

Antiviral activity and RNA polymerase degradation following Hsp90 inhibition in a range of negative strand viruses

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Abstract

We have analyzed the effectiveness of Hsp90 inhibitors in blocking the replication of negative-strand RNA viruses. In cells infected with the prototype negative strand virus vesicular stomatitis virus (VSV), inhibiting Hsp90 activity reduced viral replication in cells infected at both high and low multiplicities of infection. This inhibition was observed using two Hsp90 inhibitors geldanamycin and radicicol. Silencing of Hsp90 expression using siRNA also reduced viral replication. Hsp90 inhibition changed the half-life of newly synthesized L protein (the large subunit of the VSV polymerase) from >1 h to less than 20 min without affecting the stability of other VSV proteins. Both the inhibition of viral replication and the destabilization of the viral L protein were seen when either geldanamycin or radicicol was added to cells infected with paramyxoviruses SV5, HPIV-2, HPIV-3, or SV41, or to cells infected with the La Crosse bunyavirus. Based on these results, we propose that Hsp90 is a host factor that is important for the replication of many negative strand viruses.

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Introduction

There is significant scientific and public health interest in viruses that utilize negative strand RNA as genomes. Many of these viruses are important pathogens, including some of the most significant recurring infectious disease problems (influenza, respiratory syncytial virus) as well as some of the most alarming emerging infectious diseases (Lassa, Marburg, Hanta, Rift Valley Fever). All of these viruses encode an RNA dependent RNA polymerase (RDRP). The RDRP is required both for viral transcription and for replication of the viral genome (Whelan et al., 2004). In addition to RNA polymerization, the RDRPs are also involved in 5' and 3' end modification and in recognizing *cis*-acting sequences that govern transcription and replication. Functionally, RDRPs from different viruses appear to utilize diverse strategies to accomplish these tasks, but

a repeating theme is that the RDRP requires either cooperating viral proteins (Gupta et al., 2003; Whelan et al., 2004) or host proteins (Das et al., 1998; Shen and Masters, 2001; Strauss and Strauss, 1999) to properly accomplish replication and/or transcription. Understanding these virus/virus and virus/host interactions that govern negative strand virus replication are important for developing ways to combat important infectious agents by blocking their ability to replicate.

Heat Shock Protein 90 (Hsp90) is a cellular chaperone that has been the focus of intense research for many years (for reviews see Terasawa et al., 2005; Young et al., 2001). Knockout models have shown that Hsp90 is essential for viability in yeast and *Drosophila* (Lange et al., 2000; Panaretou et al., 1998; Rutherford and Lindquist, 1998). Hsp90 displays ATP-dependent folding capacity (Panaretou et al., 1998), and is notable among chaperone proteins because unlike its promiscuous cousin chaperone HSP70 (Agashe and Hartl, 2000), Hsp90 appears to have a specific set of client proteins *in vivo* (Pratt and Toft, 2003). These client proteins include steroid receptors, a number of transcription factors, and several protein

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kinases (Terasawa et al., 2005). Hsp90 forms a complex with co-chaperones (i.e. AHA1, Hop/Sti1, FKBP51, and 52) which then act to fold client proteins. Several known oncogenes are client proteins of Hsp90 (Pratt and Toft, 2003), which has sparked interest in using Hsp90 inhibitors to block the folding of these oncogenic proteins in an effort to drive cancer cells into apoptosis (Whitesell and Lindquist, 2005).

Hsp90 involvement in viral replication has been reported for several different viruses; however, these reports have suggested different roles of Hsp90 in a variety of virus and host systems. During vaccinia virus infection, Hsp90 inhibition blocks viral replication (Hung et al., 2002). The mechanism for this inhibition is not well established, but Hsp90's localization is restricted to the cytoplasm following VV infection, where it interacts with the viral core protein 4a, presumably as a client protein (Hung et al., 2002). Hsp90 activity has also been reported to be important for proper cleavage of newly synthesized hepatitis C NSP2/3 protein (Waxman et al., 2001), and is required for the activity of the hepatitis B reverse transcriptase (Hu and Seeger, 1996; Hu et al., 1997).

Recently, there have been several reports implicating a role for Hsp90 in the control of viral polymerase function. In a minireplicon system, Hsp90 binds to the PB2 subunit of the influenza virus RNA polymerase, and stimulates viral polymerase activity (Momose et al., 2002). In Herpes virus infection, blocking Hsp90 activity significantly inhibited viral replication (Burch and Weller, 2005; Li et al., 2004), possibly due to the improper localization of the viral polymerase in drug-treated cells (cytoplasmic rather than nuclear). Similarly, it has been shown that Hsp90 activity is important for the proper stability and localization of the RNA polymerase (protein A) of the insect virus flock house virus (Kampmueller and Miller, 2005).

There is still a great deal to be discovered about Hsp90's role in virus replication, such as whether Hsp90 is likely to play a role in the life cycle of specific viruses or whether it is involved in the life cycle of entire classes of viruses. In an attempt to address this question, we have studied the effects of Hsp90 inhibitors on the replication of several different prototype negative strand RNA viruses from the rhabdovirus, paramyxovirus, and bunyavirus families. We found that replication of viruses from each of these families was impaired by the ansamycin Hsp90 inhibitor geldanamycin, a second Hsp90 inhibitor radicicol, and by siRNA knockdown of Hsp90 protein levels. This inhibition of replication was most apparent at early stages of the virus life cycle and resulted in the selective destabilization of the L protein of the virus polymerase. Based on this and other data in the literature, we propose that viruses from multiple families and classes have all evolved to require the use of Hsp90 for the proper folding of their polymerases. This suggests that inhibitors of Hsp90 activity could act as broad-range antiviral agents.

Results

To determine whether Hsp90 inhibition has an effect on the replication of the prototype negative strand virus vesicular

stomatitis virus (VSV), we determined viral growth in cells treated with increasing concentrations of geldanamycin, an ATP-competitor that acts as a specific inhibitor of Hsp90 activity (Roe et al., 1999). We utilized chemically synthesized geldanamycin (provided by the DTP service of NCI) in these experiments because a recent report has shown that geldanamycin purified from *S. hygroscopicus* contains contaminating activities that inhibit intracellular trafficking but that the NCI synthesized compound does not (Barzilay et al., 2004). BHK cells infected with VSV at an MOI of 0.01 were treated with increasing concentrations of drug, and virus yield at 12 hpi was determined by plaque assay. As shown in Fig. 1A, geldanamycin was a potent inhibitor of VSV growth, reducing virus replication by 3 orders of magnitude at 100 nM and 5 orders of magnitude at 5 μ M.

