Constitutive overexpression of the major inducible 70 kDa heat shock protein mediates large plaque formation by measles virus


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Induction of the cellular stress response elevates cytoplasmic levels of heat shock proteins (HSPs) belonging to multiple families. When infected with canine distemper virus or measles virus (MV), cells containing elevated HSPs support increased viral gene expression and cytopathic effect. The present work tests the hypothesis that increases in the major inducible 70 kDa HSP (hsp72) are sufficient to mediate the effect of stress response induction on infection phenotype. Human astrocytoma cells (U373) were stably transfected with the human hsp72 gene under control of the β-actin promoter. Constitutive overexpression of hsp72 was demonstrated in multiple clones by Western blot analysis of cytoplasmic total protein. Southern blot analysis of cell DNA confirmed the recovery of genetically distinct clones. Infection of these clonal populations with MV resulted in increased viral transcript production relative to infected control cell lines. Increased transcript production was associated with increased viral membrane glycoprotein expression and cytopathic effect (i.e., mean plaque area). Increases in cytopathic effect were due to the emergence of a large plaque phenotype from a small plaque-purified inoculum, mimicking the effect of cellular stress response induction upon viral infection phenotype. Large plaque phenotypic variants reported in the literature are associated with enhanced neurovirulence, a fact that highlights the potential significance of physiologic elevations in hsp72 (e.g., fever-induced) that accompany in vivo viral infection.

Introduction

The cellular stress response is elicited by a wide variety of non-lethal insults, including hyperthermia (Li et al., 1992), exposure to heavy metals (Kato et al., 1997), hypoxic injury (Vass et al., 1988) and membrane perturbations such as those accompanying transient transfection (Andrews et al., 1997). The response is defined by the selective production of heat shock proteins (HSPs) belonging to multiple families defined by molecular mass. In their function as molecular chaperones, HSPs mediate recovery of the cell by facilitating either refolding or degradation of denatured protein and mediating reassembly of multimeric protein complexes (Marchesi & Ngo, 1993). Elevated cellular levels of HSPs persist following induction and may protect cells from subsequent protein-denaturing injuries that are otherwise lethal. The cellular protection mediated by prior HSP induction is known as stress conditioning and is not specific to a particular stressor (Marchesi & Ngo, 1993; Perdrizet et al., 1989). Stress conditioning using transient hyperthermia is protective against exposure to heavy metals and such in vivo insults as myocardial and cerebral ischaemia (Mestril et al., 1994; Plumier et al., 1995, 1997).

In contrast to the cellular protective role of HSPs, stress conditioning also increases in vitro gene expression and cytopathic effect of the morbilliviruses canine distemper virus (CDV) and measles virus (MV) (Oglesbee et al., 1990, 1993; Vasconcelos et al., 1998). Cytoplasmic nucleocapsid of CDV and MV from stress conditioned Vero cells supports elevated cell-free transcriptional activity relative to nucleocapsid from non-conditioned control cells. Increased nucleocapsid transcriptional activity is correlated to increased cytoplasmic accumulation of viral transcripts and increased expression of viral protein, particularly the membrane glycoprotein mediating cell-to-cell fusion (F). Accordingly, virus-induced mean plaque area is increased in stress conditioned cell monolayers when the extent of syncytium formation is measured in parallel...
plaque assays. Analysis of plaque area distribution shows that increased mean plaque areas reflect the emergence of large plaque phenotypes from small plaque purified inocula (Heller et al., 1998; Vasconcelos et al., 1998). The large plaque phenotype is an in vitro predictor of neurovirulence in rodent models of morbillivirus encephalitis (Cosby et al., 1981; Gould, 1974; Rapp, 1964), suggesting that stress conditioning may influence viral infection phenotype in neural cell types.

