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Heat Shock Response to Vaccinia Virus Infection

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We have investigated the induction of heat shock proteins (HSPs) in mice infected with vaccinia virus. Vaccinia virus replicates to high levels in the ovaries of infected mice and causes a significant inhibition of host cell DNA, RNA, and protein synthesis. Many HSPs are constitutively expressed in murine ovarian tissue at low levels, consistent with their obligatory role in normal physiological events. In contrast with these events, HSP expression was augmented in virus-infected mouse ovaries 6 days postinfection. In particular, there was a dramatic increase in the expression of a protein identified as the inducible 72-kDa HSP. Analysis of cellular mRNA confirmed this protein to be the major mouse inducible HSP70 and demonstrated its presence within virus-infected cells. Hence, we have demonstrated the expression of stress proteins during poxvirus infection in vivo.

The production of heat shock proteins (HSPs) is characteristic of all organisms in response to a variety of stresses. These include physiological stress, environmental stress, and infection by microorganisms (19). A number of viruses have been shown to cause induction of HSP expression in vitro; however, unlike many bacteria and parasites, viruses are not known to encode genes for HSPs, and hence, HSP expression in cells infected with these viruses represents cellular HSP.

It is clear that both DNA and RNA viruses are capable of inducing the expression of HSPs and that this is independent of whether the virus replicates primarily in the nucleus or cytoplasm of the infected cell. However, previous studies have indicated that there is no uniform pattern of virus-induced HSP expression in vitro. Different virus infections result in the expression of particular HSPs, depending on the virus and the type of cell that is infected (6, 15, 22, 25). Furthermore, there are conflicting accounts of transcriptional activation of HSP70 genes by viruses. For example, infection of CV1 cells with adenovirus type 5 and herpes simplex virus type 1 results in induction of HSP70 at a transcriptional level, but there are varying reports of the ability of simian virus 40 to activate HSP70 expression in these cells (13, 26).

Infection with vaccinia virus (VV) causes a significant shutdown of host cell metabolism (21), such that mRNA transcripts for host cell actin and β-tubulin decrease to nondetectable levels within 6 h postinfection (p.i.) (11). Until recently, there was no evidence that stress proteins were expressed during VV infection. In fact, a comprehensive study of the ability of a variety of DNA viruses to transcriptionally activate HSP70 family genes showed that VV infection of HeLa cells did not induce any member of the HSP70 family of genes tested (26). However, with cultures derived from freshly isolated human peripheral blood mononuclear cells, it has been shown that HSP70 was abundantly expressed after VV infection (11). Given these discordant in vitro findings, we have investigated HSP expression during VV infection in vivo.

Expression of HSPs in normal mice. The WR strain of VV grows to high titers in ovarian tissues of mice, such that approximately 10^8 PFU of virus can be recovered from these organs 6 days p.i. (29). In order to assess changes in HSP expression as a consequence of VV infection, it was necessary first to determine the constitutive levels of HSPs in normal mouse ovarian tissue. Proteins were extracted from pooled ovaries of naive 6- to 10-week-old CBA/H mice by hypotonic lysis and Dounce homogenization (35). Proteins were then separated by ion-exchange column chromatography, using DEAE cellulose (DE-52; Whatman) and a linear 20 to 350 mM NaCl gradient, in 20 ml of elution buffer (20 mM Tris-acetate [pH 7.5], 20 mM NaCl, 0.1 mM EDTA) (35). Aliquots (15 μl each) from consecutive samples were run on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels according to the method of Laemmli (14), and the total protein present in these samples was visualized by silver staining (1). HSPs present in the eluted fractions were detected by immunoblotting, with proteins from duplicate gels being transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 5% nonfat skim milk and then incubated with a cocktail of HSP-specific antibodies. This included monoclonal antibodies (MAbs) 9G10 (anti-GRP94), AC88 (anti-HSP90), and N27F3-4 (anti-HSP70), which reacts with both constitutive 73-kDa and inducible 72-kDa HSP (all MAbs were obtained from StressGen Biotechnologies). A polyclonal rabbit serum raised against mouse HSP27 was also included (supplied by Alt Zantema). Alkaline phosphatase-conjugated secondary antibodies—sheep anti-mouse immunoglobulin (Ig) (Silenus), sheep anti-rabbit Ig (Silenus), and goat anti-rat Ig (Southern Biotechnology Associates)—and then 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad) and nitroblue tetrazolium (Bio-Rad) in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris) were used to detect HSP-specific antibodies. Posttransfer gels were stained by Coomassie brilliant blue (Bio-Rad) to ensure efficient transfer of proteins. When protein was extracted from the ovaries of 10 uninfected mice, the level of HSP expression, as shown by Western blotting (immunoblotting), was barely detectable (data not shown). Consequently, proteins were extracted from the ovaries of 40 mice in order to clearly illustrate constitutive levels of HSPs expressed under normal conditions (Fig. 1A and B). The stress proteins detected in these samples were HSP90, the low-molecular-weight 27-kDa HSP, and also the glucose-regulated protein GRP94. In order to detect HSPs belonging to the 70-kDa family, the antibody cocktail included an antibody reactive with both the major constitutively expressed 73-kDa HSP and the inducible 72-kDa HSP (MAb N27F3-4). The

