate gels, in each case protein extracted from ~1 × 10⁵ cells run/lane.

Western Blotting—Protein samples were electrophoresed on polyacrylamide gels, transferred to nitrocellulose filters as described previously (19, 39), and probed with either an anti-hsp90 antibody (AC88; see Ref. 40), or antibodies to the other hsps purchased from StressGen Ltd. Cells were harvested 16 h after infection as described above, and in each case protein from ~1 × 10⁵ cells run/lane.

FIG. 3. ND7 cell survival following severe heat shock when infected with HSV vectors expressing hsps. Black bars, proportion of surviving mock-infected (ND7) and virus-infected ND7 cells incubated at 37 °C, 5% CO₂ throughout. Striped bars, proportion of surviving mock-infected (ND7) and virus-infected ND7 cells after 20 min heat shock at 8 °C with 1 h recovery at 37 °C, 5% CO₂. Cell survival was assessed by trypan blue exclusion assay. Bars represent S.E. of the means calculated from the means of three counts for each sample (n). n for all experiments = 6. *, significant difference in survival compared with mock-infected ND7 cells (p < 0.001); +, significant difference in survival compared with lacZ virus-infected ND7 cells (p < 0.001). Calculated using a Bonferroni Multiple Comparison t test after one-way analysis of variance.

FIG. 4. ND7 cell survival following simulated ischemia when infected with HSV vectors expressing hsps. Black bars, proportion of surviving mock-infected (ND7) and virus-infected ND7 cells incubated at 37 °C, 5% CO₂ in control buffer for 4 h. Striped bars, proportion of surviving mock-infected (ND7) and virus-infected ND7 cells after 4 h incubation in ischemic buffer at 37 °C, 5% CO₂. Cell survival was assessed by trypan blue exclusion assay. Bars represent S.E. of the means calculated from the means of three counts for each sample (n). n for all experiments = 6. *, significant difference in survival compared with mock-infected ND7 cells (p < 0.001); +, significant difference in survival compared with lacZ virus-infected ND7 cells (p < 0.001). Significance calculated using a Bonferroni Multiple Comparison t test after one-way analysis of variance.

or by direct addition to the cell culture (DRGs). Mixtures were incubated at room temperature for 5 min, and the proportion of cells able to exclude trypan blue was assessed.

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RESULTS

Previously an HSV-based ampiclon vector overexpressing hsp70 has been reported (16). Here a plasmid containing an HSV origin of replication and packaging sequence is propagated by transfection into susceptible cells and co-infection with a helper virus (for review of HSV vectors see Refs. 17 and 18). Progeny virions consist of a mixture of helper virus and...
packaged amplicon vector. This system suffers from the disadvantage that it produces a mixed viral population consisting of packaged amplicon and helper virus whose ratio varies between different stocks, although more recently a technique allowing low efficiency helper virus-free amplicon growth has been reported (55). We have used the alternative system in which hsp90, hsp70, hsp56, and hsp27 were introduced into a disabled HSV strain lacking the viral gene encoding ICP27. ICP27 is essential for HSV replication (42) and thus ICP27 deletion results in an efficient gene trans-

FIG. 5. ND7 cell survival over time following serum withdrawal in the presence of all-trans-retinoic acid. Proportion of surviving ND7 cells over time following serum withdrawal in the presence of 1 μM all-trans-retinoic acid. Cell survival was assessed by trypan blue exclusion assay. ND7 cells either mock-infected or infected with the lacZ recombinant HSV-1 vector are shown in all graphs. Graphs a, b, and c show the results for cells infected with viruses expressing hsp27, hsp56, and hsp70, respectively. Bars represent S.E. of the means calculated from the means of three counts for each sample (n). n = 4–6. Significant difference in the results were calculated using a Bonferroni Multiple Comparison t test after one-way analysis of variance. * indicates significant differences in survival between hsp-overexpressing ND7 cells and lacZ virus-infected ND7 cells or mock-infected ND7 cells, respectively (p < 0.001 for hsp27 and p < 0.05 for hsp70).
the cells were incubated in serum-free media containing 1 μM all-trans-retinoic acid. We have previously shown that the ability of ND7 cells to survive the removal of serum together with the addition of retinoic acid. Following plaque purification, viruses were tested for their ability to express the appropriate hsp following infection of B130/2 cells (49) which are BHK fibroblast cells stably transfected to express ICP27 and allow growth of the virus. Clear overexpression of hsp70, hsp56, and hsp27 was seen with the appropriate virus (Fig. 1) at levels comparable to those observed in stressed cells (data not shown). In contrast, the hsp90 virus did not show significant overexpression of hsp90 above endogenous levels. Similar results were also seen in parental BHK cells (data not shown) and in ND7 cells which are of neuronal origin (Fig. 2), confirming that hsps were also overexpressed without virus replication, and in particular in cells of neuronal origin.