The objective of the present study was to determine if stress response-induced alterations in MV phenotype represent a simple or complex system (i.e. attributable to elevations in one HSP or involving elevations in multiple HSPs and/or non-HSP cofactors). The specific hypothesis tested was that elevation in the predominant inducible 70 kDa HSP (hsp72) is sufficient to reproduce the effect of stress conditioning upon viral infection phenotype. Support for such a pivotal role of hsp72 was based upon the correlation between cytoplasmic concentrations of hsp72 and CDV and MV mean plaque area in Vero cells (Heller et al., 1998; Vasconcelos et al., 1990; Vasconcelos et al., 1998); hsp72 is present at low concentrations in non-stressed cells yet becomes one of the most abundant HSPs in stress conditioned cells (Mizzen & Welch, 1988). In addition, stress conditioning promotes the interaction between hsp72 and nucleocapsid (Oglesbee et al., 1990, 1993). Functional interactions between hsp72 and nucleocapsid have been shown for both CDV and MV, where hsp72 stimulates transcription (Oglesbee et al., 1996; Vasconcelos et al., 1998). In order to test our hypothesis, MV infection parameters were measured in clones of a human astrocytoma cell line (U373) stably transfected to constitutively overexpress hsp72. Astrocytoma cells were selected so that we could establish the relationship between increased hsp72 concentration and infection phenotype in cells derived from the central nervous system. Of the neural cell lineages, astrocytes support MV infection and cell death in culture, such as with non-stressed cells yet becomes one of the most abundant HSPs in stress conditioned cells (Mizzen & Welch, 1988).

Methods

**Generation and characterization of clones.** Human astrocytoma cells (ATCC HTB 17) were transfected using liposomes (Transfectam, Promega) containing vector or construct DNA. Construct DNA included the human hsp72 gene driven by a β-actin promoter in plasmid pBluescript (Williams et al., 1993). This construct contains a gene for neomycin resistance driven by the SV40 promoter and has been described previously (Williams et al., 1993). Transfectant colonies were selected with 400 µg/ml G418 (Geneticin, Sigma) and cloned by limiting dilution in 96-well plates. DNA from the plasmid vector pBluescript was used to create control cell lines which were treated identically. Use of these control cells, rather than parental cells, permitted all experiments to be performed in the same tissue culture medium as required for maintenance of the stably transfected hsp72 overexpressing clones.

Levels of hsp72 were analysed by Western blot analysis of whole cell lysates (1 x 10⁶ cells/ml) using mouse monoclonal antibody C92 (SPA-810, StressGen Biotechnologies) and mouse monoclonal antibody N27 (SPA-820, StressGen Biotechnologies) as described previously (Oglesbee et al., 1993). The latter antibody detects both hsp72 and the constitutively expressed 70 kDa HSP (hsp73 or hsc70). Included in the analyses were lysates generated from stress conditioned parental cells as a positive control for hsp72 overexpression, where the cellular stress response was induced with heavy metal. The latter cells were exposed to 240 µM sodium arsenite in medium for 1.5 h, then returned to maintenance medium and harvested for Western blot analysis 12 h post-stress (Oglesbee et al., 1990, 1993). In addition, levels of hsp90 were evaluated using rat monoclonal antibody SPA-835 (StressGen Biotechnologies).

Southern blot analysis of total cell DNA was performed to confirm integration of the construct and to identify genetically distinct clones incorporating the recombinant hsp72 gene. The DNA from whole cells was prepared for Southern blot analysis using a guanidinium isothiocyanate-based extraction system (TriReagent, Molecular Research Center). Extracted DNA was digested to completion with HindIII, resolved on a 1% agarose gel, and transferred to positively charged nylon membranes following depurination and denaturation in NaOH. Membranes were pre-hybridized in a 50% formamide solution (50% formamide, 0.12 M Na₂HPO₄, 0.25 M NaCl, 7%, w/v, SDS, 100 µg/ml herring sperm DNA) at 42 °C for 3 h. Hybridization was performed in the same solution lacking the herring sperm DNA for 42 °C overnight. An hsp72-specific cDNA probe was prepared by random primer extension using the Klenow fragment of E. coli DNA polymerase. [α³²P]dCTP and the 898 bp BglII fragment from the 5′-proximal half of the hsp72 gene as template (Radprime, Gibco BRL). In U373 cells, this probe detects both the endogenous human hsp72 gene and the transgene. Membranes were washed at room temperature for 5 min, then at 65 °C for 45 min in 1 mM EDTA, 40 mM NaH₂PO₄ and 5% SDS, and then twice for 30 min at 65°C in 1 mM EDTA, 40 mM NaH₂PO₄ and 1% SDS.