To further understand the effect of geldanamycin on virus replication, we carried out single and multiple cycle growth experiments in the absence or presence of geldanamycin at 5 μ M, a concentration previously shown to have minimal cellular toxicity (Kampmueller and Miller, 2005). As shown in Fig. 1B, geldanamycin had a significant effect on slowing virus growth in a single cycle growth experiment (MOI=10). Cells that were infected with VSV and treated with geldanamycin showed a noticeable delay in virus growth, producing lower virus titers between 4 and 16 hpi when compared to cells infected with VSV but not treated with geldanamycin. By 24 hpi, virus titers were comparable between drug-treated and untreated conditions. Inhibition of VSV replication by geldanamycin was seen in HeLa cells as well as in BHK cells (data not shown), indicating that this effect was not cell-type specific.

Inhibition of viral replication by geldanamycin was more dramatic in multi-cycle growth experiments. Geldanamycin addition delayed the appearance of progeny viruses for 8 h when compared to infected cells that were not treated with geldanamycin, and inhibited growth by more than two orders of magnitude at 24 hpi (Fig. 1C). We also determined the effect of radicicol, an Hsp90 inhibitor that is structurally distinct from geldanamycin, on low MOI VSV infection. Radicicol also inhibited VSV replication, decreasing virus titers by more than 2 orders of magnitude at 4 hpi and 4 orders of magnitude at 8 hpi. This supports the hypothesis that the mechanism of replication inhibition is through the inhibition of Hsp90 function (Fig. 1D).

To test this conclusion utilizing a different method, we determined the effect of decreasing the levels of Hsp90 through RNA silencing. Fig. 1E shows a representative western blot of HeLa cells that were either mock transfected, transfected with siRNA targeting GAPDH, or transfected with siRNA targeting Hsp90. Forty-eight hours posttransfection, cells transfected with GAPDH siRNA showed a >90% inhibition of GAPDH expression (lane 2). Cells transfected with siRNA targeting the alpha and beta forms of Hsp90 showed an 80–90% drop in Hsp90 levels at the same time posttransfection (lanes 3–6). Cell cultures transfected with either GAPDH or Hsp90 siRNA had fewer cells than controls at 72 h posttransfection suggesting that cell proliferation was

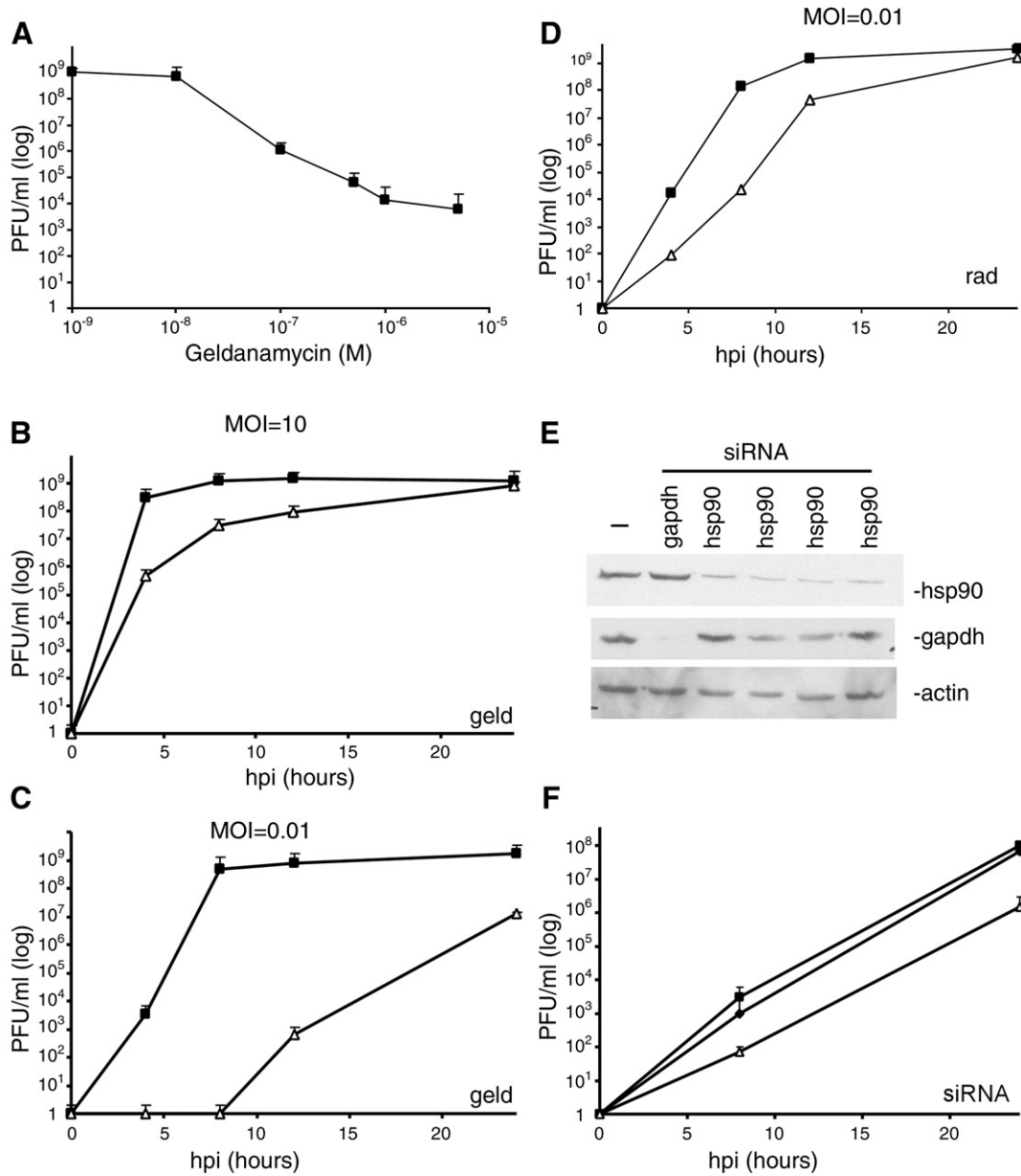


Fig. 1. Effect of Hsp90 inhibition of VSV replication. (A) Viral titers at 12 hpi from cells infected with VSV at an MOI=0.01 and treated with increasing concentrations of geldanamycin. (B) Growth curve of VSV in the presence (Δ) or absence (■) of geldanamycin (5 μM) at high MOI (= 10) and (C) growth curve at low MOI (=0.1). (D) Growth curve of VSV in the presence (Δ) or absence (■) of radicicol (5 μM). Virus titers for all panels represent the average of three different experiments. (E) Western blot analysis of 25 μg of lysate from cells that were mock-transfected, transfected with GAPDH targeting siRNA, or transfected with Hsp90 targeting siRNA. Top image shows Hsp90 levels, middle panel shows GAPDH levels, bottom panel shows actin as a loading control. (F) Growth curve of mock-transfected and siRNA-treated cells infected with VSV at an MOI of 0.01. Error bars reflect standard deviation for three experiments.

