JOURNAL OF VIROLOGY, Feb. 2002, p. 1379–1390 Vol. 76, No. 3
0022-538X/02/$04.00+0 DOI: 10.1128/JVI.76.3.1379–1390.2002
Copyright © 2002, American Society for Microbiology. All Rights Reserved.

Molecular Chaperone Hsp90 Is Important for Vaccinia Virus Growth in Cells

Jan-Jong Hung, Che-Sheng Chung, and Wen Chang*

Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan, Republic of China

Received 10 October 2001/Accepted 5 November 2001

Molecular chaperones assist protein folding, and some chaperones are induced by heat, nutrient depletion, or pathogen invasion. This study investigates the role played by Hsp90 in the life cycle of vaccinia virus. The titer of vaccinia intracellular mature virions (IMV) was reduced by 2 orders of magnitude in RK13 cells treated with geldanamycin (GA), which blocks the ATPase activity of Hsp90. GA does not affect expression from the viral early promoter, but treatment with GA delays DNA replication and intermediate gene transcription and reduces expression from the viral late promoter. Vaccinia virus infection does not induce Hsp90 expression; however, intracellular distribution of Hsp90 is altered in virus-infected cells. Hsp90 is restricted to the cytoplasm of mock-infected cells; in contrast, Hsp90 is transiently associated with virosomes in virus-infected cells although it is not incorporated into IMV. In addition, Hsp90 interacts with viral core protein 4a, the mature form of the A10L gene product, in virus-infected cells. In conclusion, these results suggest that a cellular chaperone protein, Hsp90, is important for vaccinia virus growth in cultured cells and that viral core protein 4a associates with Hsp90-containing complexes in the infected cells.

Cells induce a stress response to virus infection, which often includes expression of stress response proteins such as heat shock proteins (HSPs) (8, 35, 46, 50, 62). HSPs play important roles in biological processes such as protein folding, translocation, and cell viability (15). Several HSP families are expressed in mammalian cells, including Hsp110, -90, -70, -60, and -40 (15). The cellular response to viral infection and the induction of HSPs depend on the infecting virus and the cell type. Although many viruses have been reported to induce a heat shock response, only a few cases have been thoroughly studied. For example, during influenza virus infection, a chaperone, Hsp40, is involved in regulating the p68 protein kinase (PKR)-mediated interferon response (17, 18, 41, 42, 64, 65). Hsp40 forms an inhibitory complex with P58, an inhibitor of PKR in cells (41, 42). During influenza virus infection, these inhibitory complexes are dissociated and the released P58 binds to PKR, blocks phosphorylation of the α subunit of eukaryotic initiation factor 2, and stimulates protein synthesis in virus-infected cells (17). Another study shows that human immunodeficiency virus (HIV) infection requires the chaperone cyclophilin A for growth (16). Cyclophilin A binds to viral Gag protein and is incorporated into HIV virions during viral assembly (10). Recently, it has been shown that Hsp40 is induced by adenovirus infection and that overexpression of Hsp40 rescues the growth of Gam-1$^-$ mutant adenovirus (22). Chaperone induction or association has also been observed in many other virus systems such as Sindbis virus, herpes simplex virus, Newcastle disease virus, and rotavirus; however, the detailed mechanisms of these interactions have not been determined (8, 20, 22, 35, 45, 51, 72).

Vaccinia virus is an animal DNA virus with a wide host range that replicates in the cytoplasm of infected cells (14). Previous studies indicate that transcription of Hsp70 is induced at 8 h after vaccinia virus infection (32, 61, 62). However, vaccinia virus replication proceeds normally in a cell line deficient for Hsp70 expression, and overexpression of Hsp70 has no effect on the virulence of vaccinia virus in normal or immunocompromised mice (61). Thus, Hsp70 does not appear to be important for the vaccinia virus life cycle.

Hsp90 is a constitutive molecular chaperone present in eukaryotes and bacteria (39, 67). Unlike Hsp70, Hsp90 does not act generally in nascent protein folding, and instead, it binds to substrate proteins that are in a near-native state and thus at a late stage of folding for activation by ligand binding or interaction with other factors (31). The recently determined crystal structure of Hsp90 revealed that the N-terminal domain of Hsp90 binds ATP, which is consistent with the observation that ATP hydrolysis is required for the chaperone function of Hsp90 and for conformational changes involved in refolding protein substrates or client proteins of Hsp90 (53, 54). Unlike Hsp70, which recognizes short hydrophobic peptide segments such as those exposed in nascent polypeptides, Hsp90 is more specific in targeting its protein substrates (7, 47, 58). Most of its known substrates are proteins involved in signal transduction and cell cycle and transcriptional regulation (6, 7, 9, 30, 44). Several viral proteins, such as reovirus sigma protein, simian virus 40 T antigen, and reverse transcriptase of hepatitis B virus, have also been identified as the protein substrates of Hsp90 (21, 28, 43). So far, the exact structural features of the client proteins that are recognized by Hsp90 are not known (74).

Geldanamycin (GA) is a benzoquinone ansamycin antibiotic from *Streptomyces hygroscopicus* and is a specific inhibitor of Hsp90 (69). GA binds to the ATP-binding pocket on Hsp90 and interferes with its chaperone functions (7, 53, 54). In the presence of GA, Hsp90 client proteins do not fold properly and are degraded by a ubiquitin-dependent proteosome path-

* Corresponding author. Mailing address: 128, Sec. 2, Academia Rd., Nankang, Taipei 115, Taiwan, Republic of China. Phone: 886-2-2789-9230. Fax: 886-2-2782-6085. E-mail: mbwen@ccvax.sinica.edu.tw.

way (60). Therefore, GA has been widely used to determine whether a cellular process is regulated by Hsp90 (24, 29, 36, 59, 73).

This study investigated the role of Hsp90 in the life cycle of vaccinia virus. The inhibitory effect of GA on virus production was studied in RK13 cells and allowed us to demonstrate the essential involvement of Hsp90 in vaccinia virus growth. The distribution of Hsp90 and of the associated protein complexes was analyzed in the virus-infected cells, and a viral client protein of Hsp90 was identified.

MATERIALS AND METHODS

Viruses and reagents. Wild-type (WT) vaccinia virus (WR) was grown on BSC40 cells. Recombinant vaccinia viruses vMJ360, vP30LacZ, and vREβCAT, which express *lacZ* from early, intermediate (G8R), and late (F17R) promoters, respectively, were described previously and were provided by B. Moss (55). vREβCAT also contains the chloramphenicol acetyltransferase (CAT) gene expressed from an early promoter. Recombinant vaccinia virus expressing the A4L-enhanced cyan fluorescence protein (ECFP) fusion protein was constructed by C.-L. Lin and will be described elsewhere. GA was purchased from Sigma and dissolved in dimethyl sulfoxide at a stock concentration of 1 mM. Chemicals for electrophoresis were purchased from Bio-Rad. Other chemicals were obtained from Merck and Sigma. Cy5-conjugated phalloidin was purchased from Molecular Probes. Antibody (Ab) against zyxin (SC-6438) was purchased from Santa Cruz. Abs against Hsp90 (SPA830 and SC-1057) were purchased from Stressgen Biotechnologies and Santa Cruz. Ab against Hsp70 (SPA 810) was purchased from Stressgen Biotechnologies. A rabbit antiserum against vaccinia virus H3L protein was described previously (38). Anticore Abs were described previously and provided by J. Krijnse Locker (49).

Cell culture. RK13, HeLa, and BSC40 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 100 U of penicillin G/ml, and 100 μg of streptomycin/ml. For virus infection, cells were infected at a multiplicity of infection (MOI) of 5 PFU per cell at 37°C for 60 min. After infection, GA was added into the cell culture medium at various concentrations (0, 0.08, 0.16, 0.32, 0.64, 1.28, 2.5, 5, and 10 μM) and cells were harvested at 24 h postinfection (p.i.) for titer determination. For the cell viability test, cells were treated with the desired concentrations of GA for 24 h and harvested for trypan blue staining. The percentages of white cells that excluded dye staining were calculated as the survival population.

Plaque assays of vaccinia virus grown on RK13 cells. Freshly confluent RK13 cells were infected with a recombinant vaccinia virus, vMJ360, which expresses *lacZ* from an early promoter at roughly 200 PFU per 60-mm-diameter dish at 37°C for 60 min. These cells were further incubated in 1% soft agar with or without 0.5 μM GA for 3 days and fixed in 10% formaldehyde at room temperature for 60 min. The agar overlay was removed, and cells were stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to visualize plaques and photographed.

Immunoblot analysis. Whole-cell extracts were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with 3% nonfat milk in TTBS (0.5% Tween 20, 20 mM Tris-HCl [pH 7.4], 0.5 M NaCl) for 60 min, the membrane was washed once with TTBS and incubated with Abs against H3L (1:3,000), Hsp90 (1:1,000), or Hsp70 (1:1,000) at room temperature for 12 h. Membranes were washed three times for 10 min and incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated anti-mouse Ab (Pierce), a 1:3,000 dilution of alkaline phosphatase-conjugated anti-goat immunoglobulin G (Zymed), or a 1:2,000 dilution of anti-rabbit secondary Ab (New England Biolabs) for 120 min. Blots were washed with TTBS three times and developed with the ECL system (Amersham) or CDP-Star (Tropix) according to the manufacturer's protocols.

Expression of reporter genes from viral promoters. CAT expression from the viral early promoter was assayed as described previously (2). β-Galactosidase (β-Gal) expression from viral intermediate and late promoters was detected as described previously (2). In brief, RK13 cells were infected with vP30LacZ or vREβCAT at an MOI of 5 PFU per cell at 37°C for 60 min. After infection, the cells were incubated in media with or without 0.5 μM GA. At various times after infection, these cells were harvested and expression levels of CAT and β-Gal, respectively, were determined (2). In addition, infected cells expressing β-Gal from intermediate and late promoters were harvested and total RNA was isolated for Northern blot analyses with a ^{32}P-labeled *lacZ* DNA fragment.