As the hsp90 virus did not direct expression of hsp90 significantly above endogenous levels, it was not used further for these experiments. However, the other viruses were used to infect initially ND7 cells and subsequently primary DRG cultures. ND7 cells provide a convenient cell type of neuronal origin to initially assess the protective effect of the viruses against various insults. In these experiments, cells infected with the control virus expressing β-galactosidase showed comparable survival either before or after stress to that observed in uninfected ND7 cells (Figs. 3 and 4), showing that the virus itself did not have any significant cytopathic effect in these cells. Moreover, the hsp70-expressing virus was able to protect ND7 cells from subsequent exposure to both a severe thermal (Fig. 3) or ischemic (Fig. 4) stress, confirming our previous results obtained with ND7 cells stably transfected with a plasmid-expressing hsp70 (15). A similar protective effect was also observed with the virus expressing hsp27, but not with the virus expressing hsp56 (Figs. 3 and 4), providing for the first time data on the protective effect of overexpressing these proteins in neuronal cells.

We next tested the effect of each of these viruses on the ability of ND7 cells to survive the removal of serum together with the addition of retinoic acid. We have previously shown this treatment to induce apoptosis in ND7 cells on the basis of a number of criteria such as the morphology of cells under light and electron microscopy and DNA fragmentation (43). In these experiments (Fig. 5) cells infected with viruses expressing β-galactosidase or hsp56 showed comparable rates of survival to untreated cells, whereas cells infected with the hsp70 virus showed a marginally protective effect, but only at the 48-h time point (p < 0.05). In contrast, cells infected with the hsp27-expressing virus showed a highly statistically significant protection at both 24 and 48 h after the onset of treatment (p < 0.001 at both time points). Hence the hsp27-expressing virus had a protective effect that was specific to this virus and not observed with the other viruses. This indicates that protection was not due to a nonspecific effect of viral infection, such as an alteration in the rate of cell division.

To confirm that the protective effect of the hsp27-expressing virus was due to reduced apoptosis, we assayed for DNA cleavage, which is characteristic of apoptosis, by TUNEL labeling 48 h after serum removal and addition of retinoic acid. In these experiments (Fig. 6), the cultures infected with the hsp27-expressing virus showed a clear reduction in the number of TUNEL-labeled cells (p < 0.05) compared with control cells, whereas this was not observed in the cells infected with the other hsp-expressing viruses. These results indicate therefore that overexpression of hsp27 can protect ND7 cells from apoptosis as well as against necrosis, whereas any mildly protective effect of hsp70 against cell death does not appear to involve any reduction in the number of cells undergoing apoptosis as assayed by the TUNEL method.

To confirm the potential relevance of these results to the in vivo situation, we carried out similar infections of cultured neonatal rat dorsal root ganglion neurons. In these experiments, the hsp70- and hsp27-expressing viruses clearly protected DRG neurons from both thermal (Fig. 7) and ischemic (Fig. 8) stress at both time points tested. Interestingly, in contrast to the result in ND7 cells, some protection against both stresses was also observed with the hsp56-expressing virus, although the effect was less than that observed with hsp70 or hsp27. As before cells infected with the β-galactosidase-expressing virus showed generally similar survival to untreated cells following exposure to either stress.