slowed (Supplemental Fig. 1). However, there were no significant differences in cell viability or gross morphological changes in cells transfected with Hsp90 siRNA. To determine the effect of the lower levels of Hsp90 protein levels on VSV infection, cells were infected with VSV at a low MOI (0.01). Analysis of viral growth by plaque assay (Fig. 1F) showed that cells where Hsp90 was silenced showed a decrease in virus titer compared to a mock-transfected control at both 8 and 24 hpi, but that there was a negligible effect of GAPDH silencing on virus titer. These results support the conclusion

that Hsp90 is an important host factor in the replication of VSV.

Inhibition of virus replication is at early stages of viral replication

The time-courses shown in Fig. 1 indicated that geldanamycin inhibited VSV at early times in the VSV life cycle, and that the inhibitory action diminished at later times postinfection. In a single-cycle growth experiment, drug added coincident

with infection had a strong effect on viral replication, but drug added 4 h postinfection had almost no effect (data not shown). This suggested that the antiviral activity of geldanamycin was due to an effect on an early, rate-limiting step of viral replication. To determine how the addition of geldanamycin affected viral gene expression, cells were infected with VSV and drug was added at 0, 2, or 4 hpi (see Fig. 2A for time line). At 5 hpi, cells were labeled with ^{35}S methionine for 1 h and lysates were analyzed by SDS-PAGE and phosphorimaging. In cells that were not treated with drug, high level VSV gene expression was evident through the appearance of four major bands representing the L, G, N/P (comigrating), and M proteins (Fig. 2B, lane 1). Also apparent was the inhibition of host protein synthesis normally seen following a productive VSV infection. Addition of geldanamycin coincident with virus infection blocked viral gene expression by VSV, but host protein synthesis was still detectable (lane 2). Cells treated with drug 2 hpi showed some decrease in viral gene expression, and cells treated with drug at 4 hpi showed strong viral gene expression, demonstrating that geldanamycin inhibition of viral gene expression required addition soon after initiating the infection. As shown in Fig. 2B, the addition of drug at 4 hpi did result in one change from non-treated cells: the viral L protein, the catalytically active component of the RDRP accumulated to much lower levels in these drug-treated cells.

Inhibition of stability of newly synthesized polymerase

To investigate whether the lower levels of L protein were due to an increased incorporation of L into budded virions, we determined the extent to which newly synthesized L was incorporated into budding virions. At 4 hpi, infected cells were treated with 50 nM, 500 nM, or 5 μM geldanamycin, proteins were labeled with ^{35}S methionine for 1 h and the fate of the newly synthesized proteins was determined through analysis of virions budded into the supernatant and labeled protein in the cell after a 1 h chase with unlabeled methionine. While most proteins produced by VSV were unaffected by addition of geldanamycin, the level of L protein in cell lysates decreased with increasing geldanamycin

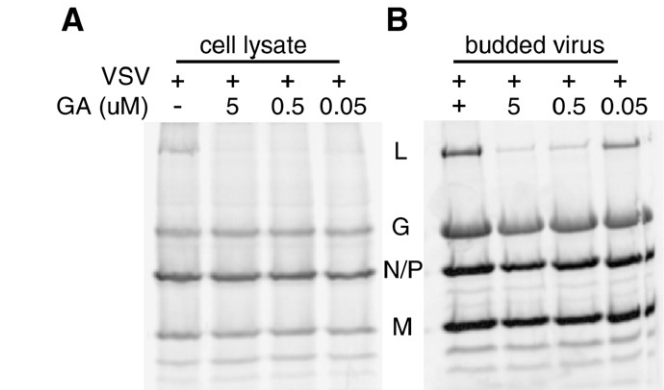


Fig. 3. Assembly of VSV in geldanamycin-treated cells. Cells were infected with VSV for 5 h, and then proteins were radiolabeled and followed using a 1 h pulse and 1 h chase protocol. Upon addition of radioactive methionine, different concentrations of geldanamycin were added to individual samples. Following a 1 h chase in the presence of drug, (A) intracellular radiolabeling of protein was determined by SDS-PAGE electrophoresis phosphorescence imaging. (B) The amount of newly synthesized protein that was incorporated into the virus was determined by virion purification from the media followed by SDS-PAGE and phosphorescence analysis.

concentration (Fig. 3A). Drug addition at concentrations as low as 50 nM decreased the amount of L in lysates, consistent with the disappearance of the L protein being linked to the effect of the drug on viral replication (Fig. 1A). In budded virions purified from the supernatant of these same cells, the concentration of geldanamycin did not have an effect on the amounts of radiolabeled G, N, P, and M protein that were incorporated (Fig. 3B), demonstrating that blocking Hsp90 does not affect any of the assembly function of the virus. In the case of the L protein, less radiolabeled L protein was incorporated into virions as geldanamycin concentration increased. This result shows that geldanamycin treatment did not result in the preferential movement of newly synthesized L protein into virions. Some newly synthesized L was found in virions from cells treated with lower concentrations of drug, indicating that some of the newly synthesized L protein remains in the cell long enough to be incorporated into virions.

