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SHORT COMMUNICATION

Which Is Induced during Virus Infection

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HSP72 is dramatically induced in the ovaries of vaccinia virus (VV)-infected mice and associates with VV proteins. In order to investigate the role of HSP72 during vaccinia virus replication, we have constructed a recombinant vaccinia virus encoding the major inducible cellular HSP72 (VV-HSP72+) and examined the replication characteristics of this virus. VV-HSP72+ exhibited growth kinetics identical to and peak titers very similar to those of control viruses, both *in vitro* and *in vivo*. In particular, replication of VV-HSP72+ was identical to that of control viruses in the HSP72-negative cell line Y3.Ag.1.2.3, and overexpression of HSP72 had no effect on the virulence of VV infection in normal or immunocompromised mice. We conclude that while VV infection results in the induction of the major inducible 72-kDa HSP, VV replication proceeds normally in the absence of this protein. It is unclear whether another celluar chaperone is required to facilitate virus replication in place of HSP72 in Y3.Ag.1.2.3 cells or whether HSP expression plays no role in virus replication, but is simply a component of the generalized stress response to virus infection.

The elevated expression of stress proteins or heat shock proteins (HSP) has been described in a number of different cells after infection by a variety of eukaryotic viruses (*1–5*). While both the mechanism(s) of HSP induction during virus infection and the subsequent role(s) HSPs play during infection remain mostly unknown, some have speculated that the function of HSPs expressed during virus infection is to act as molecular chaperones, facilitating the correct folding and/or assembly of viral proteins (*6, 7*). Clearly, many stress proteins function as molecular chaperones to assist correct protein folding (*8*), translocation across intracellular membranes (*9*), and assembly of multmeric protein complexes (for reviews see *10–12*). That stress protein expression is increased during virus infection and that many of these HSPs can associate with viral proteins (*13–21*) support this hypothesis. Moreover, some viruses have been shown to encode sequences with significant homology to stress proteins, including the poxvirus *Molluscum contagiosum* which encodes an open reading frame with significant homology to DnaJ (a cellular HSP40 homologue) (*22*) and certain closteroviruses which encode HSP70 homologues (*23–25*). In prokaryotic systems, bacterial HSPs have been clearly shown to be critical for productive bacteriophage infection. For example, stress proteins DnaK (70-kDa HSP), DnaJ (40-kDa HSP), and GrpE (a 22-kDa dimer) are essential for replication of bacteriophage λ DNA in *Escherichia coli* (*26, 27*), and GroEL (an HSP60 homologue) together with its accessory protein GroES (a 10-kDa HSP) is crucial for correct morphogenesis and assembly of bacteriophages λ, T4, and T5 (*26, 28*). The bacteriophage T4 also encodes a cochaperonin which can substitute for GroES (29). However, for eukaryotic viruses there exist only few examples where stress proteins have been observed to facilitate the processing of particular viral proteins. A nascent viral glycoprotein from vesicular stomatitis virus (VSV) (*30, 31*) and human cytomegalovirus (*32*) transiently associates with calnexin, during transit and processing through the ER, and monomeric forms of the influenza hemagglutinin molecule (*33*), as well as the VSV glycoprotein, have been observed to associate transiently with the glucose-responsive protein GRP78 or BiP. However, there is no evidence that such interactions are necessary requirements for productive eukaryotic virus replication.

We have previously shown that HSP72 is dramatically induced in the ovaries of vaccinia virus (VV)-infected mice (*5*). Furthermore, HSP72 associates with VV proteins, since HSP72 can be coimmunoprecipitated together with VV proteins (*17*). Although the precise function(s) of the

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highly stress inducible HSP72 has not been definitively shown, it has been attributed functions similar to those of the constitutively expressed 73-kDa HSP due to extensive nucleotide and amino acid homology and biochemical properties. That these two proteins have been evolutionarily conserved in most cells, suggests, however, that HSP72 possesses characteristics both similar to and unique from those of HSP73. In this study, we have attempted to address whether cellular HSP72, the most abundant stress protein expressed during VV infection, functions to facilitate poxvirus replication. First, we have overexpressed HSP72 during VV infection *in vitro* and *in vivo.* Second, we have assessed virus replication in HSP72-deficient cells in the presence or absence of virus-expressed HSP72.