Surface expression of CD46 was measured in transfected cells by flow cytometry (Coulter EPICS® Elite) following immunostaining of fresh cells with mouse monoclonal antibody 14/48 and FITC-conjugated secondary antibody as previously described (Schneider-Schaulies et al., 1995). Isotype control antibody was mouse IgG1 (MOPC 21, Sigma). Signal from 10,000 cells was evaluated in each group.

**Viral infection parameters.** The effect of hsp72 overexpression on mRNA production by the Hallé strain of MV (ATCC VR-804) was examined. This strain uses a defined cellular receptor (i.e. CD46), exhibits distinct in vitro large and small plaque phenotypes, and was isolated from the brain of a subacute sclerosing panencephalitis patient (Horta-Barbosa et al., 1971). The small plaque purity of the inoculum was based upon infection of non-stressed Vero cells; both large and small plaque phenotypes can be identified from this inoculum only when stress conditioned cells are infected (Vasconcelos et al., 1998). Northern blot analysis of total cell RNA was performed as previously described (Oglesbee et al., 1993), using the nucleic acid extraction system described for the Southern blot analysis (TriReagent). Cells were infected at a m.o.i. of 1:0 and harvested at 20 h post-infection (p.i.). Total RNA was resolved on a 1% formaldehyde–agarose gel where sample loading reflected equivalent cell number. The RNA was then transferred overnight to a positively charged nylon membrane, and pre-hybridized at 65 °C for 3 h in NorthernMax (Ambion) hybridization solution. Plus strand-specific [α³²P]UTP-labelled riboprobes were prepared from Edmonston (Ed)-MV.
N, F and H cDNAs using the MaxiScript (Ambion) kit. The generation and characterization of the cDNA templates has been previously described (Rota et al., 1994). Briefly, gene-specific cDNA fragments were ligated into Bluescript KS(+) and SK(+) transcription vectors, where the T7 promoter drives production of antisense RNA. The N gene riboprobe represented nucleotides 100–470, the F gene riboprobe represented nucleotides 546–1267, and the H gene riboprobe represented nucleotides 21–501. Overnight hybridization of the blots with individual riboprobes was followed by two low stringency washes for 5 min at room temperature and two high stringency washes for 15 min at 65 °C in NorthernMax (Ambion) post-hybridization wash solutions. Equivalent loading was confirmed based upon cellular GAPDH transcript signal from a 387 base riboprobe derived from the mouse GAPDH coding sequence (NorthernMax, Ambion).

Viral protein expression was measured by radioimmunoprecipitation assay (RIPA) as previously described (Sheshberadaran et al., 1983). Cells were infected at an m.o.i. of 1.0 and harvested at 21 h p.i., with uninfected control cells processed in parallel. Proteins were metabolically labelled with [35S]methionine by culturing cells in methionine-deficient medium from 14 h p.i. until harvest. A rabbit anti-MV hyperimmune serum was used to detect N and H protein. A mixture of mouse monoclonal antibodies against MV-F (16EE8, 16AG5, 19BG4 and 19FF4) (Sheshberadaran et al., 1983) was used to precipitate the F glycoprotein. As negative controls, uninfected cell lysates were incubated with the same antibodies, and infected cell lysates were subjected to the conditions for RIPA but without the addition of antibody. Specific bands were identified by their molecular mass and their relative signal intensities were determined using phosphorimage analysis (Phosphorimager 445SI).