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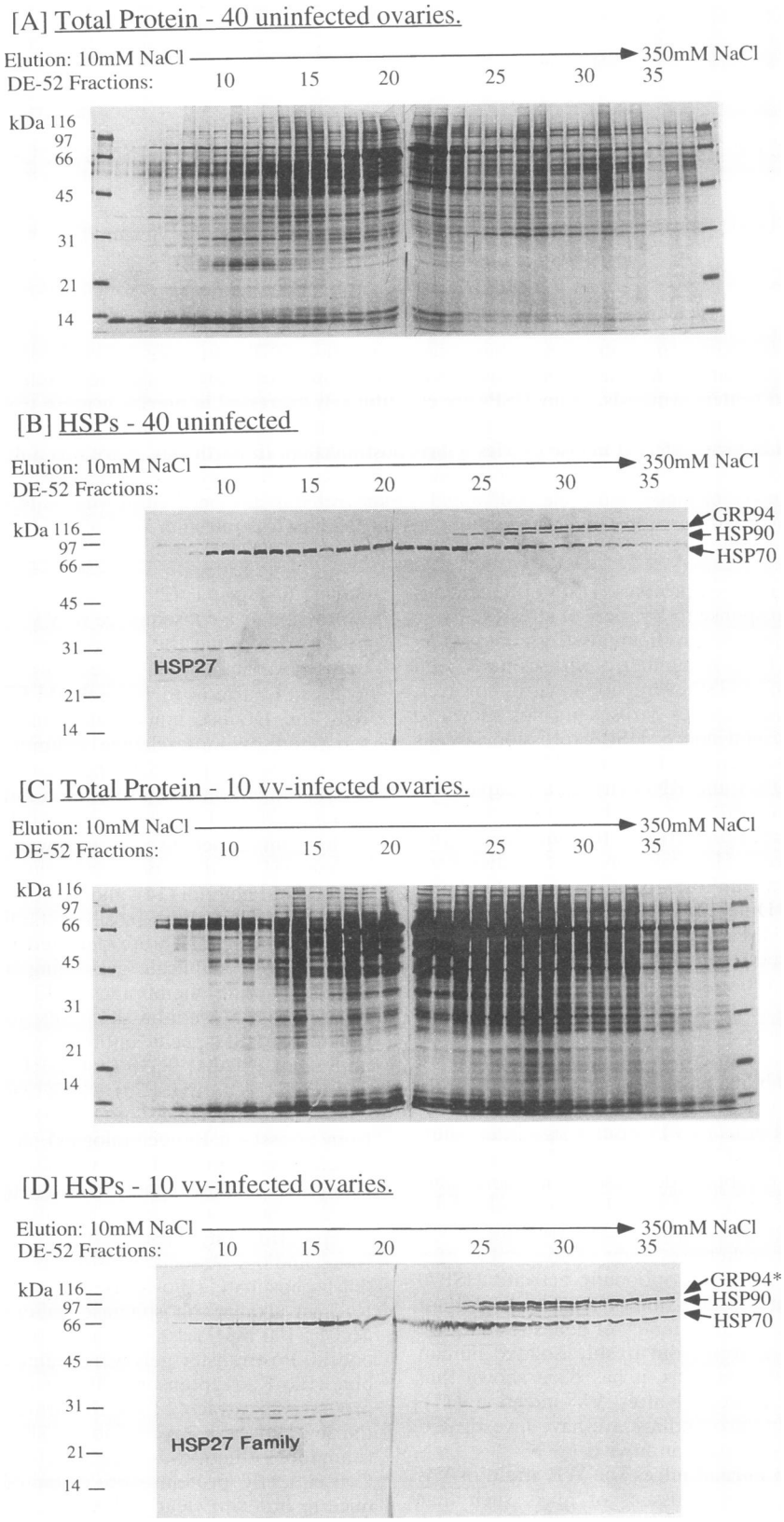