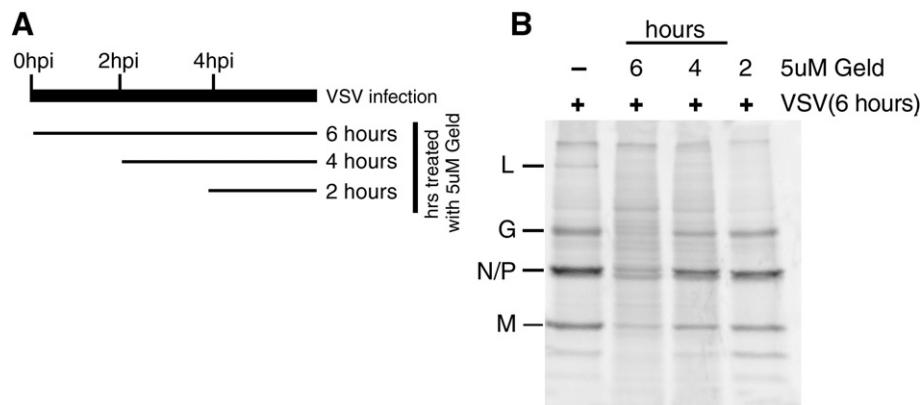


Fig. 2. Effect of geldanamycin on viral protein synthesis. (A) Schematic of experimental procedure showing times of geldanamycin treatment after viral infection (top) and total times of geldanamycin treatment (right). (B) Phosphorescence image of cell lysates from cells that were infected with VSV and treated with geldanamycin as described, followed by labeling with ^{35}S methionine for 1 h.

To determine how the synthesis and stability of L were changed following Hsp90 inhibition, we performed pulse-chase analysis in the presence and absence of geldanamycin. Radiolabeled cells were chased for 10, 20, or 30 min, and then lysates from these cells were analyzed by phosphorimaging (Fig. 4A). The resulting image showed that in both geldanamycin-treated and untreated cells infected with virus significant amounts of ³⁵S methionine were incorporated into the viral L protein. The newly synthesized L protein was stable in untreated cells but disappeared rapidly in cells treated with the drug and was almost completely absent after a 30 min chase. Quantitation of these data (Fig. 4B) over multiple experiments showed that the half-life of the L protein was greater than 60 min in the absence of drug but was reduced to less than 20 min in the presence of drug. This was not seen for the other viral proteins as analysis of the decay of viral M protein (Fig. 4C) and the other viral proteins (Fig. 4A) showed little evidence of degradation in the presence and absence of drug treatment.

L protein is degraded by the proteasome

To investigate a possible mechanism for the observed destabilization of L protein following HSP90 inhibition, we determined whether the L protein was being degraded via the proteasome. This possibility is particularly plausible because other Hsp90 substrates, such as the estrogen receptor, have been shown to be shunted to the proteasome pathway following Hsp90 inhibition (Bagatell et al., 2001; Fan et al., 2005). To test whether the proteasome is involved in the degradation of newly synthesized L protein, we repeated the experiments shown in Fig. 4, but added proteasome inhibitor (proteasome inhibitor II, Calbiochem) to infected cells treated with geldanamycin. At 10 min of chase, there were similar levels of incorporation of ³⁵S methionine into L protein in untreated, geldanamycin-treated, and geldanamycin+proteasome inhibitor-treated conditions (Fig. 5A). At 30 min chase, there was a significant decrease in the amount of L protein in the geldanamycin-treated cells similar to that seen in previous experiments. There was a

much smaller decrease in the cells treated with both geldanamycin and proteasome inhibitor showing that proteasome inhibition decreased the degradation of the L protein. Addition of proteasome inhibitor alone did not significantly alter the stability of the L protein (Fig. 5B). The quantitation of multiple experiments (Fig. 5C) showed that the addition of the proteasome inhibitor increased L protein half-life in the presence of geldanamycin from less than 20 min to close to that of L in control cells that did not receive geldanamycin (dotted line, Fig. 5B). This argues that the primary cause of the disappearance of the newly synthesized L polymerase following Hsp90 inhibition is the targeting of the newly synthesized polymerase to the proteasome for degradation.

Hsp90 inhibitors regulate the replication and stability of multiple negative strand viruses

Based on our findings that Hsp90 inhibitors decreased the replication and destabilized the L polymerase protein of the prototype negative strand virus VSV, we were interested in whether this behavior was unique to VSV or whether it was a more general phenomenon. To test the hypothesis that Hsp90 activity is required for replication and polymerase stability across negative strand virus families, we initially tested the paramyxoviruses simian virus 5 (SV5) and HPIV-2. To determine the effect of Hsp90 inhibition on viral replication, geldanamycin was added to cells that were infected at a low multiplicity with a GFP-expressing SV5 (rSV5-GFP) or HPIV-2. As shown in Fig. 6A, geldanamycin inhibited the growth of these viruses, reducing virus yield by more than 3 logs for SV5 and more than 2 logs for HPIV2.

Both geldanamycin and radicicol blocked paramyxovirus spread in a low MOI infection. Cells infected at an MOI of 0.05 with were all infected with rSV5-GFP and expressing GFP by 72 hpi (Fig. 6B left panels). Consistent with the effect of HSP90 inhibitors on virus production, cells infected with rSV5-GFP but treated with 0.5 μM geldanamycin showed dramatically lowered viral spread, with only a few cells showing GFP