Cell-free transcriptional activity of cytoplasmic nucleocapsid (NC) was measured in the insoluble cytoplasmic fraction of infected and uninfected control cell lines as previously described (Vasconcelos et al., 1998). Briefly, cells were infected at an m.o.i. of 1.0 and harvested at 21 h p.i. Cytoplasmic extracts were fractionated on discontinuous glycerol gradients and the NC-containing insoluble fraction used in 50 µl transcription reactions consisting of 1 × transcription buffer, 1 mM CTP and GTP, 2 mM ATP, 10 µM UTP, 25 µCi [α-32P]UTP, 15 µl insoluble fraction (representing 1.25 × 106 cells), 20 µg/ml actinomycin C, and 1 U/µl human placental RNase inhibitor. The protein concentration within insoluble fractions from each clone, both infected and uninfected, was equivalent based upon total protein determination using the bicinchoninic acid reagent (BCA protein assay, Pierce). Reaction products were detected by electrophoresis on 1% agarose–formaldehyde gels. Virus-induced plaque areas were measured in parallel with the Northern blot and RIPA, using vector- and construct-transfected U373 cells and shocked/non-shocked U373 parental cells. Cell monolayers were infected, maintained under methylcellulose overlays for 72 h, and then formalin-fixed and Giemsa stained. Plaque area distributions were determined using light microscopy coupled to a quantitative image analysis system (Oglesbee et al., 1990). Viral progeny release was measured in parallel by infection of cells at an m.o.i. of 0.01, with harvest for titration at 24, 36, 48 and 72 h.p.i. The titre of progeny virus was expressed as p.f.u./ml on Vero cell monolayers. The U72 clones were also infected with Onderstepoort (Ond)-CDV in order to characterize the relationship between hsp72 overexpression and CPE for a closely related morbillivirus.

Results

Characterization of U373 clones

Three cell lines were identified that constitutively expressed elevated hsp72 relative to parental U373 cells or vector transfected U373 cell lines (designated UpH) based upon Western blot analysis of cytoplasmic total protein (Fig. 1A). Clone UpH-6 was selected as the control cell line since its growth characteristics were identical to the hsp72 overexpressing clones. The latter were designated U72-1, U72-2 and U72-7. Levels of hsp72 were increased in the U72 clones compared to UpH-6 based upon staining with monoclonal antibody N27 (SPA-820). The identity of the hsp72 band was confirmed in separate Western blots using hsp72-specific mouse monoclonal antibody C92 (SPA-810). Levels of other
HSPs were equivalent between UpH-6 and the U72 clones based upon Western blot analysis, including the constitutively expressed 70 kDa HSP (hsp73 or hsc70) (Fig. 1A) and hsp90 (data not shown). Collectively, hsp72, hsp73 and hsp90 represent the majority of cytoplasmic HSP in the mammalian cell (Welch et al., 1989).

Cell viability following exposure to high thermal dosages was used to confirm the functional capacity of the overexpressed hsp72 since selective overexpression of hsp72 confers thermotolerance (Angelidis et al., 1991). Tissue culture flasks were placed in a 45 °C chamber for 4±5 h and then cell viability measured in a colony forming assay, wherein serial cell dilutions were cultured for 7 days in 24-well plates. Cell viability relative to non-stressed cells was 6% for UpH-6, 17% for U72-1, 41% for U72-2 and 39% for U72-7. These results were confirmed by measuring cell viability following exposure to lower thermal dosages (i.e. 1-5 h at 45 °C).

Southern blot analysis of total cell DNA for hsp72 sequences was used to confirm the existence of genetically distinct clones of U72 for subsequent virological studies. The banding profile characteristic of the endogenous 70 kDa HSP gene(s) was illustrated by the parental U373 cell line and was identical to the UpH-6 vector-transfected controls (Fig. 1B). One additional band was identified in U72-2 and two additional bands were detected in U72-1 and U72-7, indicating that at least two genetically distinct hsp72 overexpressing lineages were represented.

Fig. 2. Hsp72 overexpressing clones (U72-1, U72-2, U72-7) support increased accumulation of MV gene-specific transcripts compared to vector-transfectant cells (UpH-6). Illustrated are Northern blot analyses of total cell RNA for MV N, F and H plus-strand RNA. Signal intensity derived from monocistronic transcript was directly quantified using a phosphorimager, corrected for background defined by uninfected controls (Uninf.), and represented graphically to the right. Equivalent sample loading was ensured by comparison of the GAPDH signal within the N and F blots. The GAPDH was measured separately in a replicate blot for H (data not shown).