In order to test the effect of these viruses on survival following an apoptotic stimulus, we measured their effect on the survival of DRG neurons following withdrawal of nerve growth factor (NGF) which is a well characterized means of inducing

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**Figure 6.** Number of ND7 cells undergoing programmed cell death following 48 h of serum withdrawal in the presence of all-trans-retinoic acid. ND7 cells were pretreated by mock infection or by infection with lacZ, hsp27-, hsp56-, and hsp70-expressing viruses. One day later the cells were incubated in serum-free media containing 1 μM all-trans-retinoic acid for 48 h. The cells were fixed, and fragmented DNA was nick end-labeled in situ with fluorescein-conjugated dUTP, indicating programmed cell death, or apoptosis. Fluorescent cells were counted in confluent fields at a magnification of 100 ×. Three fields were counted for each sample (n), n = 4 for each group. Bars represent 1 S.E. above the mean of each n. +, significant difference in survival compared with lacZ virus-infected ND7 cells (p < 0.05). Significance was calculated using Bonferroni’s Multiple Comparison t test after one-way analysis of variance.
apoptosis (44). As illustrated in Fig. 9, the viruses expressing hsp70 and hsp56 had no protective effect in this assay, whereas the virus expressing hsp27 had a significant protective effect.

To confirm that the hsp27-expressing virus was protecting against apoptotic death, we carried out TUNEL assays in the primary DRG neurons infected with each of the viruses and then exposed to NGF withdrawal. As illustrated in Fig. 10, the hsp27-expressing virus had a clear protective effect in this assay (p < 0.05), whereas no protective effect was observed with the hsp70-expressing virus, paralleling the result obtained with the assay of total cell survival.

DISCUSSION

Although a number of studies in different types of non-neuronal cells have clearly established the protective effect of overexpressing individual hsps (see, for example, Refs. 45–48), such studies have been less extensive and less informative in primary neuronal cells. Thus whereas overexpression of hsp70 has been shown to protect against various different types of cultured neuronal cells from subsequent thermal (12, 13, 16) or ischemic (13) stress, it failed to protect them from subsequent exposure to glutamate (16) or apoptosis induced by withdrawal of nerve growth factor (14), even though a mild heat shock sufficient to induce the hsps does protect against these stimuli (6, 7, 15).

Similarly, overexpression of hsp90 has been shown to protect cultured sensory neurons from thermal and ischemic stress but not against NGF withdrawal (13, 14), whereas the other hsps have not been tested for their effects in neuronal cells.

These problems reflect in part the difficulty of successfully delivering genes to neuronal cells at high efficiency by standard transfection procedures, as well as the inability to prepare stably transfected cell lines expressing the gene of interest as can be done for dividing cell populations. Moreover, even if dividing cell lines of neuronal origin, such as the ND7 cell line (36), are used it is still necessary to laboriously prepare stably transfected cell clones so as to allow analysis of a homogeneous population expressing an individual hsp. Hence, we have used a herpes simplex virus (HSV)-based vector to deliver individual hsp genes with high efficiency (90–100% of cells are transduced at the virus multiplicities used, data not shown and see Refs. 49 and 50) to primary neurons and ND7 cells allowing assays of protection to be rapidly carried out in cells expressing the hsp. Moreover, the data presented here and elsewhere (49, 50) demonstrate that the disabled virus vector we use does not produce significant toxic effects or effects on cell division which would prevent its use in studying the protective effects of specific genes. An HSV-based vector was previously used to overexpress hsp70 in hippocampal neurons, although the other hsps were not investigated.
Moreover, the HSV vector system used by Fink et al. (16) is an amplicon system in which the gene to be overexpressed is cloned into a vector containing an HSV origin of replication and packaging signal. Packaging of this amplicon DNA into an infectious viral particle thus requires a helper virus to provide the necessary HSV proteins in trans (for review of HSV vectors see Refs. 17 and 18). Hence the resulting viral stock for use in experimental studies is a mixture of two types of infectious particles (packaged amplicon and helper virus) whose proportion varies between different stocks making it difficult to perform quantitative comparisons.