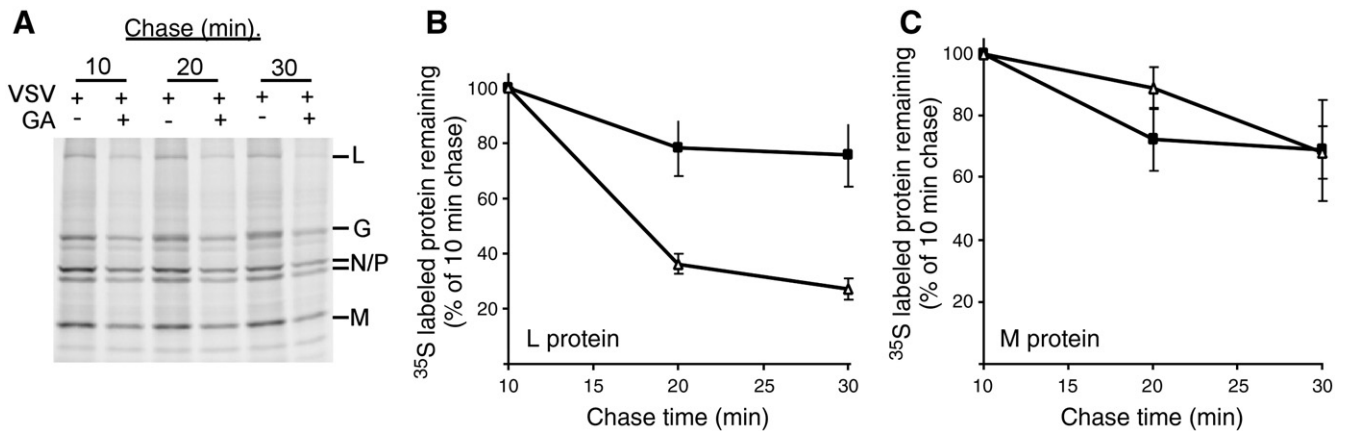


Fig. 4. Effect of geldanamycin on viral protein stability. (A) BHK cells infected with VSV for 5 h were starved for methionine, and then protein synthesis and stability were determined by pulse-chase analysis (see Materials and methods). Results were determined by SDS-PAGE and phosphorescence imaging. Viral proteins are identified to the right of the gel. Levels of protein remaining are quantities for viral L (B) and M (C) proteins presence (Δ) or absence (■) of 5 μM geldanamycin. Values represent the average of three separate experiments ± standard deviation.

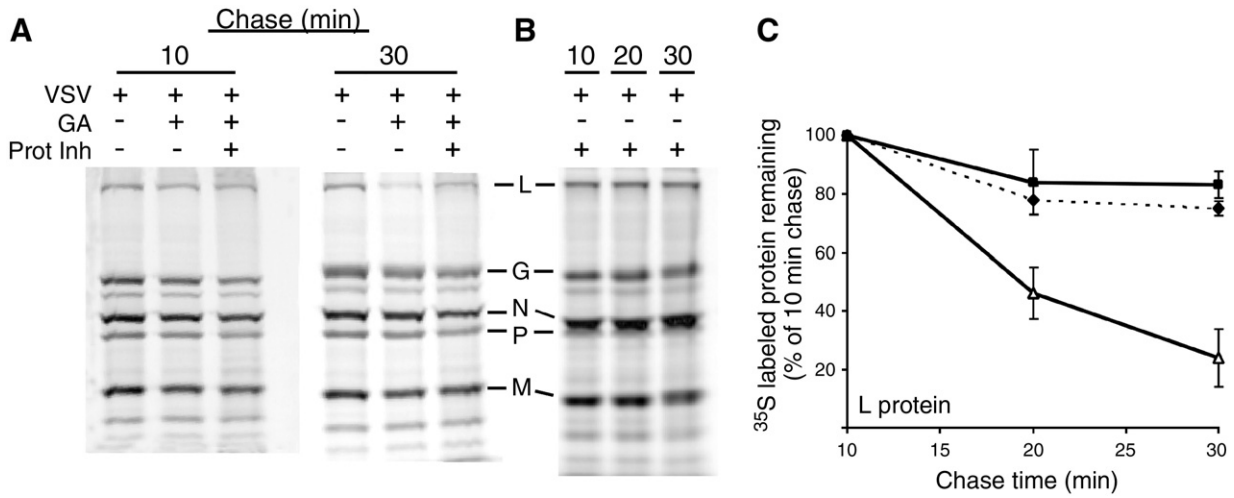


Fig. 5. Geldanamycin destabilization of VSV L protein requires proteasome function. (A) BHK cells were infected with VSV, and 5 hpi left untreated, treated with geldanamycin, or treated with both geldanamycin and proteasome inhibitor. Pulse-chase results for 10 min and 30 min chases are shown. (B) Stability of L protein in the proteins presence (Δ) or absence (\blacksquare) of 5 μ M geldanamycin and in the presence of geldanamycin and a proteasome inhibitor (\blacklozenge , dotted line). Levels are expressed as a percentage of the signal that was present at the 10 min chase. Values are the average of three separate experiments \pm standard deviation.

