The cellular stress response increases measles virus-induced cytopathic effect

Daphne Vasconcelos, Erling Norrby and Michael Oglesbee

1 Department of Veterinary Biosciences, The Ohio State University, 1925 Coffey Road, Columbus, OH 43210-1093, USA
2 Royal Swedish Academy of Science, Box 50005 SE, 10405 Stockholm, Sweden

Plaque area is a measure of the degree of cytopathic effect and a predictor of neurovirulence for tissue culture adapted morbilliviruses. In the present work, the cellular stress response was shown to be a determinant of the expression of distinct measles virus large plaque phenotypes in Vero cells. The emergence of these large plaque phenotypes was associated with increased mean viral transcriptional activity and expression of the viral fusion glycoprotein, but not upregulation of the virus receptor CD46.

Syncytium formation, a characteristic form of cytopathic effect (CPE) following infection by the morbilliviruses measles (MV) and canine distemper (CDV), reflects expression of the viral fusion glycoprotein (F) in conjunction with interactions between the viral H glycoprotein and the cellular receptor (Wild et al., 1991). The ability of tissue culture adapted MV and CDV isolates to form syncytia in Vero and HeLa cells (i.e. plaque phenotype) varies both within and amongst virus strains (Carrigan, 1986; Cosby et al., 1981; Gould, 1974; Gould et al., 1976; Rapp, 1964). Although stable genotypic large and small plaque variants have been described (Gould, 1974; Gould et al., 1976), interconversion between plaque phenotypes may occur spontaneously by unknown mechanisms (Carrigan, 1986). The potential biological significance of this virus plasticity is illustrated by the observation that distinct plaque variants have been associated with unique sequelae to infection in the hamster model of morbillivirus encephalitis; syncytiogenic and/or large plaque variants of both MV and CDV exhibit enhanced neurovirulence (Carrigan, 1986; Cosby et al., 1981).

The objective of the present work was to establish the relationship between the cellular stress response and MV plaque phenotypic variation, broadening the relevance of previous findings with CDV, where the relationship between stress response induction and virus receptor expression is defined. The cellular stress response is a highly conserved reaction of cells to loss of homeostasis, being induced in vivo under a wide spectrum of physiological states frequently associated with enhanced virus virulence, including fever (Li et al., 1992), malnutrition (Fischer et al., 1996) and psychological stress (Fukudo et al., 1997). In vitro induction of the cellular stress response prior to infection by CDV increases mean plaque area (Oglesbee et al., 1990), although emergence of distinct large plaque variants has not been described. These stress-mediated increases in CPE are correlated with elevated cellular levels of stress proteins, also known as heat shock proteins (HSPs), at the time of virus infection. Induction of the major inducible 70 kDa HSP (hsp72) is a hallmark of the cellular stress response. Elevated cellular HSPs may directly contribute to increased CPE, based upon the finding that hsp72 increases CDV transcriptional activity (Oglesbee et al., 1996).

However, work with CDV is limited in that changes in CPE have not been interpreted in the context of virus receptor expression. It is possible that stress-induced increases in virus plaque area reflect increased virus receptor expression, diminishing the importance of viral transcriptional activity to enhanced syncytium formation.

For these experiments, a small plaque isolate of the Hallé strain of MV was used to infect Vero cells where the stress response was previously induced by transient hyperthermia (43 °C, 1.5 h) or heavy metal exposure (80, 160 or 240 µM sodium arsenite, 1.5 h) as previously described (Oglesbee et al., 1993). By this approach, elevated cellular levels of HSPs are present at the time of virus challenge (i.e. 12 h after stress response induction). Cellular metabolism is otherwise equivalent between stressed and non-stressed cells at this time, based upon profiles of cellular protein synthesis (Mizzen & Welch, 1988) and cell cycle compartmental distribution (i.e. G_{1} / G_{0}, S, G_{2}/M). Alterations in protein synthesis associated with the stress response are transient following removal of non-lethal stressors, while the long half-life of HSPs allows their persistence into the post-stress interval (Mizzen & Welch, 1988). Levels of cell-surface virus receptor [i.e. CD46 (Dörg et al., 1993)] were measured in stressed and non-stressed cells to
Cellular stress response induction reduced virus receptor (CD46) expression at 12 h post-stress. Ten thousand cells were examined by flow cytometry for surface CD46. The cells were labelled using monoclonal antibody 13/42. The intensity of cell staining was graphed, and the percentage of cell staining derived from the area under the curve for each distribution. Ninety-five per cent of non-stressed cells (NS) expressed CD46 based upon comparison with cells stained with isotype control antibody (iso). In contrast, only 85% of cells exposed to 160 µM sodium arsenite and 57% of cells exposed to 43 °C (HS) expressed CD46. These results are representative of two experimental trials.