FIG. 1. Protein was extracted from the ovaries of 40 uninfected mice (A and B) or 10 VV-infected mice 6 days p.i. (C and D). Proteins were separated by DE-52 ion-exchange column chromatography, and 15-μl aliquots of consecutive fractions were run on SDS–10% polyacrylamide gels. The elution profile and fraction numbers are indicated. (A and C) Total protein present in the respective samples, as visualized by silver staining; (B and D) immunoblots of duplicate gels, detecting the presence of HSPs. The immunoblots were developed with a cocktail of MAbs reactive to the following stress proteins: HSP90, HSP72/73, HSP27, and GRP94. HSPs which were constitutively expressed in the ovaries of 40 uninfected mice are shown in panel B. Constitutive expression of HSPs in ovaries of 10 uninfected mice was barely detectable and is not shown here. Increased expression of stress proteins in ovaries from 10 VV-infected mice 6 days p.i. is shown in panel D.

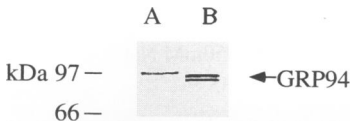

FIG. 2. Immunoblot analysis of the GRP94 stress proteins. Protein extracted from 40 normal mouse ovaries (DE-52 fraction 36) shows a single GRP94 species with an apparent molecular mass of approximately 97 kDa (lane A). Protein from 10 VV-infected ovaries (DE-52 fraction 36) shows GRP94 resolving as two distinct bands, both with slightly greater electrophoretic mobilities (lane B). The immunoblot was developed with a rat MAb specific to GRP94 (9G10) and then alkaline phosphatase-conjugated goat anti-rat antibody, as described in the text.

presence of each HSP identified in the cocktail Western blot was confirmed by individual blots with each specific antibody (data not shown). The broad elution profile for HSP70 proteins as shown here and in other studies (35) may reflect the physical characteristics of HSP70 proteins. A number of HSPs form multimers both in vitro and in vivo (20). Colocalization and immunoprecipitation studies have recently shown that the constitutive HSP73 and inducible 72-kDa forms of HSP70 interact or associate with one another (2). Complexes such as heterodimers or oligomers of HSP72-73 may form, and their elution by ionic strength competition might first require the dissolution of the putative complex, before dissociation from the charged gel matrix. Clearly, HSP70 family proteins were the most abundant stress proteins found in normal mouse ovarian tissue.

Expression of HSPs in vaccinia virus-infected mice. Next, we determined which HSPs were present in the ovaries of 10 mice after infection with VV. CBA/H mice were infected with 10^7 PFU of a recombinant VV (WR strain, encoding influenza virus A/PR/8/34 hemagglutinin and herpes simplex virus thymidine kinase; VV-HA-TK) (28). We have used this construct widely as a control virus and have found its growth very similar to that of wild-type virus in vitro and in vivo (29). Ovaries were taken from these animals 6 days p.i., and protein was extracted as described above. This time marks the peak cellular immune response to VV, but high titers of virus are still recoverable from the ovaries of infected mice (29). Despite the severe inhibition of host cell protein synthesis which occurs during VV infection, high levels of proteins were detected in extracts from the ovaries of 10 VV-infected mice, as illustrated by silver staining (Fig. 1C). In VV-infected ovaries there is marked inflammation, and both virus and inflammatory factors contribute the increased protein. High levels of HSPs were also demonstrated in these VV-infected samples (Fig. 1D), contrasting with the very low levels of HSPs in the same number of ovaries from uninfected mice (data not shown). HSPs detected in infected samples were HSP90, the 94-kDa GRP, both constitutive 73-kDa and inducible 72-kDa HSPs, and a number of 27- to 32-kDa related proteins.