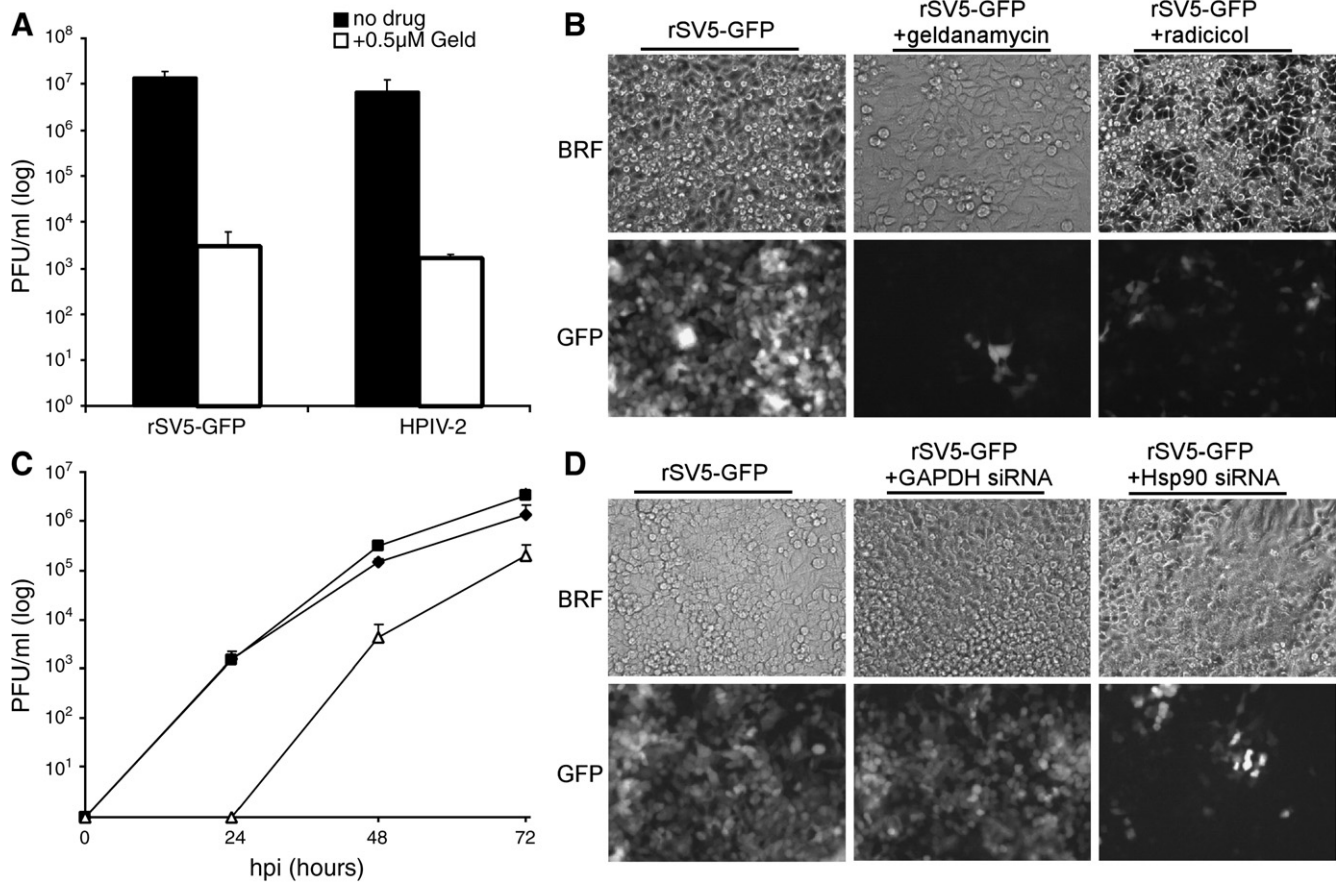


Fig. 6. Effect of geldanamycin on paramyxovirus growth and L stability. (A and B) Cells were infected at an MOI of 0.05 with rSV5-GFP or HPIV-2 and incubated with or without 0.5 μ M geldanamycin. At 72 hpi, virus titers were determined by plaque assay (A) or cells were examined for GFP expression (B). Results are representative of three independent experiments. (C and D) Cells that were mock-transfected or transfected with either GAPDH targeting siRNA or Hsp90 targeting siRNA were infected with SV5-GFP at an MOI of 0.05. (C) Shows SV5-GFP growth curve (done in triplicate) untransfected (\blacksquare), GAPDH siRNA transfected (\blacklozenge), and Hsp90 siRNA transfected (\blacksquare); (D) shows representative images showing cell spread of the virus in different transfection conditions.

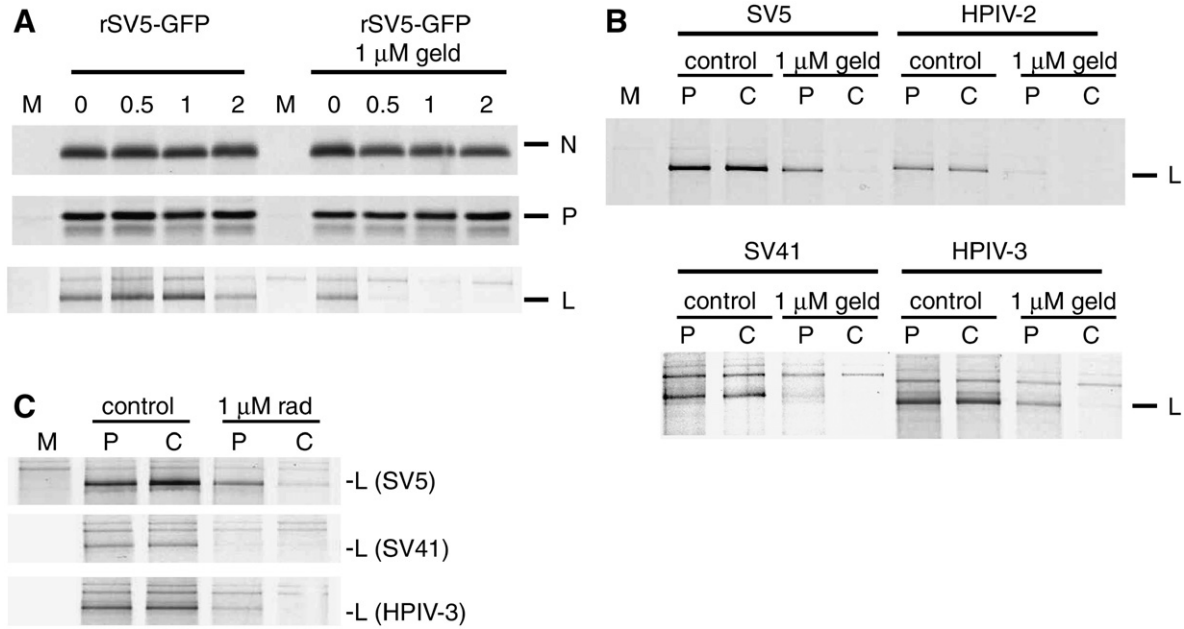


Fig. 7. Hsp90 inhibition destabilizes newly synthesized L proteins from multiple paramyxoviruses. (A) Cells were mock infected (M lane) or infected at an MOI of 10 with rSV5-GFP. At 13 hpi, cells were treated for 1 h with or without 1 μM Geldanamycin. Cells were radiolabeled for 20 min with ³⁵S-amino acids and incubated in nonradioactive media for the indicated times before lysis and analysis by immunoprecipitation with antibodies specific for NP, P, or L. Geldanamycin was included at 1 μM during radiolabeling and chase periods. (B) Stability of L proteins. Cells were mock infected or infected at an MOI of 10 with SV5, HPIV-2, SV41, or HPIV-3. 13 hpi, cells were treated with 1 μM geldanamycin, radiolabeled for 20 min and lysed (Pulse) or chased using unlabeled methionine for 1 h (Chase). Following cell lysis, samples were immunoprecipitated using L-specific antibodies. (C) Experiments similar to B were carried out, but 1 μM radicicol was used instead of geldanamycin.

expression (middle panels). A similar limitation of spread was seen in cells treated with radicicol, with few cells showing the intense GFP signal seen in mock-infected cells (right panels).