Cell parameters that could non-specifically influence the magnitude of viral gene expression or cytopathic effect were also examined, including growth rate, cell-surface expression of CD46 and cell size. Levels of cell-surface CD46, the receptor for tissue culture-adapted strains of MV (Dörig et al., 1993), were measured by immunocytochemical staining of fresh cells followed by FACS. Expression of CD46 was detected to the same level in UpH-6 and the U72 clones, based upon comparison to negative signal defined by cells stained with isotype control antibody. In addition, cell sizes were equivalent based upon both FACS analysis, where profiles of forward and side scatter were compared, and light microscopic appearance of cell monolayers.

Viral gene expression

Cells were infected at an m.o.i. of 1-0 so that individual virus–cell interactions could be analysed. Cell monolayers were harvested at 20–21 h p.i., a time when syncytia appeared in parallel plaque assays. Northern blot analysis of total RNA from Hallé MV-infected cells showed increased levels of N, F and H signal consistent with monocistronic mRNA in the U72 clones compared to UpH-6 (Fig. 2). Yields of RNA were equivalent between infected and uninfect ed clones and GAPDH signal confirmed equivalent sample loading for the analysis. Using gene- and plus strand-specific riboprobes, the N gene signal was increased over that detected in UpH-6 by 5-3-fold (i.e. 5·3 ×) in U72-1, 6·4 × in U72-2 and 7·3 × in
Hsp72 increases measles virus plaque area

Fig. 3. (A) RIPA of Hallé MV protein from cytoplasmic extracts of infected UpH-6 and U72-1 was performed using MV hyperimmune rabbit serum and a mixture of anti-F monoclonal antibodies. The antiserum yielded a prominent N and H signal that was greater in U72-1 than in UpH-6 (left panel). A faint signal corresponding to P can be resolved below the H band in the U72-1 lane. Using the anti-F monoclonal antibodies, the F1 subunit of the F glycoprotein was detected in U72-1 but not in UpH-6 or in uninfected controls (right panel). Positions of molecular mass markers are indicated on the left and expressed in kDa. (B) Cell-free nucleocapsid transcriptional activity was measured in the insoluble cytoplasmic fraction of infected UpH-6 and U72-1 cells. Extracts from uninfected cells were used as controls. The [32P]UTP-labelled reaction products were resolved on 1% agarose gels under formaldehyde denaturation. An approximately 1.6 kb RNA was detected in UpH-6 and U72-1 reactions consistent with the N and/or P monocistronic transcript. The position of RNA size standards are indicated on the left and expressed in kb.

U72-7 based upon phosphorimager signal intensities. Increase in F signal relative to control was 1.9 x in U72-1, 2.8 x in U72-2 and 3.2 x in U72-7. Increase in H signal relative to control was 1.9 x in U72-1, 2.3 x for U72-2 and 3.4 x for U72-7. Results of the accompanying plaque analysis are illustrated in Fig. 4. Results of the Northern blot analysis were reproduced in two additional experimental trials.

Subsequent experiments showed that increases in intracellular levels of MV mRNA in U72-1 were translated into increased viral protein. Immunoprecipitation of viral proteins metabolically labelled with [35S]Met showed increased levels of N, H and F in U72-1 compared to UpH-6 at 21 h p.i. (Fig. 3A). Using a rabbit antiserum raised against gradient purified Chicago-1 MV, N signal was increased by 4.4 x and the H signal 2.7 x. The identity of the H band was confirmed by RIPA using monoclonal antibody 366 (Bellini et al., 1986); antibody 366 precipitated a protein doublet where migration of the lower component corresponded to the major band identified as H in Fig. 3 (data not shown). Resolution of the P protein band was obscured by the H signal when using the hyperimmune serum and so P signal intensity was not quantified. An F1-specific signal was not detected in UpH-6 extracts using a mixture of anti-F monoclonal antibodies, although prominent signal was detected in U72-1. The RIPA detects the larger F1 subunit of the F glycoprotein, but labels F2 very poorly because of the small size and paucity of methionine residues in the latter. Lack of signal in UpH-6 was interpreted to reflect the inherent difficulty in detecting low levels of this protein (i.e. the protein was likely present since syncytial development had commenced, but that level was below the threshold of detection). In a separate experiment, F protein was also detected in infected U72-2 and U72-7, although levels were 65 and 49%, respectively, of that observed in U72-1 (data not shown).