Dot blot analysis for viral DNA replication. Viral DNA replication was detected by a modification of previously published procedures (13, 26). In brief, RK13 cells were infected with vMJ360 at an MOI of 10 PFU per cell and harvested at various times in the absence or presence of 0.5 μM GA. Cells were washed in phosphate-buffered saline (PBS) and lysed with digestion buffer (100 mM NaCl, 10 mM TrisCl [pH 8], 25 mM EDTA [pH 8], 0.5% SDS). The lysate was digested with proteinase K (100 μg/ml) and extracted with phenol-chloroform, and total DNA was isolated by isopropanol precipitation. After RNase treatment, DNA samples were applied to nitrocellulose paper using a microsample filtration manifold (Schleicher & Schuell). The filters were hybridized with a ^{32}P-labeled 3-kb *lacZ* DNA fragment, washed, and autoradiographed. The experiments were repeated twice, and the resulting blots were scanned, quantitated, and averaged with a PhosphorImager (Molecular Dynamics).

Electron and confocal microscopy. RK13 cells were cultured in 60-mm-diameter dishes, infected with WT vaccinia virus at an MOI of 5 PFU per cell for 1 h, incubated with medium containing 0.5 μM GA, and fixed at 24 h p.i. for electron microscopy as described previously (27).

For confocal microscopy, HeLa cells were seeded on round glass slides in 12-well plates and infected with a recombinant vaccinia virus expressing A4L-ECFP at an MOI of 5 PFU per cell at 37°C for 1 h. The infected cells were incubated in media with or without 0.5 μM GA for 12 and 24 h and fixed in 4% paraformaldehyde at 4°C for 20 min. Cells were rinsed in PBS three times and permeabilized in 1% Triton X-100 for 5 min and in 0.5% Tween 20 for 15 min. These cells were blocked with 1% bovine serum albumin–PBS for 60 min at 25°C and incubated with Abs against Hsp90 (SC-1057) at a dilution of 1:200 for 1 h and with Cy5-conjugated donkey anti-goat immunoglobulin G (Jackson ImmunoResearch Laboratories) for 1 h. Alternatively, these cells were incubated with Cy5-conjugated phalloidin (1:200) to stain intracellular actin. The samples were washed with PBS, mounted in 50% glycerol, and analyzed using a Zeiss LSM 510 confocal microscope.

Immunoprecipitation. Cells were infected with WT vaccinia virus at an MOI of 5 PFU per cell and incubated for 8 h. Cells were rapidly chilled on ice, washed twice with ice-cold PBS, and then lysed in lysis buffer (20 mM HEPES-KOH [pH 7.9], 5 mM EDTA, 10 mM EGTA, 5 mM NaF, 0.1 μg of microcystine-LR/ml, 10% glycerol, 1 mM dithiothreitol, 0.4 M KCl, 0.4% NP-40, and protease inhibitors as follows: 5 μg of leupeptin/ml, 5 μg of aprotinin/ml, 5 μg of pepstatin/ml, 1 mM benzamidine, 50 μg of phenylmethylsulfonyl fluoride/ml) for 10 min on ice. Insoluble material was removed by centrifugation (10,000 × *g*, 20 min, 4°C). Protein concentration of the cell lysate was determined using a bicinchoninic acid protein assay kit (Pierce), and an equal amount of protein was used in each experiment. Cell lysates were preincubated with protein A/G-Sepharose (Santa Cruz) for 2 h at 4°C and centrifuged. The supernatant was transferred to new tubes and incubated with either control Abs (antizyxin) or anti-Hsp90 Abs at a dilution of 1:200 for 12 h at 4°C. The immune complexes were subsequently incubated with protein A/G-Sepharose, washed five times with lysis buffer, and separated on an SDS–10% polyacrylamide gel. After electrophoresis, the gels were processed for silver staining as previously described (5). The 65-kDa protein was excised out, and the identity was determined by mass analysis performed by EverNew Biotech. Alternatively, the immunocomplexes were separated by SDS-PAGE and subjected to immunoblot analyses with anti-Hsp90 (1:1,000) and anticore Abs (1:10,000) as described previously (49).

RESULTS

GA inhibits growth of vaccinia virus in RK13 cells. GA is a specific inhibitor of Hsp90 that interferes with ATP binding and inactivates Hsp90 chaperone activity (69). In the following experiments, GA was used to determine whether Hsp90 chaperone function is required during the vaccinia virus life cycle. The cytotoxicity of GA was tested in mock-infected RK13 cells treated with various concentrations of GA for 24 h. Figure 1A showed that cell viability was unaffected in medium containing GA up to 2.5 μM, but cytotoxicity increased when the GA concentration was higher than 5 μM. To determine the effect of GA on growth of vaccinia virus, cells were infected with WT vaccinia virus at an MOI of 5 PFU per cell and incubated in medium lacking GA or containing GA from 0.08 to 1.28 μM. Cells were harvested at 24 h p.i., and titers of cell lysates were

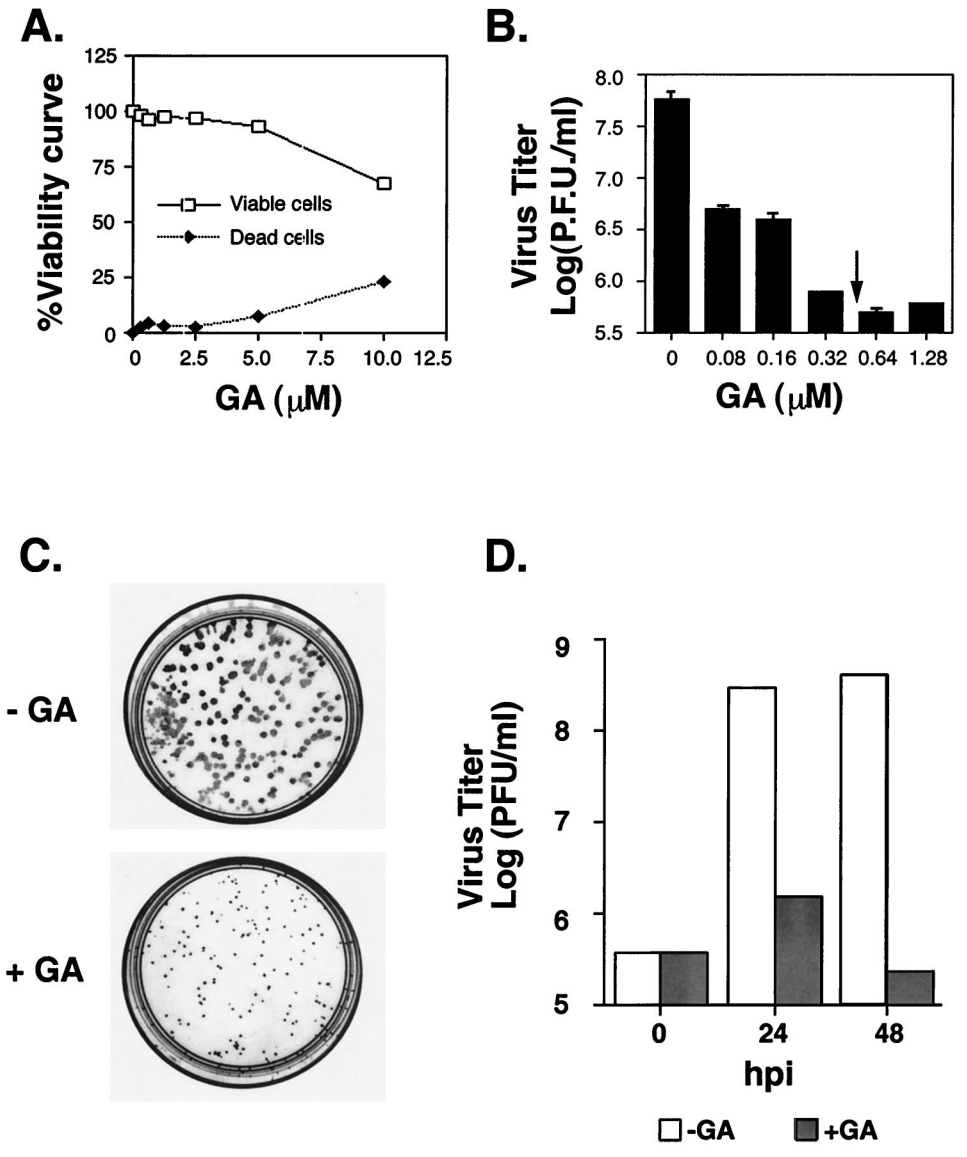


FIG. 1. Inhibition of vaccinia virus growth in RK13 cells treated with GA. (A) Cell viability in medium containing GA. RK13 cells were cultured in medium supplemented with different concentrations of GA and incubated for 24 h. Cells were trypsinized and stained with trypan blue, and the percentage of cells that exclude trypan blue was determined. (B) Inhibition of vaccinia virus by different concentrations of GA. RK13 cells were infected with WT vaccinia virus at an MOI of 5 PFU per cell for 1 h and incubated in medium with or without GA for 24 h, and cell lysates were harvested for IMV titer determination as described previously (2). (C) Plaque morphology of vaccinia virus on RK13 cells in normal medium (−GA) or medium with 0.5 μM GA (+GA). RK13 cells were infected with roughly 150 PFU of vaccinia virus vMJ360 per plate, changed into medium with or without GA for 3 days, fixed, stained with X-Gal, and photographed. (D) Reduction of vaccinia virus IMV titers by GA is not a delayed phenotype. RK13 cells were infected and incubated in normal medium (−GA) or medium containing 0.5 μM GA (+GA) for 24 and 48 h, cell lysates were harvested, and IMV titers were determined as described for panel B.