Using siRNA to decrease the level of Hsp90 protein in cells also had a significant effect on paramyxovirus replication. Shown in Fig. 6C are SV5-GFP virus titers at 24, 48, and 72 hpi from HeLa cells that were either mock transfected, transfected with siRNA targeting GAPDH, or transfected with

siRNA targeting Hsp90. Silencing GAPDH minimally decreased the replication of SV5-GFP, but silencing Hsp90 reduced SV5-GFP replication significantly. Progeny viruses were not detected at 24 hpi in Hsp90-silenced cells infected with SV5-GFP, a 3 log order difference compared to control and GAPDH-silenced cells. Virus replication was observed at both 48 and 72 hpi, but at lower levels than was seen in controls. Silencing of Hsp90 expression but not silencing of

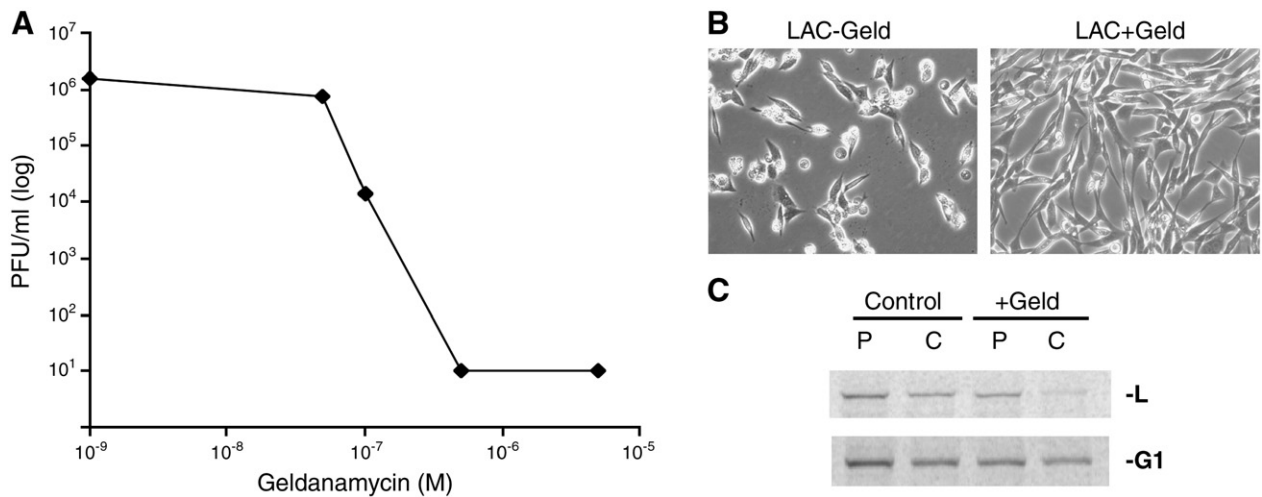


Fig. 8. Geldanamycin inhibition of La Crosse virus replication. (A) La Crosse virus titers at 24 hpi from cells infected at an MOI=0.01 and increasing concentrations of geldanamycin at time of infection. (B) Light microscope images of cells infected with La Crosse virus in the absence (left) and presence of 0.5 μM geldanamycin. (C) Protein synthesis and stability of La Crosse L and G1 proteins. 16 hpi with Lacrosse virus at an MOI of 1, cells were labeled with ³⁵S-methionine for 20 min and then lysed (P) or incubated in non-radioactive media for 1 h. Cells were then lysed, and incorporation of radioactivity into viral proteins was determined by SDS-PAGE followed by phosphorescence imaging.

GAPDH expression inhibited the spread of SV5 GFP (Fig. 6D) in these cells.

This inhibition of paramyxovirus growth was accompanied by changes in the stability of the viral polymerase. Fig. 7A shows the results of pulse-chase (0.5, 1 and 2 h chase, followed by immunoprecipitation of indicated protein) experiments that were used to follow the stability of individual SV5 viral proteins. In cells infected with rSV5-GFP but not treated with geldanamycin, L, N and P proteins showed very stable profiles, with the polymerase protein L showing a half-life in excess of 1 h. In geldanamycin-treated cells infected with SV5-GFP, both the N and P proteins remained stable, but the half-life of the L protein dropped to less than half an hour following treatment with geldanamycin, similar to the polymerase destabilization observed in the case of the VSV polymerase.

In addition to showing antiviral activity against SV5 and HPIV-2, both geldanamycin and radicicol showed antiviral activity against SV41 and HPIV3 (data not shown). Analysis of the viral polymerase of each of these viruses (Fig. 7B) directly following pulse labeling (P) for 20 min or after a 1 h chase (C) showed that (1) all of these polymerase proteins were stable in the absence of geldanamycin, and (2) all displayed a marked decrease in the half-life of the L polymerase protein following geldanamycin addition. (3) The amount of labeled L protein following the 20 min pulse was reduced in the presence of geldanamycin, suggesting that turnover was occurring during the pulse period. The stability of SV41 L protein appeared to be most significantly decreased in the presence of geldanamycin, suggesting a very rapid turnover of the L protein in this virus following HSP90 treatment. These effects of destabilization of these viral L proteins were attributable to Hsp90 inhibition, as treatment of cells infected with SV5, SV41 or HPIV-3 with radicicol also caused the rapid degradation of newly synthesized viral polymerase (Fig. 7C).

HSP90 inhibitors block the replication of La Crosse virus

We also tested the impact of Hsp90 inhibition on the replication of Bunyaviruses, a family of negative-strand viruses containing segmented RNA genomes. In the case of the pediatric encephalitis virus, La Crosse virus (H78 strain (Chandler et al., 1998), a kind gift from B.J. Beaty), virus growth was also inhibited by geldanamycin (Fig. 8A) and radicicol (data not shown) at concentrations as low as 100 nM. In cells not treated with drug, La Crosse virus infection resulted in the development of noticeable cytopathic effects (Fig. 8B, left panel). Infection with La Crosse in the presence of geldanamycin prevented the development of cytopathic effect (Fig. 8B, right panel), consistent with the observed inhibition of virus replication. Similar to VSV and paramyxovirus family members, HSP90 inhibition appeared to destabilize the viral polymerase. Pulse-chase analysis of La Crosse virus proteins in infected cells (30 min pulse followed by a 1 h chase) showed that La Crosse L protein is normally stable, but there was a distinct decrease in L protein stability following the addition of geldanamycin (Fig. 8C, top panel), while other La Crosse virus proteins such as the glycoprotein G1 remained stable (bottom panel).