determine if stress response induction increased receptor expression. Flow cytometric evaluation of surface CD46 in uninfected cells was performed upon removal of the thermal or heavy metal stress and at 12, 24 and 36 h post-stress using indirect immunocytochemical staining with monoclonal antiprimate CD46 antibody as previously described (Schneider-Schaulies et al., 1995). Results were compared to staining of non-stressed control cells. Induction of the stress response reduced CD46 expression at all time-points. The magnitude of CD46 reduction was proportional to the degree by which hsp72 was induced over basal levels at 12 h post-stress; 95% of non-stressed cells expressed CD46 whereas prior exposure to 160 µM sodium arsenite reduced expression to 85%, and prior hyperthermia reduced expression to 57% (Fig. 1) at 12 h post-stress. When the stress response is induced by hyperthermia, increased hsp72 levels are observed in 96% of the population at 12 h post-shock based upon immunocytochemical staining (Oglesbee et al., 1993). This and the normal distributions of CD46 staining indicate that stress response-mediated reductions in mean CD46 expression reflect a change that uniformly affects the cell populations. These results also suggest that the cellular stress response may contribute to the down-regulation of CD46 associated with MV infection (Naniche et al., 1993), since MV infection can also induce the stress response (Sheshberadaran & Norrby, 1984).

When Vero monolayers were infected with Hallé MV, mean plaque area at 48 h post-infection (p.i.) was increased by 50–220% in stress response-induced versus non-stressed cells, where the mean plaque area was proportionate to the magnitude of hsp72 expression at the time of virus challenge (Fig. 2A). Plaque area was determined by image analysis as previously described (Oglesbee et al., 1990). The distribution of areas was unimodal in non-stressed cells. In contrast, cells infected following induction of the stress response exhibited a predominantly bimodal distribution, reflecting the emergence of a large plaque phenotypic variant (Fig. 2B). Total plaque number was relatively constant between cell treatment groups (average of 18% increase in stress response-induced cells, n = 5 experimental trials), although progeny release from infected stressed cells was increased at 24, 30, 36 and 48 h p.i.; peak progeny release was observed at 36 h p.i. and was 1.7 × 10³ p.f.u./ml for heat stressed cells compared to 4 × 10³ p.f.u./ml for non-stressed cells. The large plaque phenotype was not stable, based upon propagation of large and small plaque progeny harvested from stress response-induced infected cell monolayers. When individual plaques were harvested from the methylcellulose overlays, large plaque progeny formed small plaques on non-shocked monolayers and yielded a bimodal distribution on stress response-induced cells. Conversely, small plaque progeny formed small plaques in both stress response-induced and non-shocked monolayers. The ability of the stress response to induce MV large plaque variants was independent of cell line, being demonstrated also in human astrocytoma cells (U373, ATCC HTB 17). Stress response induction of MV large plaque variants was also independent of virus strain, being demonstrated with the Edmonston MV. The mean plaque area was increased 3.8-fold following infection of shocked Vero cells with Edmonston MV, reflecting an increase in the mean small plaque area and the emergence of a distinct large plaque variant; the ratio of large to small plaques on shocked cells was 1:3.