determined using a plaque assay (Fig. 1B). The titer of intracellular mature virions (IMV) was reduced 10-fold in the presence of 0.08 and 0.16 μM GA and 100-fold in the presence of 0.32 and 0.64 μM GA. No further reduction of virus titer was seen even when the concentration of GA was increased to 1.28 μM. Subsequent experiments were carried out using 0.5 μM GA, a concentration at which the toxicity of GA for mock-infected or virus-infected RK13 cells was minimal. Titration of GA concentration for cell viability is critical, since different cell types may exhibit different ranges of drug toxicity. For example, BSC40 cells appeared more tolerant of GA and could survive at higher concentrations of GA than could RK13 and HeLa cells (Table 1). This difference in GA toxicity may reflect the endogenous levels of Hsp90 in cells. Although GA did affect RK13 cell growth, it had a greater inhibitory effect on vaccinia virus growth (Fig. 1C and D). Vaccinia virus normally formed large plaques on confluent RK13 monolayer cells at 3 days p.i.; however, in the presence of GA only small plaques were produced (Fig. 1C). Consistently, the titers of IMV grown in GA were reduced by 2 to 3 logs of magnitude (Fig. 1D). Longer incubation times did not increase the plaque size or the virus yields, indicating that GA inhibition was not simply a

TABLE 1. Inhibition of vaccinia virus growth in cell lines[a]

Cell type	GA concn (μM)	Virus titer (PFU/ml)		Titer difference (+GA/−GA)
		+GA	−GA	
RK13	0.5	5.6×10^5	7.3×10^7	7.7×10^{-3}
HeLa	0.5	3.3×10^6	1.1×10^8	3×10^{-2}
BSC40	2	7.1×10^6	1.6×10^8	4.4×10^{-2}

[a] Cells were infected with vaccinia virus at an MOI of 5 PFU per cell and harvested at 24 h p.i. The virus titers obtained for cells with (+GA) or without (−GA) GA are shown.

delayed phenotype. These results indicate that GA, a specific inhibitor of Hsp90, exerts an antiviral effect during vaccinia virus infection. The inhibitory effect of GA on virus growth is not limited to RK13 cells and was also observed on other cell lines such as HeLa and BSC40 cells (Table 1). Thus, we conclude that GA inhibition reflects a general requirement for Hsp90 for vaccinia virus to grow in cultured cells.

Since vaccinia virus IMV titer was significantly reduced in cells treated with GA, electron microscopy was used to investigate whether a particular stage of virion morphogenesis was interrupted by GA (Fig. 2). In untreated cells, numerous IMV as well as some immature virions (IV) were readily accumulated at 24 h p.i. (Fig. 2A). At the same time, only a few IV associated with viral nucleoprotein mass were detected in GA-treated cells and no IMV were observed (Fig. 2B). Many of these IV exhibited dark staining with a polarity, in contrast to the light and uniform staining normally seen for IV in the control cells. While so few IV and IMV were detected, the sizes and the numbers of viral crescents were also largely reduced, with no accumulation of other recognizable viral intermediate structures in GA-treated cells, indicating that an early phase of IMV morphogenesis was already affected by the drug.

GA interferes with viral intermediate and late but not early gene transcription. The above results indicate that either GA directly acts on virion assembly stage or it interrupts viral gene expression and consequently affects virion assembly. The effect of GA on viral gene expression at different stages of the vaccinia virus life cycle was investigated using recombinant viruses that contain reporter genes regulated by an early, intermediate, or late promoter (55). RK13 cells were infected with each recombinant virus and incubated in medium with or without GA, and the kinetics of reporter gene expression was analyzed quantitatively (Fig. 3). In the absence of GA, reporter activity was readily detected from early (E), intermediate (I), or late (L) viral promoters in virus-infected cells. In GA-treated RK13 cells, the kinetics and level of CAT reporter activity from an early promoter were similar to those for the control cells (Fig. 3A). In contrast, the activity of β-Gal reporter protein expressed from an intermediate promoter was significantly delayed in GA-treated cells. In untreated RK13 cells, the reporter β-Gal activity reached half-maximal expression by 3 h p.i., but in GA-treated cells, half-maximal activity was delayed up to 6 h p.i. (Fig. 3B, left panel). Furthermore, both the kinetics and level of β-Gal from a late promoter were clearly attenuated in GA-treated cells, reaching only 50% even at 24 h p.i. (Fig. 3C, left panel).

In order to demonstrate that reduction of β-Gal activity was due to a blockage at transcriptional level, total RNA was prepared from GA-treated infected cells and the kinetics of the *lacZ* transcripts from intermediate and late viral promoters was analyzed by Northern blot analyses (Fig. 3B and C, right panels). In parallel with β-Gal activity profiles, *lacZ* transcription from the intermediate promoter was significantly delayed and that from the late promoter was both delayed and reduced in GA-treated cells. Thus, GA interferes with intermediate and late viral promoter activities.

Delayed viral DNA replication in RK13 cells treated with GA. Viral DNA replication is essential for activation of intermediate promoters; thus, agents that block DNA replication, such as hydroxyurea or cytosine arabinoside, inhibit intermediate promoter activity (3, 33). It is therefore possible that GA delays intermediate gene transcription as an indirect consequence of its inhibition of viral DNA replication. To examine this possibility, RK13 cells were infected with vaccinia virus, treated with GA, and harvested at different time points to quantify viral DNA replication using dot blot analysis (Fig. 4A). In untreated cells infected by vaccinia virus, viral DNA replication initiated at 2 h p.i., continued synthesis between 2 and 6 h p.i., and became saturated at approximately 8 h p.i. (Fig. 4B). The kinetics of viral DNA replication is consistent with previous reports (26). Interestingly, in cells treated with GA, viral DNA replication was significantly delayed and reached roughly 70% at 10 h p.i. This result suggests that GA delays onset of viral DNA replication and may explain the delayed activation of intermediate and subsequent late viral promoters in GA-treated cells.

Hsp90 is not induced after vaccinia virus infection and not incorporated in IMV. The results described above suggest that Hsp90 is essential for vaccinia virus growth in cells. Hsp90 forms complexes with different cochaperones and facilitates conformational maturation of specific proteins. To understand the role of Hsp90 in the virus life cycle, examination of endogenous Hsp90 expression in virus-infected cells becomes necessary. Because our Ab recognizes human Hsp90 and does not cross-react with a rabbit Hsp90 homologue in RK13 cells, we therefore used HeLa cells in all subsequent experiments. Besides, from the data in Table 1 we know that GA inhibition is not cell type specific, so the information that we obtain from HeLa cells should be applicable to other cells as well.

HeLa cells were infected with vaccinia virus at an MOI of 5 PFU per cell and harvested at different times for Western blot analysis (Fig. 5). The level of Hsp90 was not induced by vaccinia virus and remained relatively constant up to 4 h p.i. At 24 h p.i., Hsp90 migrated slightly faster on SDS-polyacrylamide gels, but the reason was not known. A minor increase of Hsp70 expression was noticed early after infection, but an increase of the control actin protein was also observed. Thus, no significant induction of Hsp90 and Hsp70 was detected after vaccinia virus infection. Furthermore, unlike cyclophilin A, which is packaged into HIV virions, Hsp90 and Hsp70 were not incorporated into vaccinia virus particles and were not detected in purified IMV (Fig. 5). Viral late H3L protein served as a positive control and was present in virus-infected cells and purified virions as expected (38).

Subcellular distribution of Hsp90 is altered and transiently colocalized with virosomes in infected cells. The intracellular localization of Hsp90 was examined in uninfected and virus-infected HeLa cells under a confocal microscope (Fig. 6). Since vaccinia virus A4L protein is an abundant core protein known