Analysis of cytoplasmic nucleocapsid (NC) transcriptional activity supported transcription as the basis for increased viral mRNA expression within hsp72 overexpressing cells (Fig. 3B). Nucleocapsid-containing insoluble cell fractions were isolated from infected UpH-6 and U72-1 at 21 h p.i. Corresponding extracts were generated from uninfected cells for use as controls. Total protein concentration was equivalent between all insoluble cell fractions. However, Western blot analysis of the insoluble cell fraction using a MV N-specific monoclonal antibody (16AG5) (Sheshberadaran et al., 1983) showed an approximately 4 x increase in N protein within the insoluble cell fraction of infected U72-1 cells compared to infected UpH-6. Gel analysis of [32P]UTP-labelled reaction products from infected UpH-6 and U72-1 revealed a ≈ 1.6 kb band consistent with the N and/or P monocistronic transcript. Phosphorimager signal intensity of this product was 1.8 x greater in U72-1 reactions than in UpH-6. Specific signal was not identified from uninfected control reactions.
Virus-induced CPE and progeny release

Infection of the UpH-6 vector-transfectants with plaque-purified Hallé MV resulted in a unimodal distribution of small plaque areas at 3 days p.i., based upon quantitative image analysis of infected cell monolayers (Fig. 4). The mean small plaque area on UpH-6 was 0.42 ± 0.04 mm² (SEM), consistent with that obtained on parental U373 cells (0.55 ± 0.03 mm²) and the uncloned pool of vector-transfected cells (0.49 ± 0.02 mm²). This uncloned pool was the source of individual UpH clones, the latter being generated by limiting dilution in 96-well plates. Infection of the hsp72 overexpressing U72 clones with the same viral inoculum resulted in the emergence of large plaque phenotypes while total plaque number was unchanged from that measured on UpH-6. By defining large plaques as having areas ≥ the small plaque mean + two standard deviations, 82% of the plaques on U72-1 were large, shifting the mean of all plaques to 1.41 ± 0.10 mm², a 3.4 × increase over the small plaque mean on UpH-6. In this same trial, 72% of plaques on U72-2 were large, with the mean of all plaques being increased by 2.99 × (1.23 ± 0.12 mm²) over the small plaque mean, and 84% of the plaques on U72-7 were large, with a total mean plaque area that was increased by 3.3 × (1.37 ± 0.13 mm²). Differences in percentage of large plaques and mean plaque areas between U72 clones were not significant based upon results of five subsequent experimental trials that were performed in conjunction with measurements of viral gene expression (above). The emergence of large plaques on the U72 clones was similar to that observed following infection of the uncloned U72 pool or stress conditioned parental U373 cells (i.e. cells exposed to a 1.5 h 160 µM sodium arsenite shock 12 h prior to infection). However, the magnitude of change observed in the uncloned pool and stress conditioned parental cells was less. For uncloned U72 cells, 38% of the plaques were large, resulting in a 1.8 × increase in the mean plaque area over that observed on the uncloned UpH pool. For stress conditioned parental U373 cells, 27% of the plaques were large and the mean plaque area was increased by only 29% (representative of two experimental trials); the mean of all plaques on stress conditioned U373 was 0.72 ± 0.04 mm² compared to 0.56 ± 0.03 mm² on non-stressed controls. The increased plaque areas on the U72 clones, uncloned U72 or shocked U373 parental cells were evident when syncytia first appeared at approximately 18–20 h p.i., with the magnitude of difference from controls increasing at later times p.i.

The emergence of large plaque variants of MV on the U72 clones was correlated to increased production of infectious viral progeny (Table 1). Whole cell lysates combined with culture supernatant were harvested from duplicate 25 cm² flasks at different times p.i. and progeny from each flask titrated in duplicate. The U72-1 and U72-2 cell lines were selected for these assays since they were shown to be distinct transfectant genotypes. Significant progeny release was not observed in UpH-6 clones through 72 h p.i., the time at which plaque areas were analysed. In contrast, infectious viral progeny were