By comparison with the constitutive expression of HSPs, some forms of stress proteins were detected only after infection. In VV-infected samples, the GRP94 protein appeared as a doublet (Fig. 1D). A higher resolution photograph of immunoblots developed with antibody specific to GRP94 (Fig. 2) showed that in VV-infected samples (Fig. 2, lane B) GRP94 appeared as two bands, with both exhibiting electrophoretic mobilities greater than that for uninfected samples. This may indicate posttranslational proteolysis of GRP94 or differential glycosylation. Changes in glycosylation states of stress proteins have been previously reported after virus infection (25). Also, there were four proteins between 27 and 32 kDa which were identified by the HSP27 antiserum (Fig. 1D), compared with a single 27-kDa stress protein in uninfected mouse ovaries (Fig. 1B). It is possible that different forms or family members of these stress proteins exist in inflammatory cells present in the VV-infected samples but are absent in normal mouse ovarian tissue. The 32-kDa proteins evident in VV-infected ovaries may represent the oxidation-specific stress protein (12). This protein is known to be induced in phagocytes which produce toxic metabolites as a consequence of the potent factors which they secrete, such as tumor necrosis factor (27). Importantly, control immunoblots with only secondary antibodies showed that the mouse Ig heavy chains of IgG (approximately 50 kDa) and IgM (approximately 70 kDa) were only very faintly detectable in this system, unlike light chain Ig (data not shown).

Expression of the inducible 72-kDa HSP. To determine the levels of the major inducible form of the HSP70, we performed Western blots using an antibody specific for the 72-kDa HSP (MAb C92F3A5; StressGen). HSP72 was present at very low levels in protein extracted from the ovaries of 10 uninfected mice (Fig. 3A). The presence of this HSP in normal mouse tissue was confirmed with protein extracted from 40 mice (data not shown). In contrast, there was a dramatic increase in the expression of the 72-kDa HSP in ovaries from 10 VV-infected mice 6 days p.i. (Fig. 3B). Unlike the constitutively expressed HSPs, the inducible 72-kDa HSP is found in most cells only after exposure to stress; however, our results demonstrate that this protein is detectable in normal mouse ovarian tissue. The synthesis of this protein by murine cells under normal conditions has not been described previously, although primate cells are known to express low levels of HSP72 constitutively (34). The expression of HSP after infection with the recombinant VV was not due to expression of the foreign proteins per se, since we have used a variety of other viruses, including other VV recombinants, wild-type VV (WR strain), and also the virulent mousepox ectromelia virus, to demonstrate in vitro the induction of HSP72 expression after virus infection (data not shown).

Transcription of the 72-kDa HSP within VV-infected cells. It was important to establish whether the 72-kDa HSP was expressed within the virus-infected cell and which member of the HSP70 multigene family it represented. Primary murine ovarian fibroblast cultures were established from the ovaries of normal mice and examined for the presence of HSP70 transcripts after VV infection. Briefly, a single cell suspension was prepared by pushing ovaries through a fine metal grid. The cells were washed and then incubated with 0.08% trypsin in 0.01% EDTA–0.05% HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) for 1 h at 37°C with stirring. The trypsin was removed by washing, and cells were cultured in MLC (5) containing 10% calf serum with 5% CO_2 and used between the fourth and eighth passages. Cultures were infected at a multiplicity of infection of 10, and total cellular RNA was extracted by the method of Cathala et al. (4) at various times p.i. We determined the presence of the HSP72 message by Northern (RNA) blotting using the 3′ untranslated region of the mouse heat-inducible HSP70 as a probe. This was derived from a *Stu* I–*Bgl*II–*Hin*dIII digest of plasmid pM70.1 (10), which was extracted from low-melting-point agarose and labelled with ^{32}P by random oligonucleotide primers, as described elsewhere (31). This DNA fragment specifically recognizes the inducible HSP72 mRNAs of 3.1 and 2.6 kb and does not hybridize with the constitutive HSP73 message of 2.3 kb (10). HSP72 mRNA was detected by Northern analysis 8 h after VV infection and in greater abundance at 16 and 24 h (Fig. 4). By 32 h p.i., there was a significant decline in HSP70