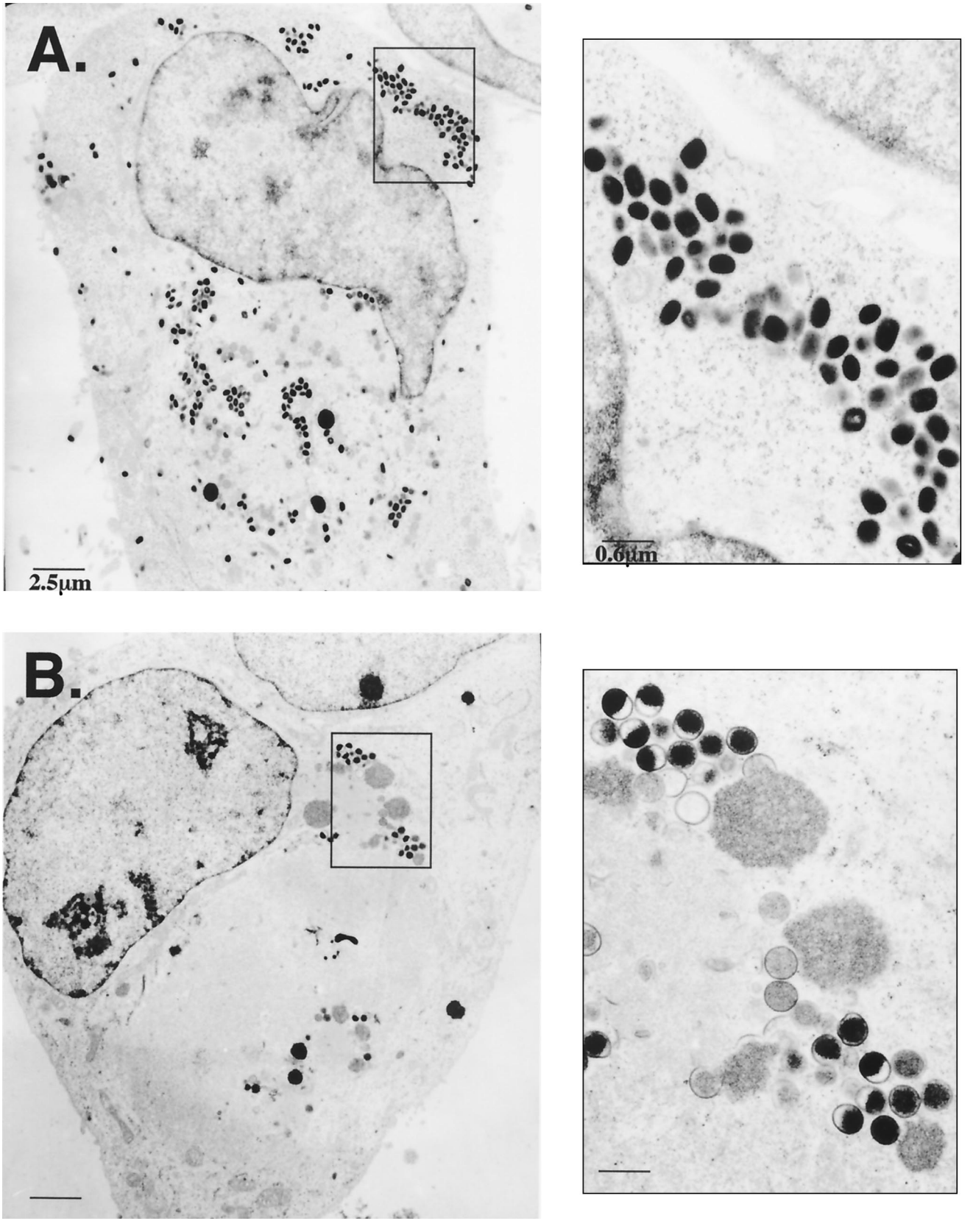


FIG. 2. Electron micrographs of RK13 cells infected with vaccinia virus in the presence of GA. RK13 cells were infected with WT vaccinia virus, incubated for 24 h in the absence (A) or presence (B) of GA, and processed for electron microscopy as described previously (38). Panels A and B are ×3,000 magnifications, and the panels at right are higher magnifications (×30,000) of the boxed areas in panels A and B.

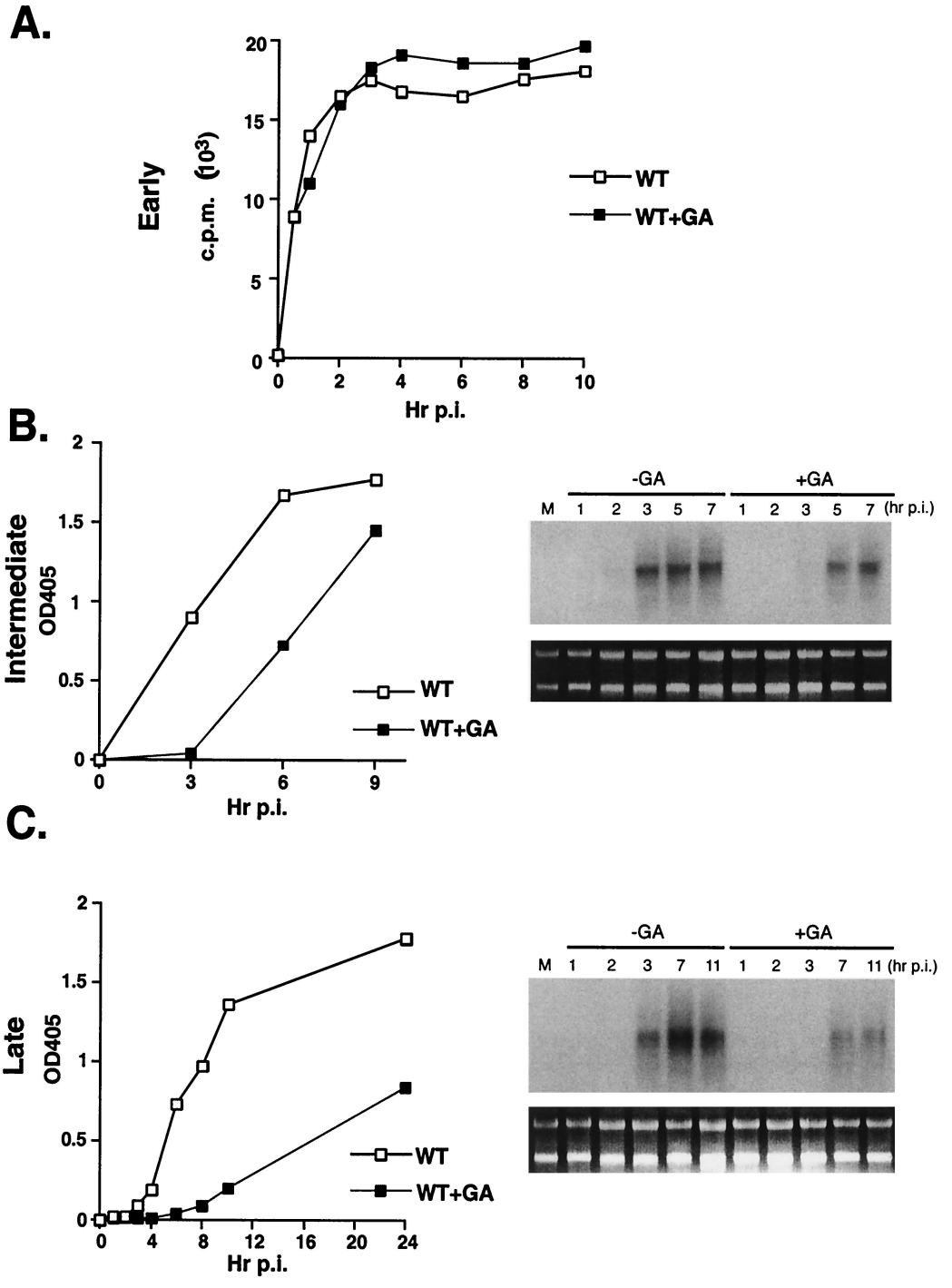


FIG. 3. Reporter gene expression from viral promoters in GA-treated RK13 cells. RK13 cells were infected with individual recombinant vaccinia viruses that express CAT or *lacZ* reporter genes driven by early (A), intermediate (B), or late (C) promoters as described in Materials and Methods; incubated in the absence (WT) or presence (WT+GA) of 0.5 μM GA for various times; and harvested for reporter enzyme assays as described previously (55). Also, Northern blot analyses of reporter gene *lacZ* transcripts expressed from intermediate and late promoters are shown at the right of panels B and C, respectively.

to be present on incoming cores, virosomes, and virion progeny, staining of A4L protein in cells will faithfully reflect these viral structures in infected cells (49, 57). For technical convenience, we used a recombinant vaccinia virus that expresses A4L core protein fused to an ECFP to facilitate a direct visualization of virosomes and intracellular virions in virus-infected cells (Fig. 6g, k, and s).

In mock-infected cells, Hsp90 was distributed throughout the cytoplasm but was specifically excluded from the nucleus (Fig. 6b). In virus-infected cells, the cytoplasmic staining of

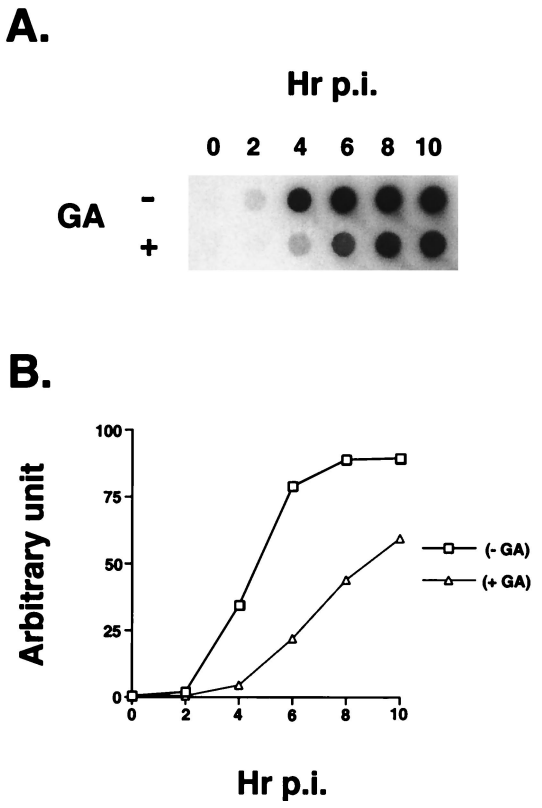


FIG. 4. Viral DNA replication in RK13 cells is affected by GA. (A) RK13 cells were infected as described in Materials and Methods and harvested at 0, 2, 4, 6, 8, and 10 h p.i. After cell lysis, viral DNA was extracted and a DNA sample was applied to nitrocellulose paper using a filtration manifold and hybridized with a ^{32}P-labeled *lacZ* fragment as a probe. (B) Quantitative analysis of viral DNA replication. Nitrocellulose filters as shown in panel A were scanned with a phosphorimager and quantitated by computer program analysis according to the manufacturer's instructions (Molecular Dynamics, Inc.). The data are the averages of two independent experiments.

Hsp90 was altered and some areas of stronger fluorescence staining were observed at 12 h p.i. (Fig. 6f). These strongly staining regions became partially overlapped with intracellular virosomes near the nuclear periphery (Fig. 6f to h). Translocation of Hsp90 toward the peripheral region of virosomes is specific, since other cellular proteins such as actin did not accumulate at virosomes (Fig. 6r to t). At 24 h p.i., many infected cells harbored large numbers of intracellular virions that spread throughout the cytoplasmic area; however, no colocalization of Hsp90 with virions was seen (Fig. 6j to l). Thus, distribution of Hsp90 is consistent with the data in Fig. 5 indicating that Hsp90 is not present in IMV. In conclusion, translocation of Hsp90 around virosomes in infected cells indicated that it plays a transient role during vaccinia virus replication in cells.