Cellular stress response-mediated increases in Hallé MV mean plaque area were associated with increased transcriptional activity of total nucleocapsids isolated from the cytoplasm of infected cells at 24 h p.i., a result consistent with previous findings for CDV (Oglesbee et al., 1996). The stress response was induced in this and subsequent experiments by transient hyperthermia (1.5 h, 43 °C). Cells were infected at an m.o.i. of 0.1 at 12 h post-stress and the transcriptional activity of cytoplasmic NC measured in cell-free assays described previously for CDV (Oglesbee et al., 1996), where newly formed transcripts incorporate [α-32P]UTP. When infecting monolayers at this m.o.i., syncytial coverage is complete at 24 h p.i. in both stressed and non-stressed cells, and equivalent amounts of NC are recovered from both cell treatment groups (Oglesbee et al., 1996); variation in plaque phenotype was assessed in parallel plaque assays. Transcription reaction products were resolved by 6% PAGE under urea denaturation and the amount of a given reaction product based upon autoradiographic signal intensities of banded material (PhosphorImager 445SI). Nucleocapsid from non-shocked cells produced a 1.6 kb band compatible with the N and/or P...
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Fig. 2. Mean plaque area was proportionate to hsp72 expression at the time of virus challenge, reflecting the emergence of large plaque phenotypic variants. (A) Top panel: cells were infected following induction of the cellular stress response by exposure to 80 or 160 µM sodium arsenite (NaAs), or by incubation at 43 °C, for 1±0.5 h. Mean plaque areas were measured at 48 h p.i. Bottom panel: cytoplasmic extracts of cells were prepared at 12 h post-stress (i.e. the time of virus challenge) and examined for hsp72 (lower band) and hsp73 (upper band) by Western blot analysis, demonstrating an association between hsp72 level and mean plaque areas. Loading levels of cytoplasmic extract reflected equivalent cell volume between treatment groups, where signal intensities were within the linear range of detection. (B) Assessment of plaque area distributions showed that infection of non-stressed cells produced a unimodal distribution of small plaque areas. Infection of cells following induction of the stress response promoted the emergence of large plaque phenotypic variants, creating a predominantly bimodal distribution. The proportion of large plaque phenotypic variants correlated with the level of hsp72 expression at time of virus challenge.

Fig. 3. (A) NC derived from cells infected 12 h post-stress (3) produced greater amounts (30% increase) of a 1.6 kb N and/or P transcript relative to NC derived from non-stressed cells (2), based upon 6% PAGE analysis of transcription reaction products. This result was representative of three trials. NC from CDV-infected non-stressed cells was used as a positive control for the transcription reactions (1), producing [α-32P]UTP-labelled products corresponding to N/P, M, F and H monocistronic transcripts. Uninfected control cell extracts (4) were used as negative controls for the reactions. (B) Cells infected after induction of the cellular stress response produced greater quantities of F glycoprotein than non-stressed infected cells. Viral protein was metabolically labelled with [35S]Met, immunoprecipitated with monoclonal antibody 16EE8 (recognizing MV F), and the recovered product resolved by 12% SDS–PAGE. A 78% increase in yield of the F1 subunit was observed in cells infected after induction of the stress response (S) compared to non-stressed infected cells (NS). This procedure does not label the F2 subunit. Uninfected control cell extracts (UI) were used as negative controls for the immunoprecipitations. Molecular mass marker positions are at left.

monocistronic transcript. The same amount of total NC from cells infected after induction of the stress response produced 30% more of the 1.6 kb reaction product (Fig. 3A).