In contrast, we have introduced cDNAs encoding individual hsp genes directly by recombination into a defective HSV vector lacking a functional viral gene encoding ICP27 and therefore unable to replicate lytically (42). We have used these vectors to replicate our earlier experiments (13, 15), and we show that
hsp70 can protect both ND7 cells and DRG neurons from both thermal and ischemic stress. Hence, protective effects can be achieved with these vectors in the same manner as in conventional transfection experiments. Moreover, we have extended these studies to other hsps whose effect has not previously been tested in neuronal cells. Thus, we have shown that overexpression of hsp27 has a similar protective effect against thermal and ischemic stress in both ND7 cells and DRG neurons, whereas hsp56 has some protective effect against both these stresses in DRG neurons but not in ND7 cells. This is of particular interest since we have previously shown that hsp70, hsp27, and hsp56 are all overexpressed during cerebral ischemia in vivo (51).

Most importantly, we have shown that hsp27 can protect both ND7 cells and DRG neurons against stimuli which would otherwise induce apoptosis as well as against stimuli which induce necrosis. In contrast, hsp56 had no protective effect, and hsp70 produced only a minimal effect against an apoptotic stimulus at one time point only in ND7 cells when total cell numbers were measured and no effect at all on the number of TUNEL-labeled cells. This suggests that hsp70 has only a minimal protective effect in this system which may be too small to be measured by the TUNEL assay or alternatively that it is directed against necrotic rather than apoptotic death. These results are in accordance with our earlier findings with hsp70 using plasmid-based systems (14–15) and suggest that, unlike other cell types (22–24), neuronal cells cannot be protected against apoptosis by overexpression of hsp70.

In contrast, our results clearly show the protective effect of overexpressing hsp27 against programmed cell death as well as against necrosis in neuronal cells. This effect was specific to the hsp27-expressing virus and was not observed with viruses expressing hsp70, hsp56, or β-galactosidase, indicating that the protection cannot be due to some nonspecific effect of virus infection. This is the first time such a protective effect of hsp7 has been shown in neuronal cells, although it has previously been observed in fibrosarcoma lines (22, 27). In these experiments the overexpression of hsp27 protected cells against apoptosis induced by the Fas/APO-1 pathway as well as by a protein kinase C inhibitor (27). Our results extend this to a completely different cell type and to models of programmed cell death involving retinoic acid treatment and neurotrophic factor withdrawal.

These findings suggest therefore a widespread protective role for hsp27 against apoptotic stimuli in different cell types, as well as against stressful stimuli such as elevated temperature or ischemia, although the mechanism of these effects remains unclear. Hsp27 has been shown to act as an actincapping protein, and overexpression will lead to a greater proportion binding to the barbed ends of the F-actin polymer (52, 53). On heat shock hsp27 is multiply phosphorylated which may cause a conformational change either stabilizing the polymer and maintaining cytoskeletal integrity or cause the hsp27 to dissociate to chaperone other denatured molecules, preventing their aggregation, and promoting correct refolding, while freeing the barbed ends of F-actin to allow polymerization and elongation. It is presently unclear, however, precisely how these changes produce a protective effect against apoptotic or necrotic stimuli.

Whatever the precise mechanism for its protective effect, it is clear that our previous studies in which prior heat shock produced a protective effect against subsequent exposure to apoptotic stimuli (14, 15) can be explained, at least in part, by the enhanced expression of hsp27 rather than being dependent upon a combination of hsps induced by heat shock or some other protective effect not involving hsp synthesis. Similarly, the well characterized protective effect of a prior heat shock against subsequent severe thermal or ischemic stress is likely to involve the elevated synthesis of hsp27 as well as of hsp70.

Hence our studies establish hsp27 as a protective protein with a broader effect in terms of cell type specificity and nature of the stress compared with hsp70 or other hsps tested so far such as hsp90 (14, 15) or hsp56 (this study). The known induction of this protein in cerebral ischemia in vivo (51) suggests that its artificial overexpression in vivo may confer a similar protective effect and may ultimately be of therapeutic benefit in diseases such as stroke. Similarly, its protective effect against apoptotic as well as necrotic neuronal cell death suggests that it may be of use in gene therapy procedures aimed at chronic neurological diseases such as Alzheimer's and Parkinson's disease. The availability of an HSV vector capable of efficient gene delivery in vivo (34, 49) expressing hsp27 will facilitate testing of its potential therapeutic role in minimizing apoptotic and necrotic cell death in the brain.

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