Interaction of Hsp90 with viral core protein 4a in virus-infected cells. Confocal microscope analysis indicated that Hsp90 interacts with virosomes in infected cells. To extend this biological observation further, coimmunoprecipitation experiments were performed to determine whether Hsp90 interacts with any viral protein in virus-infected cells. Cell lysates were prepared from mock-infected, virus-infected, or GA-treated infected cells, and the Hsp90-containing immunocomplexes were analyzed on SDS–10% polyacrylamide gels (Fig. 7A). A protein of approximately 65 kDa was specifically immunoprecipitated from virus-infected but not from mock-infected cells, indicating that it could be a viral gene product. The 65-kDa protein was excised from the gel and used for peptide mass analysis. The protein sequence database was searched with the resulting peptide mass data, and we identified the 65-kDa protein as vaccinia virus core protein 4a, a processed form of p4a that is encoded by the A10L gene (Fig. 7B) (23).

In order to confirm that the interaction of Hsp90 and core protein 4a is specific, we obtained an anticore Ab that reacts with 4a-4b core proteins that are present on purified viral cores (49). Cell lysates were first subjected to immunoprecipitation with control Ab or anti-Hsp90 Ab, and the immunocomplexes were analyzed by Western blot analyses with the anticore or anti-Hsp90 Abs, respectively (Fig. 7C). It is clear that anti-Hsp90 Ab, but not the control Ab, specifically immunoprecipitated Hsp90 from infected-cell lysates. More importantly, a 65-kDa protein that was recognized by the anticore Ab was present only in virus-infected cells and not in mock-infected cells. The immunoreactivity of the 65-kDa protein was consistent with our peptide results in Fig. 7B and demonstrated that Hsp90 binding to core protein 4a is not an artifact generated from nonspecific interaction of Abs with infected-cell lysates.

DISCUSSION

Previous studies of HSPs in vaccinia virus infection indicate that Hsp70 and Hsp72 are dispensable (32, 61, 62). One report showed a dramatic induction of Hsp70 after vaccinia virus infection of primary macrophages at 12 h p.i. (32). Another study showed that vaccinia virus grows in cells that do not express Hsp72 and that overexpression of Hsp72 in these cells does not increase virus titers (61). In our experiment, Hsp70 was not induced after vaccinia virus infection from 30 min up to 24 h p.i., although it was easily induced in 15 min after heat

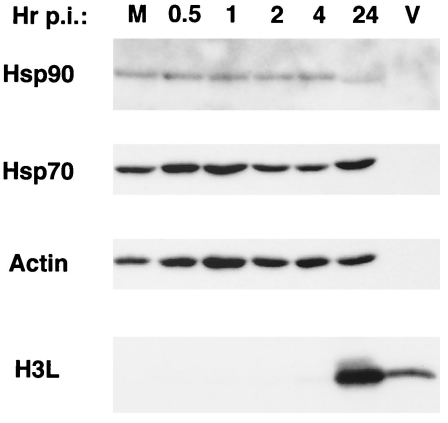
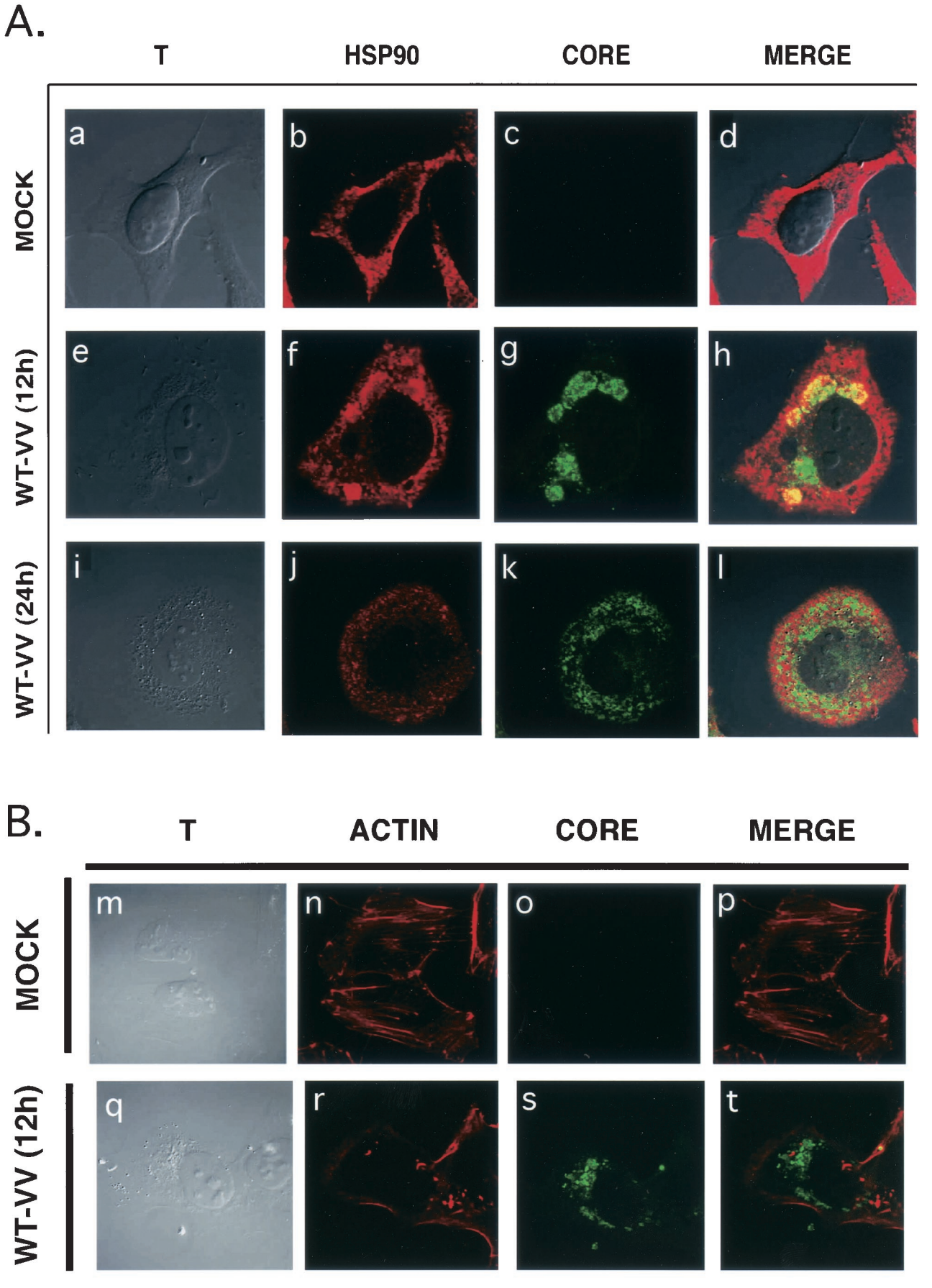


FIG. 5. Expression of HSPs in HeLa cells infected with vaccinia virus. HeLa cells were either mock infected (M) or infected with WT vaccinia virus at an MOI of 5 PFU per cell and harvested at 0.5, 1, 2, 4, and 24 h p.i. Cell lysates were harvested for Western blot analyses with Abs against Hsp90, Hsp70, actin, or vaccinia virus H3L protein as described in Materials and Methods. Lane V indicates purified vaccinia virus IMV.


A.
T
HSP90
CORE
MERGE
MOCK
WT-VV (12h)
WT-VV (24h)
a
b
c
d
e
f
g
h
i
j
k
l
B.
T
ACTIN
CORE
MERGE
MOCK
WT-VV (12h)
m
n
o
p
q
r
s
t

treatment. The discrepancy between our results and previous data may be due to different cell types in use.

This study demonstrates that Hsp90 is essential for vaccinia virus growth in cells. Our conclusion is based on the evidence that GA strongly inhibits vaccinia virus growth in several cell lines. The advantages of using GA in experiments are several. First of all, it has a well-defined chemical structure, a benzoquinone ansamycin moiety; specifically binds to Hsp90, as documented elsewhere (63, 74); and is easy to obtain commercially (69). Crystal structure analysis of the Hsp90 complex with GA revealed that GA binds to the nucleotide-binding pocket within the N-terminal domain of Hsp90 (53, 54). Most importantly, numerous studies have shown that GA specifically interferes with Hsp90 chaperone functions, making GA a convenient and reliable reagent to assess Hsp90 functions in various biological systems (12, 24, 29, 36, 73). In our analysis, although we favor the interpretation that GA inhibition of vaccinia virus growth reflects an essential role of Hsp90 in the virus life cycle, we cannot completely exclude the possibility that GA also inhibits other targets in virus-infected cells. More work is needed to clarify this issue in the future.

One objection to using inhibitors like GA is that, despite specific targets being known, these drugs could be pleiotropic due to cytotoxicity, which could complicate the interpretation of data. To avoid such problems, we carefully titrated drug concentrations on freshly confluent cells so that monolayer cells remained viable up to 3 days. At the same time, virus plaques growing on these drug-treated cells became very small and difficult to visualize. Thus, we conclude that, while GA exhibits minimal toxicity to cells, it blocks virus growth to a great extent.

An alternative way to demonstrate the effect of GA on vaccinia virus growth is to use a genetic approach. Genetic selection for mutant cells that are resistant to GA would complement the above approach that we used. If vaccinia virus growth in GA-resistant cells is blocked, it demonstrates that the cellular target of GA is genetically linked with the cellular activity necessary for vaccinia virus growth. However, selection for such resistant cells could be difficult, since it would be for the Hsp90 null mutation, which is lethal in eukaryotes (74). Indeed, there was one report describing a breast cancer cell line which, after continuous passage in GA-containing medium for 6 months, was adapted in 0.1 μM GA but, nevertheless, remained sensitive to GA at higher concentrations (4). Therefore, a genetic approach to isolate GA-resistant cells, though sound in theory, may not be feasible.