In order to assess the influence of murine HSP72 on virus replication, a recombinant VV was engineered to encode the major murine heat-inducible HSP72 gene (*34*). Murine HSP72 cDNA was amplified by PCR and cloned into the *Sma*I site of the VV thymidine kinase-insertion vector pSC11 (*35*) in either 5′-3′ sense or 3′-5′ anti-sense orientation, immediately downstream of the p7.5 early/late poxvirus promoter. Sequence analysis of both strands of the PCR product revealed one nucleotide change from the published sequence (*34*) corresponding to an amino acid substitution from arginine to threonine at residue 5. This is a highly divergent region in HSP70 family proteins, and given that the threonine is fairly similar to other residues found at this position, while other HSP72 homologues have quite dissimilar amino acids at this position, we consider it unlikely to alter function. Recombinant VV were constructed by homologous recombination with the L929 cell-adapted WR strain of wild-type vaccinia virus (*36*) using standard techniques (*37*). These viruses were designated VV-HSP72+ (5′-3′), VV-HSP72− (3′-5′), and VV-pSC11 (vector only). To test for HSP72 expression by the recombinant VV, murine L929 cells were infected with each of the recombinant VV and were harvested 24 hr postinfection (p.i.), and cell lysates were prepared and examined by Western immunoblotting using the anti-HSP72 antibody C92F3A5 (StressGen Biotechnologies Corp., Victoria, Canada) as described previously (*5*). Murine L929 cells infected with VV-HSP72+ expressed abundant HSP72 compared to cells infected with control construct VV-HSP72− or VV-pSC11 (Fig. 1A). Western immunoblot analysis of lysates from L929 cells infected with VV-HSP72− indicated that this 3′-5′ antisense construct did not block cellular HSP72 induced by VV infection (Fig. 1A), which could be due to a variety of factors (*38*). As expected, cellular HSP72 induced by VV infection was also detected in L929 cell lysates from VV-pSC11-infected cultures (Fig. 1A).

We then examined whether the abundant expression of HSP72 influenced viral replication by titrating virus production on 143B cells. When murine L929 cells were infected with VV-HSP72+ at a range of multiplicities of

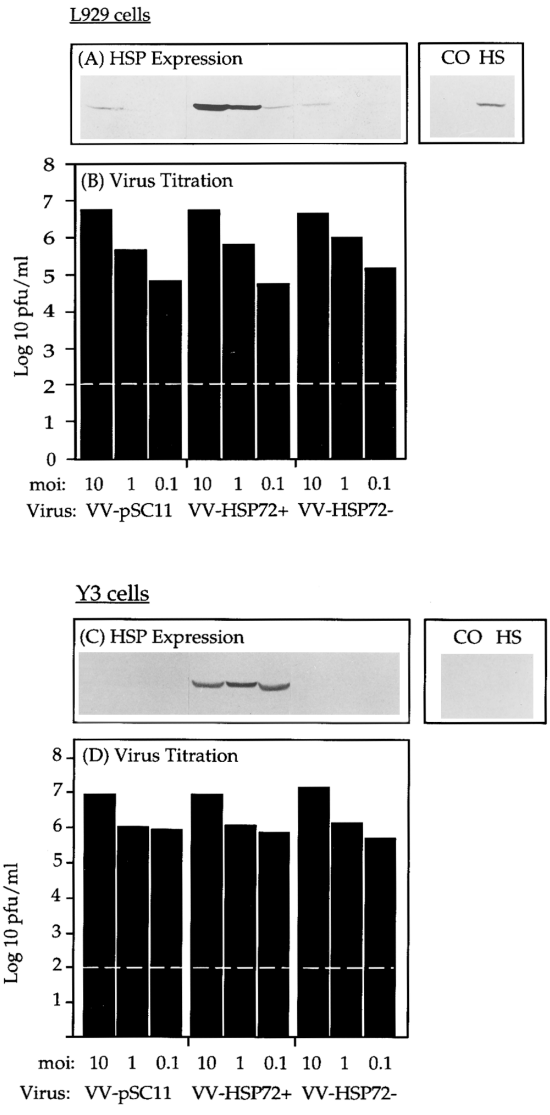

FIG. 1. Western immunoblot analysis of HSP72 expression and virus replication 24 hr p.i. with VV-HSP72+ VV-HSP72−, or VV-pSC11 in L929 cells (A and B) and HSP72-deficient Y3-Ag.1.2.3 (Y3) cells (C and D). Lysates were prepared from 10^6 cells per sample, 24 hr p.i. at the m.o.i. shown, and HSP72 was detected using HSP72-specific antibody C92, as described previously (5). HSP72 expression in control uninfected cells (CO) and after heat shock for 2 hr at 42° (HS) is also shown. Virus replication, 24 hr p.i., was determined by titrating virus from lysates of L929 cells or Y3 cells (10^6 cells per sample) on human 143B cells. The limit of detection of 10^2 PFU in the virus plaque assay is indicated by the dotted line, and the data shown are representative of repeat experiments.