Table 1. Titres of Hallé MV infectious progeny produced in hsp72 overexpressing and vector-transfectant cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Time p.i. (h)</th>
<th>Titre (p.f.u./ml)</th>
</tr>
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<tbody>
<tr>
<td>UpH-6 (vector-transfectant)</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>U72-1 (hsp72 overexpressing)</td>
<td>36</td>
<td>950</td>
</tr>
<tr>
<td>U72-2 (hsp72 overexpressing)</td>
<td>48</td>
<td>31500</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>10000</td>
</tr>
</tbody>
</table>

Values reflect average progeny release from flasks infected and titrated in duplicate, and are representative of two experimental trials.
detected in U72-1 and U72-2 beginning at 36 h p.i., with peak titres obtained in U72-1 at 48 h p.i. (3-1 x 10^4 p.f.u./ml).

The effect of hsp72 overexpression on CDV-induced CPE was also defined. The U375 cell line did not readily support CDV-induced CPE following infection at an m.o.i. of 0-5, requiring serial passage of infected cells before CPE was observed. By 5 days following the first pass, plaques were present on both UPH-6 and U72 monolayers, although plaques on the U72-6 cells were rare. An equivalent proportion of UPH-6 and U72 cells were infected at this time (>90%) based upon immunocytochemical staining for CDV total protein. However, only two plaques (mean area = 0.05 mm^2) were detected in a 25 cm^2 confluent UPH-6 monolayer in one of two experimental trials. In contrast, confluent monolayers of U72-1 and U72-2 contained 33 and 26 plaques, respectively. The distributions of plaque areas in the U72 clones were multimodal, where 27% of U72-1 plaques and 19% of U72-2 plaques were >0.07 mm^2, two standard deviations above the small plaque mean. All CDV infections were poorly productive (10–100 p.f.u./ml) and differences in progeny release between UPH-6 and U72 clones were not observed.

**Discussion**

This work demonstrates that overexpression of hsp72 alone is sufficient to mimic the effect of stress response induction upon Hallé MV infection phenotype in human astrocytoma cells. Increased cytoplasmic concentrations of hsp72 were correlated to increased production of N, H and F mRNAs in the U72 clones relative to the vector transfected control. This finding is consistent with our previous observation that infection of stress conditioned cells with Hallé-MV or Ond-CDV results in the production of cytoplasmic nucleocapsid possessing increased transcriptional activity in cell-free assays (Oglesbee et al., 1996; Vasconcelos et al., 1998). The increased transcriptional activity is directly attributable to hsp72–nucleocapsid interaction, since the latter is promoted by increased cytoplasmic hsp72 concentrations and since purified hsp72 stimulates transcriptional activity of nucleocapsid isolated from the cytoplasm of non-stress conditioned cells (Oglesbee et al., 1990, 1993, 1996). These results do not rule out the involvement of other HSP cofactors in the hsp72-mediated enhancement of viral transcription, although such putative cofactors must be constitutively expressed in order to support the effects of hsp72 overexpression on viral infection phenotype. To date, we have been unable to detect the participation of additional HSPs that are known to support hsp72 function in other viral and non-viral systems (i.e. hsp90, hsp40, hsp73) (Brown et al., 1993; Georgopoulos et al., 1989; Schumacher et al., 1996). Purification of Hallé-MV nucleocapsid under conditions that support hsp72–nucleocapsid complex formation does not result in recovery of hsp90 or hsp40 by Western blot analysis (M. J. Oglesbee, unpublished observation). Hsp73 is detected in these preparations, although antibody against hsp73 fails to inhibit cell-free nucleocapsid transcriptional activity, challenging the functional significance of hsp73–nucleocapsid interaction (Oglesbee et al., 1996; Vasconcelos et al., 1998).

The cytoplasmic level of hsp72 required to stimulate viral gene expression in the stably transfected cell lines was low. These low levels contrast to the higher levels achieved by cellular stress response induction. However, induction of hsp72 by thermal or heavy metal stress is mediated by protein denaturation (Baler et al., 1992), rendering much of the induced hsp72 unavailable due to the high affinity association between hsp72 and irreversibly malfolded protein targets. In Vero cells treated with a similar stress conditioning protocol, only 10–30% of cytoplasmic hsp72 is capable of ATP-dependent target interactions (Oglesbee et al., 1996). Thus, the level of functional hsp72 in both stably transfected and stress-conditioned cells is likely similar. In addition to being better tolerated by the cell, low levels of hsp72 may actually be optimal for promoting viral gene expression. For CDV, 2 µg/ml of purified functional hsp72 stimulates CDV cell-free NC transcriptional activity whereas higher levels inhibit transcription (Oglesbee et al., 1996).