An ATP-dependent association of hsp72/73 with Hallé MV NC was demonstrated, identical to findings with CDV (Oglesbee et al., 1996) and characteristic of functional HSP–substrate interactions (Macejak & Sarnow, 1992). Cytoplasmic MV NC was purified using CsCl isopycnic density centrifugation, where the ATP content of crude cell extracts was manipulated prior to gradient centrifugation (Oglesbee et al., 1996); the ATP concentration was adjusted to 2.5 mM by addition of ATP, or ATP was depleted by incubation with 10 U/ml apyrase (4 °C, 15 min). ATP-depleted extracts yielded NC of two densities, a light NC of 1.307 g/ml and a dense NC of 1.313 g/ml. Both the constitutively expressed member of the 70 kDa HSP family (i.e. hsp73) and hsp72 were detected in both NC variants by Western blot analysis using N27 monoclonal antibody (StressGen, data not shown). Identical extracts supplemented with ATP yielded only a dense NC (density 1.313 g/ml) where the hsp72/73 and hsp72 were dissociated. Total NC yields were unaffected by ATP depletion or supplementation, based upon measurement of total NC protein (Pierce Micro BCA). For CDV, complexes between hsp72/73 and NC exhibit increased transcriptional activity (Oglesbee et al., 1996), and infection of cells expressing elevated levels of HSP results in a greater degree of HSP–NC complex formation (Oglesbee et al., 1990, 1993).
Increased cell-free transcriptional activity of NC from cells infected following induction of the cellular stress response correlated with increased intracellular accumulation of (+)-strand MV N and F gene-specific RNA at 30 h p.i. Total RNA from cells infected at an m.o.i. of 0·1 was examined by slot blot analysis using strand and MV gene-specific [α-32P]UTP-labelled riboprobes (Oglesbee et al., 1993). The generation of cDNA templates for riboprobe synthesis and subcloning into transcription vectors has been previously described (Rota et al., 1994). Autoradiographic signal intensities (PhosphorImager 445SI) corresponding to 25, 50 and 100 thousand cells were compared for each treatment group (stressed and non-stressed infected and uninfected cells) and probe. Both N and F gene-specific signals were increased (by 51 and 53% respectively) in cells in which the stress response had been induced, compared to non-stressed cells.

Expression of fusion glycoprotein (F) was further examined because of its relevance to cell–cell fusion. The increase in F-specific mRNA signal correlated with increased expression of fusion glycoprotein at 34 h p.i., based upon immunoprecipitation of [35S]Met-labelled viral protein. Anti-F monoclonal antibody 16E8 (Sheshberadaran et al., 1983) was used in immunoprecipitation of extracts of cells infected after induction of the cellular stress response and in non-stressed infected and uninfected control cells. Recovered proteins were resolved by 12% SDS–PAGE and resultant autoradiographic signal intensities derived from the [35S]Met-labelled F1 subunit of the glycoprotein measured by image analysis (PhosphorImager 445SI). Levels of F protein were increased by 78% (1·8-fold) relative to non-shocked cells (Fig. 3B). This result is representative of changes in viral protein expression where, on accompanying plaque assays, the cellular stress response mediates a 2·0-fold increase in mean plaque area. The increase in mean area reflects primarily the emergence of large plaque variants and, to a much lesser degree, increases in the small plaque mean area. Since the level of F protein measured by radioimmunoprecipitation is an average of protein expression mediated by relatively non-stress responsive (i.e. small plaque) and stress responsive (i.e. large plaque) variants, the degree of stimulation in the latter group is likely under-represented. To date, we have not observed parallel increases in N protein expression, despite increased production of N gene transcripts. This result is consistent with equivalent nucleocapsid recovery from infected shocked and non-shocked cells reported here and in previous work with CDV (Oglesbee et al., 1993, 1996). The basis for the disparity between increased N gene transcript levels and constant levels of protein is unknown, although reminiscent of translational attenuation of N gene products in cases of vaccine-associated CDV encephalitis (Nesselar et al., 1997).

These results show that the cellular stress response is a basis for Hallé and Edmonston MV plaque variant interconversion and that changes in virus receptor expression do not account for the stress response-induced change in infection phenotype. Down-regulation of the virus receptor and increased NC transcriptional activity support the hypothesis that the basis for large plaque formation is transcriptional, although additional effects of the stress response upon viral mRNA half-life or translation may also be operative. The association between MV NC and hsp72 suggests a functional interaction, by analogy to CDV, and both transcriptional activity and CPE are increased when hsp72 is elevated within cells at the time of virus challenge. However, the emergence of bimodal distributions of large and small plaques illustrates variability in the virus stress responsiveness even when using plaque purified inocula. Current efforts will determine whether stress response–induced alterations in both CDV and MV infection phenotype represent a simple or complex system (i.e. attributable to one HSP such as hsp72, or involving multiple HSPs or non-HSP cofactors). Only then can we examine potential differences in the stress-responsiveness of NC associated with unique plaque variants.

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