infection (m.o.i.), the extent of virus replication by 24 hr p.i. was found to be similar to that of control viruses VV-HSP72− or VV-pSC11 (Fig. 1B). Since infection by control viruses induced cellular HSP72 in L929 cells (Fig. 1A), the effect of HSP72 expression on VV growth was also assessed in an HSP72-deficient rat myeloma cell line, Y3-Ag.1.2.3 (*39*), denoted here as Y3 cells. These cells did not express HSP72 after heat shock (*40*) (Fig. 2A), and unlike L929 cells, infection of Y3 cells with VV-pSC11

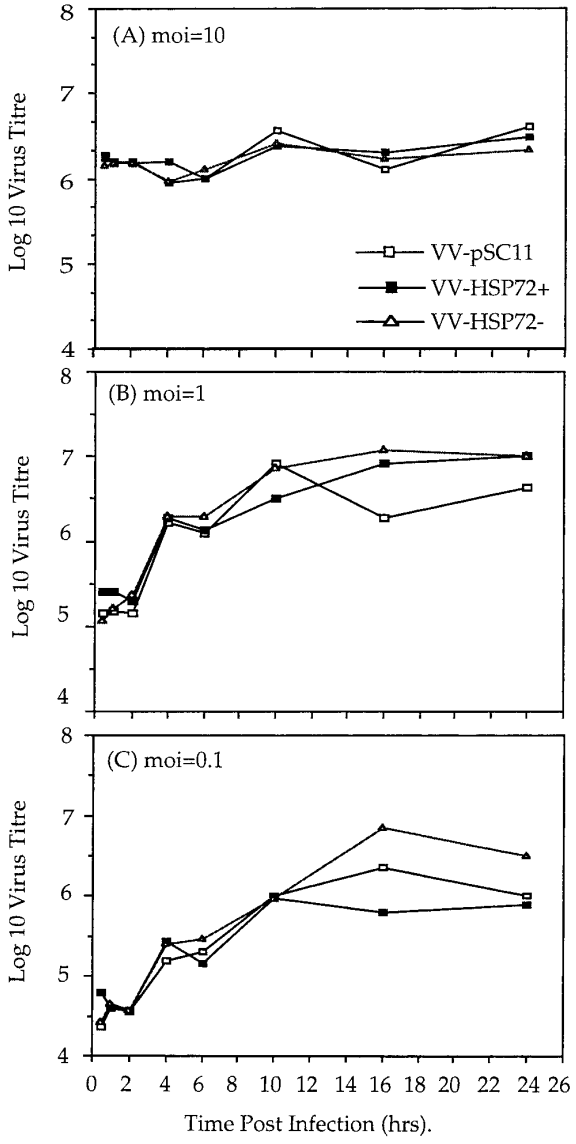

FIG. 2. Kinetics of virus growth in Y3 cells. Replication of VV-HSP72+, VV-HSP72−, and VV-pSC11 in HSP72-deficient Y3-Ag.1.2.3 (Y3) cells, infected at m.o.i. of 10 (A), 1 (B), or 0.1 (C). Virus replication was determined by titrating virus from lysates of Y3 cells (10^6 cells per sample) taken at various times p.i., on human 143B cells. Data shown are representative of repeat experiments.

or VV-HSP72− did not result in expression of cellular HSP72 (Fig. 1C). Hence, neither heat shock nor VV infection induced HSP72 in Y3 cells. Infection of Y3 cells with VV-HSP72+ resulted in abundant production of virus-encoded HSP72 24 hr after infection (Fig. 1C). However, VV-HSP72+ and the control constructs, VV-HSP72− and VV-pSC11, grew to identical titers, irrespective of the presence or absence of HSP72 or the m.o.i. (Fig. 1D). In order to define whether HSP72 influences early events during virus infection, we also looked for differences in virus replication from the onset of infection through to 24 hr p.i. At all times investigated, we found that VV-HSP72+ replication was very similar to VV-pSC11 and VV-HSP72− replication in HSP72-defective Y3 cells (Fig. 2). Since HSPs also play an important role in the quality control mechanisms of normal and abnormal protein folding and processing, the morphogenesis of virus particle formation from early through to late times p.i. was examined at an ultrastructural level using scanning electron microscopy. Y3 cells were infected with VV-HSP72+ or VV-HSP72− at a m.o.i. of 5 PFU/cell, and then cells were washed and incubated for various times through to 24 hr p.i. No differences in virus particle formation or the morphology of Y3 cells infected with VV-HSP72+ or VV-HSP72− were observed (data not shown). Together, these results indicate that VV replication proceeded normally at all times throughout infection irrespective of the presence or absence of HSP72.