GA does not inhibit early events such as viral early gene expression. However, GA delays viral DNA replication and therefore slows down intermediate viral gene transcription. GA also inhibits viral late gene transcription, which may be an indirect effect, since vaccinia virus regulates gene transcription through the cascade mechanism. Similarly, because most structural proteins are expressed to lesser extents virion assembly could simply be halted due to a lack of building blocks. Since in all our experiments GA was added to cells immediately after virus infection and remained in the medium throughout the virus life cycle, these analyses revealed viral DNA replication only as the earliest stage at which the Hsp90 function became rate limiting for virus growth.

There are two possible mechanisms to explain the above GA inhibition of viral DNA replication. One possibility is that Hsp90 is required for proper folding and maturation of enzymes in the viral DNA replication machinery; thus, in the presence of GA, the viral replication machinery may include misfolded proteins and therefore be defective. However, this possibility seems unlikely, since viral DNA replication is delayed but otherwise fully active in GA-treated cells. Alternatively, Hsp90 may regulate uncoating events such as conformational alterations of viral core proteins so that they could be released from the viral genome and the viral DNA would become accessible to the replication machinery. Such conformational change of core proteins is slowed down when GA is present, resulting in delay of DNA replication. As we discuss below, we prefer the latter hypothesis, since our results showed that Hsp90 binds to core protein 4a.

Hsp90 is localized in the cytoplasm of uninfected cells. Although the protein level of Hsp90 is not induced in virus-infected cells, its distribution changes. Some staining of Hsp90 seems to concentrate around cell edges early after virus infection. Most interestingly, Hsp90 interacts with virosomes where active DNA replication occurs. By confocal microscopy, consecutive sections of Hsp90 images in infected cells were reconstructed in three dimensions. These images nicely reveal that the location of Hsp90 on virosomes is concentrated on their outside surface toward the cytosolic face (data not shown). Such colocalization of Hsp90 with virosomes is not observed when GA is added, indicating that such association is related to the functions of Hsp90. The colocalization is transient and appears to be restricted to certain stages of virosomes. For example, in Fig. 6h there are four virosomes coated with Hsp90 in a single cell. These virosomes have clear boundaries with no release of virus particles. However, the only virosome with much less Hsp90 costaining is surrounded by scattering virus particles. This implies to us that after virus infection Hsp90 is recruited to virosomes and remains associated. This association is transient, and Hsp90 disappears when virosomes mature to produce virion particles. Indeed, at 24 h p.i. when numerous IMV were produced no colocalization of Hsp90 with IMV was seen in infected cells. Consistently, no Hsp90 was detected in purified IMV particles. Translocation of Hsp90 to virosomes is not simply a nonspecific crowding of cytoplasmic protein at virosome borders, since other cytoplasmic proteins such as actin do not show a similar staining pattern. It is interesting to speculate on the molecular mechanism that regulates Hsp90

FIG. 6. Confocal microscope images of Hsp90 (A) or actin (B) and viral cores in mock-infected or virus-infected HeLa cells. HeLa cells were seeded on glass slides and either mock infected (a to d and m to p) or infected with a recombinant vaccinia virus expressing A4L-ECFP fusion protein (e to l and q to t). At 12 and 24 h p.i., cells were fixed and processed for confocal imaging analysis as described in Materials and Methods. T, transmission microscope for cell morphology; HSP90, Hsp90 staining as visualized with Cy5-conjugated secondary Abs; ACTIN, cytoplasmic actin staining with Cy5-conjugated phalloidin; CORE, the intracellular virosomes and viruses as visualized by fluorescent A4L-ECFP fusion protein; VV, vaccinia virus.

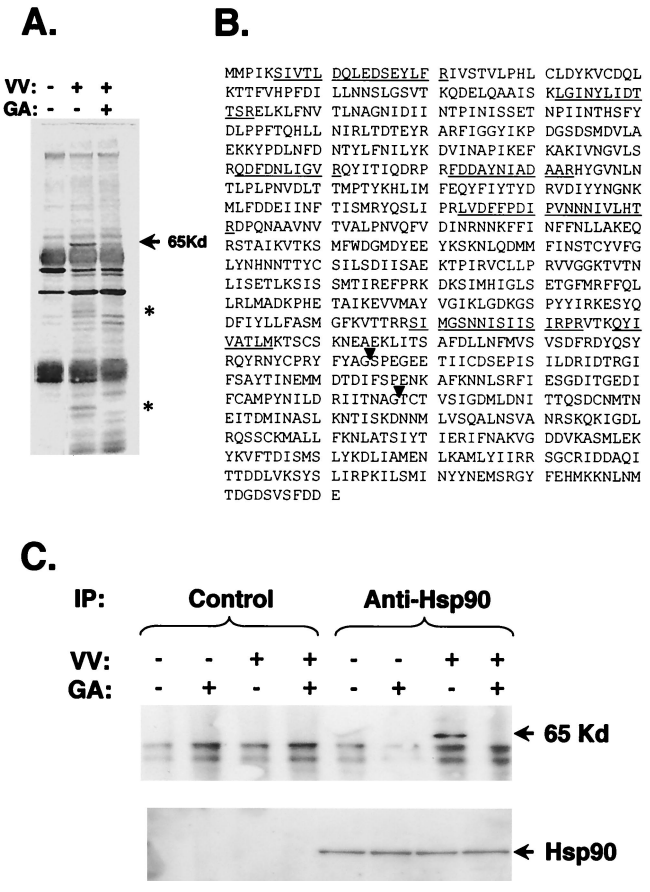


FIG. 7. Identification of core protein 4a as the 65-kDa protein associated with Hsp90 in virus-infected cells. (A) Silver staining of a 65-kDa protein in Hsp90-containing immunoprecipitates in infected cells. The position of a 65-kDa protein in the silver-stained gel is indicated by an arrow. Two smaller proteins marked by asterisks are proteolytic fragments of the Abs. (B) The p4a precursor amino acid sequences encoded by the vaccinia virus A10L gene. Two arrowheads indicate proteolytic cleavage sites between aa 614 and 615 and aa 697 and 698, respectively, to generate processed core 4a protein and p23. The core protein 4a contains the N-terminal sequence of 614 aa. The underlined regions are the peptide mass data obtained by mass spectrometry analysis and are all present in the core protein 4a region (EverNew Biotech). (C) Specific interaction of core protein 4a with Hsp90. HeLa cells were either mock infected or infected with vaccinia virus (VV) at an MOI of 5 PFU per cell, and cells were incubated in medium with or without GA as indicated above the panel. Cell lysates were harvested, and the immunocomplexes obtained with either goat antizyxin (control Ab) or anti-Hsp90 Ab were separated by SDS–10% PAGE and analyzed with anticore Ab or anti-Hsp90 Ab. The positions of the 65-kDa protein and Hsp90 are marked by arrows. IP, immunoprecipitation.

translocation, which could be mediated by microtubules. Recently, the literature has reported a direct interaction of Hsp90 or its homologues with microtubule components in vitro as well as in cells (11, 12, 19, 56).

From these observations, we reasoned that Hsp90 should interact with viral proteins on virosomes in infected cells, and when such interaction is interrupted by GA, the virus life cycle is blocked. Coimmunoprecipitation experiments revealed that viral core protein 4a is specifically associated with Hsp90 in virus-infected cells, although such an association could be indirect.

Core protein 4a is processed from a precursor core protein, p4a, which is encoded by the viral A10L gene (71). p4a protein is 102 kDa in size and is cleaved at sites between amino acids (aa) 614 and 615 and aa 697 and 698 to generate core protein 4a and p25, respectively (66, 68). Both p4a and 4a proteins interact with A4L protein during virion morphogenesis (57). A recombinant virus that expresses the A10L gene under isopropyl-β-D-thiogalactopyranoside (IPTG) regulation indicated that core proteins p4a-4a are important for virion morphogenesis (25). Since we showed that Hsp90 is translocated to virosomes in infected cells, the relationship of such movement with core protein 4a and morphogenesis could be further explored with this virus. For example, when cells are infected with the IPTG-regulated A10L virus, in the absence of IPTG, we expect that Hsp90 will remain cytoplasmic. This result will demonstrate that expression of core protein 4a is critical for Hsp90 translocation in infected cells.

Core protein 4a was also suggested to have some roles in viral core penetration immediately after membrane fusion. Core protein 4a forms intermolecular disulfide bonds in the virions that become reduced during virus penetration into cells, suggesting a major conformational change of core protein 4a on incoming cores (40). Furthermore, core protein 4a was shown previously to mediate binding of viral cores to microtubules in vitro (52). These two properties of core protein 4a were of particular interest to us since data from GA inhibition experiments suggested a role for Hsp90 in viral core uncoating. One explanation is that Hsp90 facilitates core protein 4a structural alteration that couples with initiation of DNA replication. Another, more interesting, idea is that Hsp90 binding to core protein 4a also involves microtubules, reflecting a role of Hsp90 in regulating intracellular trafficking of incoming cores. Previously, Hsp90 interaction with microtubules was shown to be important for intracellular transport of its substrate proteins such as glucocorticoid hormone receptor complexes that translocate from the cytosol to the nucleus (1, 37). As for the similar short-range trafficking of viral cores along microtubules, binding of core protein 4a to Hsp90 might be a direct mechanism for linking the incoming viral cores to the molecular motors for movement toward the microtubule organization center at the perinuclear region. If this is true, we would expect that Hsp90 would colocalize with incoming cores as well as microtubule components prior to DNA replication in cells. Unfortunately, we could not visualize the transport of incoming cores in cells under confocal microscopy due to the fact that A4L-ECFP fluorescence of the viral cores was too weak to be detected. Thus, the significance of microtubule association remains a hypothesis and needs to be tested in the future.

If Hsp90 indeed affects virus growth via its interaction with core protein 4a, how do we explain the fact that the IPTG-regulated A10L virus exhibits a mutant phenotype different from that of WT virus under GA treatment? We think the apparent discrepancy is due to the limitation of individual methods in use. The IPTG-regulated A10L recombinant virus revealed the essential role of newly synthesized p4a-4a in DNA packaging into nucleoprotein complexes during assembly, whereas GA is added in medium immediately after viral infec-

tion and affects functions of the existing core protein 4a at the early phase. The IPTG-regulated A10L mutant virus cannot be used to address functions of core protein 4a at the early phase, since the A10L gene is essential and mutant virions devoid of core 4a will not be formed (25).