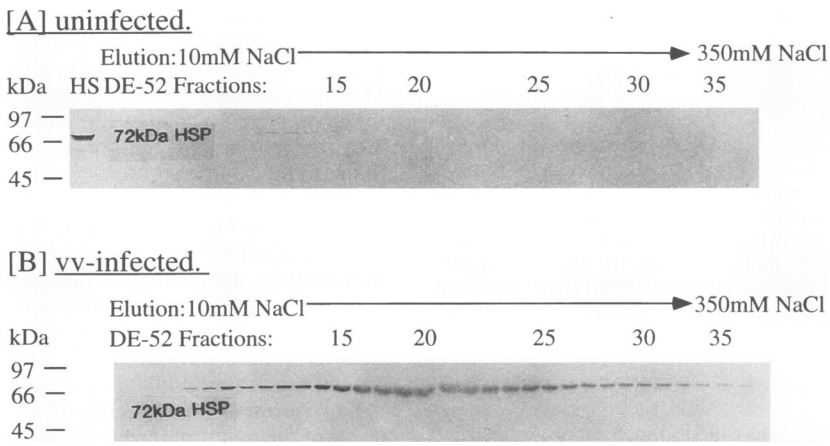

FIG. 3. Western blots showing expression of the 72-kDa HSP in ovarian tissues of 10 mice before (A) and after (B) VV infection. Samples were 15-μl aliquots from consecutive fractions eluted from the DE-52 ion-exchange column, as in Fig. 1. Immunoblots were developed with MAb specific to the inducible 72-kDa HSP (C92F3A5) and alkaline phosphatase-conjugated sheep anti-mouse Ig (Silenus). (A) Low basal expression of the 72-kDa HSP in ovaries from 10 uninfected mice. As a positive control, protein extracted from heat-shocked (HS) L929 cells was run. (B) Dramatic increase in expression of the 72-kDa HSP in the ovaries of 10 VV-infected mice.

mRNA, and no detectable message was found at 40 h. There were two mRNA transcripts of 3.1 and 2.6 kb apparent at 16 h p.i. and also after heat shock. Similar-size transcripts have previously been reported in mouse cells in response to either heat shock or adenovirus infection (10, 16). As a corollary to the demonstrated presence of HSP72 mRNA within VV-infected cells, we have also detected the protein product of this gene as early as 2 h after VV infection by Western blotting with an HSP72-specific antibody (C92F3A5) (data not shown). Cell types tested include primary ovarian fibroblast cultures, murine sarcoma cells (L929), and murine macrophages (RAW264). The demonstrated HSP72 expression within VV-infected cells does not negate the possibility that it is also expressed within inflammatory cells present in VV-infected tissues.

The functional significance of the induction of stress proteins during virus infection is unknown. It is interesting that HSP70 proteins have been found by immunoprecipitation studies to associate with viral proteins. For example, HSP70 has been found associated with adenovirus fiber protein (17), polyomavirus middle T antigen (33), vaccinia virus proteins (11), purified rabies virions (30), and biologically active forms of the canine distemper virus nucleocapsid (23). However, the strong affinity of molecular chaperones for unfolded or non-native proteins (7, 24, 32) suggests a high likelihood of interactions between HSPs and abundant nascent viral proteins. As such, HSP-viral protein associations may simply be unavoidable. Conversely, it has been postulated that HSPs act to chaperone viruses, and thereby facilitate viral replication (7, 9, 32). Evidence supporting this includes the action of HSP90 as a targeting and transport protein for the Rous sarcoma virus oncogene product $pp60^{src}$ (3, 36) and the role of GRP78 (BiP) in maintaining influenza virus hemagglutinin monomers in a trimer competent state (8). However, since the half-lives of some viral proteins are increased as a result of their association with HSPs, some have argued that HSPs act to interfere with the assembly and production of progeny virus. In this case, the presence of HSPs is beneficial to the cell, by precluding the cleavage and subsequent processing of precursors into mature virions (18). The induction of cellular HSP during virus infection may also have immunological significance. The data reported here serve to demonstrate that cellular HSPs are synthesized at high levels during a physiological response to poxvirus infection. In particular, the major inducible HSP72 is selectively induced, within virus-infected fibroblasts. This study, therefore, provides the basis from which we can now question the role that stress proteins play during virus infection and the mechanism(s) of their induction.

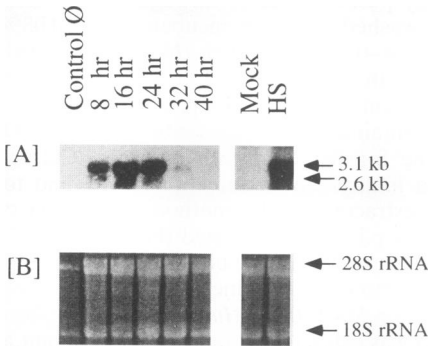

FIG. 4. (A) Northern blot analysis of HSP70 mRNA expressed in murine primary ovarian fibroblasts at various times after infection with VV. Transcripts of 3.1 kb were detected at 8, 16, 24, and 32 h p.i. A second 2.6-kb transcript was evident at 16 h p.i. Heat-shocked (HS) and uninfected (Control Ø) samples were run as controls. (B) Gels were run in parallel, and rRNA was stained with acridine orange to show even loading of lanes and integrity of RNA.

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