Next, we investigated the growth of the recombinant VV encoding HSP72 *in vivo.* Replication of VV-HSP72+ was compared to that of VV-HSP72− and VV-pSC11 in the ovaries of infected mice, since VV normally grows to very high titers in these organs (*41, 42*). VV-HSP72+ grew to peak titers of $10^{7.5}$ PFU in murine ovarian tissue by 6 days p.i., similar to VV-pSC11 and VV-HSP72− (Fig. 3). Furthermore, the kinetics of VV-HSP72+ replication and clearance from murine ovarian tissue was identical to that of control viruses (Fig. 3). Since mouse ovarian tissue contains high levels of mRNA for the transcription factor responsible for inducible HSP72 expression, muHSF1 (*43*), we also looked at the ability of these viruses to grow in the liver, where muHSF1 mRNA was not detected

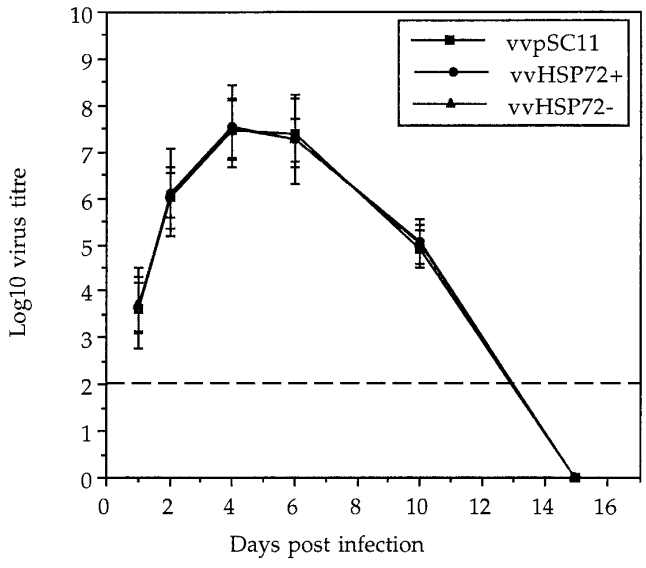

FIG. 3. Growth of HSP72 viruses in normal mice. Kinetics of VV-HSP72+, VV-HSP72−, and VV-pSC11 replication in the ovaries of normal CBA mice. Specific pathogen-free female CBA-H mice were obtained from the Animal Breeding Establishment at the JCSMR. Groups of 5 female mice were infected with 10^7 PFU of virus iv, and virus replication was measured by titration of ovary homogenates on human 143B cells. Data shown are means of $\log_{10}$ PFU/pair of ovaries ± SEM of 5 mice. The limit of detection of 10^2 PFU in this plaque assay is indicated by the dotted line.

TABLE 1

Growth of HSP72-Encoding Vaccinia Virus in Nude Mice[a]

Virus[a]	Titer[b]	% Mortality	MTD[c]
VV-pSC11	7.35 ± 0.50	ND	ND
VV-HSP72+	7.40 ± 0.45	100	14.0 (12, 13, 14, 15, 16)
VV-HSP72−	7.38 ± 0.65	100	13.6 (12, 14, 14, 14, 14)

[a] Female Swiss outbred nude mice (nu/nu), 8–10 weeks old, were obtained from the Animal Breeding Establishment at the John Curtin School of Medical Research. Ten mice per group were infected iv with 10^6 PFU or recombinant vaccinia virus VV-pSC11, VV-HSP72+, or VV-HSP72−. Five mice were sacrificed 4 days p.i. for determination of virus titers from ovary tissue homogenates, titrated on human 143B cells. The remaining five mice were used for assessment of mortality due to virus disease. ND, not tested.