The increased amount of viral transcripts in the U72 clones is translated into increased viral protein, as with infection of stress conditioned cells. There was not strict correlation between the magnitude by which transcripts were increased by hsp72 overexpression and the magnitude by which protein expression was increased. At present, it is unclear if this phenomenon represents limitations in the precision of the protein and/or RNA analyses or whether it reflects differences in the post-transcriptional processing of specific viral gene products. It is unlikely that hsp72 influences post-transcriptional events in the production of F and/or H since hsp72 plays no role in the maturation of glycoproteins within the endoplasmic reticulum (Welch et al., 1989).

Unlike infection of stress conditioned cells, increased production of the membrane glycoproteins H and F was also accompanied by increases in N in the hsp72 overexpressing clones. With lytic infection of stress conditioned cells, production of N transcripts by nucleocapsid is increased in cell-free assays and is associated with increased cytoplasmic accumulation of N mRNA (Oglesbee et al., 1993; Vasconcelos et al., 1998). However, N protein expression and the amount of cytoplasmic nucleocapsid is equivalent between infected stress conditioned and non-conditioned cells. Constant levels of nucleocapsid in cytoplasmic extracts of different treatment groups allow changes in transcriptional activity to be interpreted as changes in the activity per unit nucleocapsid. These same cell-free nucleocapsid transcriptional assays were performed using extracts of Hallé-MV infected U72-1 and UPH-6 cells; U72-1 extracts supported increased viral transcription relative to UPH-6. However, Western blot analysis of the insoluble cytoplasmic fraction showed a 4 x increase in the N protein content of U72-1 extracts relative to UPH-6, paralleling
the 4.4 × increase in total cytoplasmic N levels detected in the RIPA. Such rapidly sedimenting N protein within infected cells may represent either N protein self-complexes lacking genomic RNA (Peluso, 1988) or encapsids genome/antigenome (i.e. nucleocapsid). If increased cytoplasmic concentrations of hsp72 promote formation of nucleocapsid (e.g. by simply increasing the cytoplasmic concentration of core proteins), then hsp72-mediated increases in transcription may reflect increases in the total amount of nucleocapsid as well as increased activity per unit nucleocapsid, although at present we have been unable to establish the contribution of the former.

Increased viral F and H protein expression in U72 clones relative to vector transfected controls was manifest as increased mean plaque area. These increased mean plaque areas reflected the emergence of large plaque phenotypes from a small plaque purified inoculum, a phenomenon that also was observed following infection of stress conditioned U373 cells with Hallé-MV or stress conditioned Vero cells with Hallé-MV, Ed-MV or Ond-CDV (Vasconcelos et al., 1998; Heller et al., 1998). The small plaque-purified inoculum is derived from Vero cells expressing only basal levels of hsp72. It is our hypothesis that although the small plaque purified inoculum is phenotypically homogeneous, it represents a heterogeneous mixture of stress responsive and non-responsive viral variants, the latter yielding small plaques in the face of hsp72 overexpression. Progeny of MV small plaques isolated from stress conditioned Vero cells continue to produce small plaques on both stress conditioned and non-conditioned cells (Vasconcelos et al., 1998). Conversely, progeny of large plaques continue to produce large plaques on stress conditioned cells, although there is a high rate of reversion to the small plaque phenotype. Definitive proof of a viral genetic basis for large and small plaques is the focus of ongoing investigation. The identification of a host factor that reproducibly promotes the emergence of large plaque variants is the first step in defining the corresponding viral determinant of infection phenotype and the possible role of virus–HSP interaction as a determinant of virus virulence. In this capacity, hsp72 is an attractive candidate for a cellular protein modulating virulence since increases in hsp72 frequently accompany physiologic states associated with high morbidity, including psychological stress (Fukudo et al., 1997; Meerson et al., 1993).

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References


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