Hsp90 forms discrete subcomplexes in cells and interacts with more than 10 cochaperones before binding to its client proteins (7). Some cochaperones contain a conserved tetratricopeptide repeat (TPR) for binding to Hsp90 (48). TPR is a degenerate 34-aa repeated motif that forms an amphipathic α-helix to mediate protein-protein interactions and is present on proteins with diverse functions (34). We found no TPR-like sequences on core 4a protein or other vaccinia virus open reading frames, indicating that core protein 4a may directly interact with Hsp90 via a novel, unidentified region. Alternatively, core protein 4a binding to Hsp90 could be indirect and mediated by other cellular components present in Hsp90-containing complexes.

The finding of Hsp90 association with core protein 4a may have influence on cell biology as well. First of all, the association of Hsp90 with tubulin is important for microtubule stability (11, 12, 19, 56, 70). However, vaccinia virus infection was shown elsewhere to induce reorganization of microtubules and disruption of the centrosome in a fashion reminiscent of overexpression of microtubule-associated proteins (MAPs) (52). Furthermore, vaccinia virus proteins A10L and L4R were shown previously to have MAP-like properties and to mediate direct binding of viral cores to microtubules in vitro (52). Whether binding of core protein 4a to Hsp90 also leads to reduction of microtubule stability in infected cells remains an interesting possibility. HSP interactions with viral structural proteins are not without precedents. In cells infected by adenovirus, Hsp70 and Hsc70 proteins were associated with hexon, the major adenovirus capsid protein, soon after virus penetration into cells (45).

Viruses must evolve a delicate balance with their host cell in order to survive and proliferate. Viruses with large genomes, like vaccinia virus, could encode more essential functions and thereby gain more autonomy, but these viruses are not economical. In contrast, viruses with small genomes are more restricted by host cell activity in order to replicate. Nevertheless, all viruses depend on cells for crucial steps in their life cycles. Thus, the identification of cellular factors that play essential functions during virus replication is critical to understanding viral biology and cellular pathological response. This report demonstrates that Hsp90 is essential for vaccinia virus growth, and perhaps by diverting Hsp90 to viral substrates, such as core protein 4a, virus could pirate cellular factors and take advantage of host resources that normally are regulated by Hsp90. It is possible that many such cellular factors are present in cells and remain to be identified.

ACKNOWLEDGMENTS

We thank Sue-Ping Lee for her excellent techniques in confocal and electron microscopy and C.-L. Lin for help in computer image analyses.

This work is supported by grants from Academia Sinica and the National Science Council (NSC89-2311-B-001-212) of the Republic of China.

REFERENCES

1. **Akner, G., A. C. Wikstrom, P. E. Stromstedt, O. Stockman, J. A. Gustafsson, and M. Wallin.** 1995. Glucocorticoid receptor inhibits microtubule assembly in vitro. Mol. Cell. Endocrinol. **110:**49–54.
2. **Bair, C.-H., C.-S. Chung, I. A. Vasilevskaya, and W. Chang.** 1996. Isolation and characterization of a Chinese hamster ovary mutant cell line with altered sensitivity to vaccinia virus killing. J. Virol. **70:**4655–4666.
3. **Baldick, C. J., Jr., and B. Moss.** 1993. Characterization and temporal regulation of mRNAs encoded by vaccinia virus intermediate-stage genes. J. Virol. **67:**3515–3527.
4. **Benchekroun, M. N., E. Schneider, A. R. Safa, A. J. Townsend, and B. K. Sinha.** 1994. Mechanisms of resistance to ansamycin antibiotics in human breast cancer cell lines. Mol. Pharmacol. **46:**677–684.
5. **Blum, H., H. Beier, and H. J. Gross.** 1987. Improved silver staining of plant protein, RNA and DNA in polyacrylamide gels. Electrophoresis **8:**93–99.
6. **Borkovich, K. A., F. W. Farrelly, D. B. Finkelstein, J. Taulien, and S. Lindquist.** 1989. hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. Mol. Cell. Biol. **9:**3919–3930.
7. **Caplan, A. J.** 1999. Hsp90's secrets unfold: new insights from structural and functional studies. Trends Cell Biol. **9:**262–268.
8. **Collins, P. L., and L. E. Hightower.** 1982. Newcastle disease virus stimulates the cellular accumulation of stress (heat shock) mRNAs and proteins. J. Virol. **44:**703–707.
9. **Craig, E. A., B. D. Gambill, and R. J. Nelson.** 1993. Heat shock proteins: molecular chaperones of protein biogenesis. Microbiol. Rev. **57:**402–414.
10. **Cullen, B. R., and J. Heitman.** 1994. Human immunodeficiency virus. Chaperoning a pathogen. Nature **372:**319–320.
11. **Czar, M. J., M. J. Welsh, and W. B. Pratt.** 1996. Immunofluorescence localization of the 90-kDa heat-shock protein to cytoskeleton. Eur. J. Cell Biol. **70:**322–330.
12. **de Carcer, G., M. do Carmo Avides, M. J. Lallena, D. M. Glover, and C. Gonzalez.** 2001. Requirement of Hsp90 for centrosomal function reflects its regulation of Polo kinase stability. EMBO J. **20:**2878–2884.
13. **Ensinger, M. J.** 1987. Phenotypic characterization of temperature-sensitive mutants of vaccinia virus with mutations in a 135,000-M_r subunit of the virion-associated DNA-dependent RNA polymerase. J. Virol. **61:**1842–1850.
14. **Fenner, F.** 1990. Poxviruses, p. 2113–2133. *In* B. Fields and D. M. Knipe (ed.), Virology. Raven Press, New York, N.Y.
15. **Fink, A. L.** 1999. Chaperone-mediated protein folding. Physiol. Rev. **79:**425–449.
16. **Franke, E. K., H. E. Yuan, and J. Luban.** 1994. Specific incorporation of cyclophilin A into HIV-1 virions. Nature **372:**359–362.
17. **Gale, M., Jr., C. M. Blakely, D. A. Hopkins, M. W. Melville, M. Wambach, P. R. Romano, and M. G. Katze.** 1998. Regulation of interferon-induced protein kinase PKR: modulation of P58IPK inhibitory function by a novel protein, P52rIPK. Mol. Cell. Biol. **18:**859–871.
18. **Gale, M., Jr., S. L. Tan, M. Wambach, and M. G. Katze.** 1996. Interaction of the interferon-induced PKR protein kinase with inhibitory proteins P58IPK and vaccinia virus K3L is mediated by unique domains: implications for kinase regulation. Mol. Cell. Biol. **16:**4172–4181.
19. **Garnier, C., P. Barbier, R. Gilli, C. Lopez, V. Peyrot, and C. Briand.** 1998. Heat-shock protein 90 (hsp90) binds in vitro to tubulin dimer and inhibits microtubule formation. Biochem. Biophys. Res. Commun. **250:**414–419.
20. **Garry, R. F., E. T. Ulug, and H. R. Bose, Jr.** 1983. Induction of stress proteins in Sindbis virus- and vesicular stomatitis virus-infected cells. Virology **129:**319–332.
21. **Gilmore, R., M. C. Coffey, and P. W. Lee.** 1998. Active participation of Hsp90 in the biogenesis of the trimeric reovirus cell attachment protein sigma1. J. Biol. Chem. **273:**15227–15233.
22. **Glotzer, J. B., M. Saltik, S. Chiocca, A. I. Michou, P. Moseley, and M. Cotten.** 2000. Activation of heat-shock response by an adenovirus is essential for virus replication. Nature **407:**207–211.
23. **Goebel, S. J., G. P. Johnson, M. E. Perkus, S. W. Davis, J. P. Winslow, and E. Paoletti.** 1990. The complete DNA sequence of vaccinia virus. Virology **179:**247–266, 517–563.
24. **Grammatikakis, N., J. H. Lin, A. Grammatikakis, P. N. Tsichlis, and B. H. Cochran.** 1999. p50(cdc37) acting in concert with Hsp90 is required for Raf-1 function. Mol. Cell. Biol. **19:**1661–1672.
25. **Heljasvaara, R., D. Rodriguez, C. Risco, J. L. Carrascosa, M. Esteban, and J. R. Rodriguez.** 2001. The major core protein P4a (A10L gene) of vaccinia virus is essential for correct assembly of viral DNA into the nucleoprotein complex to form immature viral particles. J. Virol. **75:**5778–5795.
26. **Hooda-Dhingra, U., C. L. Thompson, and R. C. Condit.** 1989. Detailed phenotypic characterization of five temperature-sensitive mutants in the 22- and 147-kilodalton subunits of vaccinia virus DNA-dependent RNA polymerase. J. Virol. **63:**714–729.
27. **Hsiao, J. C., C. S. Chung, and W. Chang.** 1999. Vaccinia virus envelope D8L protein binds to cell surface chondroitin sulfate and mediates the adsorption of intracellular mature virions to cells. J. Virol. **73:**8750–8761.
28. **Hu, J., and C. Seeger.** 1996. Hsp90 is required for the activity of a hepatitis