[b] Data shown are means $\log_{10}$ PFU/pair of ovaries ± SEM of 5 mice, 4 days p.i. The limit of detection in this assay is 10^2 PFU.

[c] MTD, mean time of death, days p.i.

(*43*) and where VV does not normally grow to high titers (*41, 42*). Titration of liver homogenates from mice infected i.p. with 10^8 PFU of either VV-HSP72+ or VV-HSP72− revealed that both viruses replicated to very similar levels in this tissue, reaching peak titers of 10^4 PFU by 2 days p.i. and decreasing to approximately 10^3 PFU by Day 4 (data not shown). Hence the overexpression of HSP72 by the recombinant VV did not alter the level of virus replication in the ovaries or livers of normal mice.

Finally, we investigated virus replication and disease progression in T-cell-deficient nude mice, which normally cannot control VV replication and die (*41, 44*). By 4 days p.i., VV-HSP72+ grew to the same titer as VV-HSP72− and VV-pSC11 in the ovaries of these mice (Table 1). Consistent with this finding, there was also no difference in the mean time of death of nude mice similarly infected with VV-HSP72+ or VV-HSP72−, since mice infected with either virus died by Day 13 or 14 p.i. (Table 1). Therefore, overexpression of HSP72 did not influence the growth of VV or the virulence of infection in immunocompromised nude mice.

The demonstration that HSP72 stress proteins are expressed in abundance in virus-infected cells (*5*), can transiently associate with viral proteins (*18*), and are important cofactors for prokaryotic virus replication (*26–29*) suggests that cellular HSPs play an important role during poxvirus replication. That some viruses encode open reading frames homologous to cellular HSPs (*22–25*) adds further support to this hypothesis. However, this evidence remains circumstantial, and although examples exist where certain HSPs and chaperonins have been demonstrated to participate in the processing of particular eukaryotic virus-encoded glycoproteins (*30–33*), there remains very little evidence that this is an important or necessary requirement for viral replication. It is possible that interactions between HSPs and viral proteins may either facilitate virus protein maturation or inhibit viral protein assembly and virus morphogenesis. Alternatively, HSP expression may simply be a part of the generalized stress response to virus infection and play no role in virus replication. Our study indicates that overexpression of HSP72 within the virus-infected cell did not detectably alter VV replication. Thus, the interactions of HSPs with VV proteins demonstrated by others (*18*) may be interpreted to reflect normal physiological events, such that a plentiful supply of HSP70 with inherent affinity for nonnative protein would be highly likely to interact with abundant nascent viral proteins which are present in the same cellular compartment. The fact that we found no differences in the replication of these HSP72 recombinant vaccinia viruses does not discount the possibility that HSP72 can influence other subtle factors not reflected by viral titers. For example, the requirements for correct viral protein folding may be energetically more expensive without HSP72 participating in the folding process.

In interpreting our data, it may be important to consider that HSP genes exist as multigene families. For example, there have been estimated to be at least 8 functional human HSP70 family genes (*45–48*) and a similar number in mice (*34, 49*). It is not known to what extent functional redundancy exists within this family of heat shock proteins, and although there is clearly no expression of HSP72 in Y3 cells, it may be significant that detectable levels of the constitutively expressed 73-kDa HSP can be found in these cells before and after VV infection (data not shown). It is possible that HSP73 or another HSP70 family member may functionally compensate for the lack of HSP72 in Y3 cells. If in fact HSP72 does influence virus replication, redundancy in function of HSP70 family proteins may mean that it is impossible to delineate the role of HSP72 in vaccinia virus infection, using these systems. For example, it is possible that HSP70 proteins are important for sequestering aberrantly folded viral proteins, to preclude their packaging into mature virions, whilst optimizing functional viral protein expression. On the other hand, the presence of functional copies of both HSP72 and HSP73 in most cells argues that HSP72 may feature properties distinct from HSP73, and notably it is the expression of HSP72 which has been demonstrated to be dramatically upregulated in VV-infected tissues in mice (*5*). In this paper, we have demonstrated that VV-HSP72+ replication was identical to that of control constructs, irrespective of the m.o.i. used for *in vitro* studies and the time postinfection or target tissue examined in infected mice. We conclude that while we have previously shown that VV infection results in the induction of the major inducible 72-kDa HSP, VV replication occurs normally in the absence of this protein. It is unclear whether another celluar chaperone is required to function in place of HSP72 in Y3 cells or whether HSP expression plays no role in virus replication, but is simply a component of the generalized stress response to virus infection.

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