B virus reverse transcriptase. Proc. Natl. Acad. Sci. USA **93:**1060–1064.
29. **Imai, J., and I. Yahara.** 2000. Role of HSP90 in salt stress tolerance via stabilization and regulation of calcineurin. Mol. Cell. Biol. **20:**9262–9270.
30. **Jaattela, M., and D. Wissing.** 1992. Emerging role of heat shock proteins in biology and medicine. Ann. Med. **24:**249–258.
31. **Jakob, U., H. Lilie, I. Meyer, and J. Buchner.** 1995. Transient interaction of Hsp90 with early unfolding intermediates of citrate synthase. Implications for heat shock in vivo. J. Biol. Chem. **270:**7288–7294.
32. **Jindal, S., and R. A. Young.** 1992. Vaccinia virus infection induces a stress response that leads to association of Hsp70 with viral proteins. J. Virol. **66:**5357–5362.
33. **Keck, J. G., C. J. Baldick, Jr., and B. Moss.** 1990. Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late trans-activator genes. Cell **61:**801–809.
34. **Lamb, J. R., S. Tugendreich, and P. Hieter.** 1995. Tetratrico peptide repeat interactions: to TPR or not to TPR? Trends Biochem. Sci. **20:**257–259.
35. **La Thangue, N. B., and D. S. Latchman.** 1988. A cellular protein related to heat-shock protein 90 accumulates during herpes simplex virus infection and is overexpressed in transformed cells. Exp. Cell Res. **178:**169–179.
36. **Lewis, J., A. Devin, A. Miller, Y. Lin, Y. Rodriguez, L. Neckers, and Z. G. Liu.** 2000. Disruption of hsp90 function results in degradation of the death domain kinase, receptor-interacting protein (RIP), and blockage of tumor necrosis factor-induced nuclear factor-kappaB activation. J. Biol. Chem. **275:**10519–10526.
37. **Liang, P., and T. H. MacRae.** 1997. Molecular chaperones and the cytoskeleton. J. Cell Sci. **110:**1431–1440.
38. **Lin, C. L., C. S. Chung, H. G. Heine, and W. Chang.** 2000. Vaccinia virus envelope H3L protein binds to cell surface heparan sulfate and is important for intracellular mature virion morphogenesis and virus infection in vitro and in vivo. J. Virol. **74:**3353–3365.
39. **Lindquist, S., and E. A. Craig.** 1988. The heat-shock proteins. Annu. Rev. Genet. **22:**631–677.
40. **Locker, J. K., and G. Griffiths.** 1999. An unconventional role for cytoplasmic disulfide bonds in vaccinia virus proteins. J. Cell Biol. **144:**267–279.
41. **Melville, M. W., W. J. Hansen, B. C. Freeman, W. J. Welch, and M. G. Katze.** 1997. The molecular chaperone hsp40 regulates the activity of P58IPK, the cellular inhibitor of PKR. Proc. Natl. Acad. Sci. USA **94:**97–102.
42. **Melville, M. W., M. G. Katze, and S. L. Tan.** 2000. P58IPK, a novel cochaperone containing tetratricopeptide repeats and a J-domain with oncogenic potential. Cell Mol. Life Sci. **57:**311–322.
43. **Miyata, Y., and I. Yahara.** 2000. p53-independent association between SV40 large T antigen and the major cytosolic heat shock protein, HSP90. Oncogene **19:**1477–1484.
44. **Nathan, D. F., M. H. Vos, and S. Lindquist.** 1997. In vivo functions of the Saccharomyces cerevisiae Hsp90 chaperone. Proc. Natl. Acad. Sci. USA **94:**12949–12956.
45. **Niewiarowska, J., J. C. D'Halluin, and M. T. Belin.** 1992. Adenovirus capsid proteins interact with HSP70 proteins after penetration in human or rodent cells. Exp. Cell Res. **201:**408–416.
46. **Notarianni, E. L., and C. M. Preston.** 1982. Activation of cellular stress protein genes by herpes simplex virus temperature-sensitive mutants which overproduce immediate early polypeptides. Virology **123:**113–122.
47. **Panaretou, B., C. Prodromou, S. M. Roe, R. O'Brien, J. E. Ladbury, P. W. Piper, and L. H. Pearl.** 1998. ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. EMBO J. **17:**4829–4836.
48. **Pearl, L. H., and C. Prodromou.** 2000. Structure and in vivo function of Hsp90. Curr. Opin. Struct. Biol. **10:**46–51.
49. **Pedersen, K., E. J. Snijder, S. Schleich, N. Roos, G. Griffiths, and J. K. Locker.** 2000. Characterization of vaccinia virus intracellular cores: implications for viral uncoating and core structure. J. Virol. **74:**3525–3536.
50. **Peluso, R. W., R. A. Lamb, and P. W. Choppin.** 1978. Infection with paramyxoviruses stimulates synthesis of cellular polypeptides that are also stimulated in cells transformed by Rous sarcoma virus or deprived of glucose. Proc. Natl. Acad. Sci. USA **75:**6120–6124.
51. **Phillips, B., K. Abravaya, and R. I. Morimoto.** 1991. Analysis of the specificity and mechanism of transcriptional activation of the human hsp70 gene during infection by DNA viruses. J. Virol. **65:**5680–5692.
52. **Ploubidou, A., V. Moreau, K. Ashman, I. Reckmann, C. Gonzalez, and M. Way.** 2000. Vaccinia virus infection disrupts microtubule organization and centrosome function. EMBO J. **19:**3932–3944.
53. **Prodromou, C., S. M. Roe, R. O'Brien, J. E. Ladbury, P. W. Piper, and L. H. Pearl.** 1997. Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. Cell **90:**65–75.
54. **Prodromou, C., S. M. Roe, P. W. Piper, and L. H. Pearl.** 1997. A molecular clamp in the crystal structure of the N-terminal domain of the yeast Hsp90 chaperone. Nat. Struct. Biol. **4:**477–482.
55. **Ramsey-Ewing, A. L., and B. Moss.** 1995. Restriction of vaccinia virus replication in CHO cells occurs at the stage of viral intermediate protein synthesis. Virology **206:**984–993.
56. **Redmond, T., E. R. Sanchez, E. H. Bresnick, M. J. Schlesinger, D. O. Toft, W. B. Pratt, and M. J. Welsh.** 1989. Immunofluorescence colocalization of the 90-kDa heat-shock protein and microtubules in interphase and mitotic mammalian cells. Eur. J. Cell Biol. **50:**66–75.
57. **Risco, C., J. R. Rodriguez, W. Demkowicz, R. Heljasvaara, J. L. Carrascosa, M. Esteban, and D. Rodriguez.** 1999. The vaccinia virus 39-kDa protein forms a stable complex with the p4a/4a major core protein early in morphogenesis. Virology **265:**375–386.
58. **Sanchez, E. R., and Y. M. Ning.** 1996. Immunophilins, heat shock proteins, and glucocorticoid receptor actions in vivo. Methods **9:**188–200.
59. **Schulte, T. W., S. Akinaga, S. Soga, W. Sullivan, B. Stensgard, D. Toft, and L. M. Neckers.** 1998. Antibiotic radicicol binds to the N-terminal domain of Hsp90 and shares important biologic activities with geldanamycin. Cell Stress Chaperones **3:**100–108.
60. **Schulte, T. W., M. V. Blagosklonny, C. Ingui, and L. Neckers.** 1995. Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association. J. Biol. Chem. **270:**24585–24588.
61. **Sedger, L., I. Ramshaw, A. Condie, J. Medveczky, A. Braithwaite, and J. Ruby.** 1996. Vaccinia virus replication is independent of cellular HSP72 expression which is induced during virus infection. Virology **225:**423–427.
62. **Sedger, L., and J. Ruby.** 1994. Heat shock response to vaccinia virus infection. J. Virol. **68:**4685–4689.
63. **Supko, J. G., R. L. Hickman, M. R. Grever, and L. Malspeis.** 1995. Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent. Cancer Chemother. Pharmacol. **36:**305–315.
64. **Tan, S. L., M. J. Gale, Jr., and M. G. Katze.** 1998. Double-stranded RNA-independent dimerization of interferon-induced protein kinase PKR and inhibition of dimerization by the cellular P58IPK inhibitor. Mol. Cell. Biol. **18:**2431–2443.
65. **Tan, S. L., and M. G. Katze.** 1998. Biochemical and genetic evidence for complex formation between the influenza A virus NS1 protein and the interferon-induced PKR protein kinase. J. Interferon Cytokine Res. **18:**757–766.
66. **Vanslyke, J. K., S. S. Whitehead, E. M. Wilson, and D. E. Hruby.** 1991. The multistep proteolytic maturation pathway utilized by vaccinia virus P4a protein: a degenerate conserved cleavage motif within core proteins. Virology **183:**467–478.
67. **Welch, W. J.** 1991. The role of heat-shock proteins as molecular chaperones. Curr. Opin. Cell Biol. **3:**1033–1038.
68. **Whitehead, S. S., N. A. Bersani, and D. E. Hruby.** 1995. Physical and molecular genetic analysis of the multistep proteolytic maturation pathway utilized by vaccinia virus P4a protein. J. Gen. Virol. **76:**717–721.
69. **Whitesell, L., E. G. Mimnaugh, B. De Costa, C. E. Myers, and L. M. Neckers.** 1994. Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc. Natl. Acad. Sci. USA **91:**8324–8328.
70. **Williams, N. E., and E. M. Nelsen.** 1997. HSP70 and HSP90 homologs are associated with tubulin in hetero-oligomeric complexes, cilia and the cortex of Tetrahymena. J. Cell Sci. **110:**1665–1672.
71. **Wittek, R., B. Richner, and G. Hiller.** 1984. Mapping of the genes coding for the two major vaccinia virus core polypeptides. Nucleic Acids Res. **12:**4835–4848.
72. **Xu, A., A. R. Bellamy, and J. A. Taylor.** 1998. BiP (GRP78) and endoplasmin (GRP94) are induced following rotavirus infection and bind transiently to an endoplasmic reticulum-localized virion component. J. Virol. **72:**9865–9872.
73. **Young, J. C., and F. U. Hartl.** 2000. Polypeptide release by Hsp90 involves ATP hydrolysis and is enhanced by the co-chaperone p23. EMBO J. **19:**5930–5940.
74. **Young, J. C., I. Moarefi, and F. U. Hartl.** 2001. Hsp90: a specialized but essential protein-folding tool. J. Cell Biol. **154:**267–273.