Discussion

The experiments described here demonstrate through both gene-knockdown and cell-permeable inhibitor approaches that Hsp90 activity is important for the rapid growth of negative strand RNA viruses. Mechanistically, geldanamycin and other Hsp90 inhibitors appear to block virus replication by dramatically decreasing the stability of the L subunit of the viral RNA dependent RNA polymerase. This mode of action would explain the effectiveness of Hsp90 inhibition at early stages of viral infection where the level of RNA synthesis and genome replication would be most affected by decreased levels of the viral polymerase. Adding geldanamycin at progressively later times postinfection has progressively less effect on viral gene expression, indicating that accumulation of L protein prior to drug addition can compensate for the rapid turnover of newly synthesized L protein. Our studies show that viruses from the rhabdovirus, paramyxovirus, and bunyavirus families all require Hsp90 activity to achieve normal levels of a stable viral polymerase. Other researchers have shown that the positive-strand RNA virus FHV polymerase (Kampmueller and Miller, 2005) and the influenza virus polymerase (Momose et al., 2002) require Hsp90 activity for proper function. Together, our data and that from other laboratories (Kampmueller and Miller, 2005; Momose et al., 2002) suggest that Hsp90 is a host factor that is of central importance to viral replication for many, if not all, RNA viruses. The RNA polymerases from these different viruses do not share a high degree of sequence homology, so their dependence on Hsp90 points to convergent evolution. To our knowledge, we are the first to propose that Hsp90 activity is a conserved requirement for achieving a stable virus polymerase for a wide spectrum of RNA viruses.

For the VSV L protein, its large size (241 kDa) and the existence of several different catalytic activities within the protein (Feller et al., 2000; Grdzlishvili et al., 2006; Li et al., 2005, 2006) imply a number of independently folded domains. This is also likely true of the other viral polymerases affected by Hsp90 inhibition, as the viruses used in this study all have relatively large polymerase proteins (~240 kDa) that are likely to have independent functional domains. How Hsp90 is involved in folding these polymerases has yet to be determined, but because Hsp90's protein folding activity requires the binding and action of additional co-chaperones, the folding of viral polymerases is likely to be a coordinated effort of several cellular proteins, of which Hsp90 is the first to be identified.

Despite the attractiveness of hypothesizing that Hsp90 is directly involved in folding viral polymerases, other possibilities exist for how Hsp90 inhibition might destabilize RNA virus polymerases. For viruses of the rhabdovirus and paramyxovirus families, the L polymerase requires the binding of the viral phospho (P) protein for activity (Horikami et al., 1992). Hsp90 may facilitate the L–P interaction to promote the stability of L. Hsp90 could also act to promote the stable formation of L protein multimers (Smallwood et al., 2002). Thus, Hsp90 inhibition could lead to a disruption of L–L interactions resulting in an unstable polymerase that is quickly degraded. Additionally, Hsp90 may be more remotely involved

and impact L stability by folding or activating a cellular component that is required for polymerase stability. Further experiments will be required to determine the interaction between Hsp90 and RNA virus polymerases, as well as other cellular factors.

The importance of Hsp90 for the replication of multiple viruses opens an interesting possibility for developing new antiviral therapies. There are few compounds that display antiviral activity against major encephalitis-causing and hemorrhagic fever-causing negative strand RNA viruses (De Clercq, 2005; Domachowske and Rosenberg, 2005). Our findings suggest that the inhibition of Hsp90 activity should be investigated as a mechanism of limiting the replication and spread of these viruses *in vivo*. An advantage of this approach is that the lack of Hsp90 activity appears to hamper viral RDRP function. The RDRP represents a rate limiting enzyme step in virus replication that performs many unique and essential replication functions and is synthesized at relatively low levels in infected cells. Additionally, because of the effectiveness of this compound against a wide variety of viruses, Hsp90 inhibitors may be broad spectrum antivirals capable of inhibiting the replication of several different infectious agents.

Already, significant effort has been devoted to developing Hsp90 inhibitors. One of these compounds, 17-AAG (a geldanamycin derivative) has completed phase 1 and is currently in phase 2 clinical trials as an anticancer therapy (Goetz et al., 2005; Whitesell and Lindquist, 2005). Results from phase 1 trials showed that tolerated doses of the drug led to decreases in the levels of Hsp90 client proteins (Banerji et al., 2005; Grem et al., 2005). Thus, this compound has already passed many of the hurdles that confront successful drug development, and it or other Hsp90 inhibitors in development (Whitesell and Lindquist, 2005) are likely to be clinically viable. Regardless of their utility in cancer treatment, we propose that further experimentation should be undertaken to determine whether these compounds have a useful role as antiviral agents. Hsp90 inhibitors may also work synergistically with other antivirals such as ribavirin, a drug that has also been suggested to target the function of the viral polymerase (Graci and Cameron, 2002).

An important corollary to these findings is that these studies highlight the utility of prototype negative strand viruses as tools that can be used to screen for new antiviral compounds. Utilization of viruses that have shown minimal human pathogenicity and have been well characterized offers significant advantages over newly discovered or highly pathogenic viruses for lead compound identification and can serve as an important stepping stone to the discovery of new antiviral compounds that have a wide spectrum of antiviral activities.

Materials and methods

Reagents

Geldanamycin was synthesized at the drug therapeutics program of the NIH. Radicol and proteasome inhibitor II were obtained from Calbiochem. ³⁵S Methionine was

obtained from Amersham. siRNA targeting Hsp90 and polyclonal anti-Hsp90 antibody were obtained from Santa Cruz. siRNA transfection reagent was obtained from Mirus biotechnology. siRNA targeting GAPDH was obtained from Dharmacon, antibody recognizing GAPDH was obtained from chemicon. All other reagents were obtained from Fischer Co. La Crosse virus (H78) was a kind gift of Barry Beaty (Colorado State University).

Virus infection

Cells were plated to reach a density of between 70 and 80% in DMEM+10%FBS. Cells were infected with VSV at an MOI=10 or an MOI=0.01 as described previously (Connor and Lyles, 2002). For dose–response experiments with VSV and La Crosse virus, BHK cells were infected at an MOI of 0.01 and virus budded into culture media was determined by plaque assay using BHK cells. Drugs were added coincident with infection unless otherwise indicated.

siRNA knockdown experiments

For siRNA targeting the human alpha and beta forms of Hsp90, HeLa cells were incubated with 50 nM siRNA using Mirus siPORT transfection reagent for 48 h prior to infection with VSV. For GAPDH siRNA, 75 nM siRNA was utilized using the same transfection reagent. For both Hsp90 and GAPDH siRNA transfections, there was no obvious cytotoxicity at 48 h posttransfection.

Pulse-chase experiments

For VSV, cells were infected with VSV for 5 h. Cells were labeled with ³⁵S methionine-containing media for 1 h and then the radioactive media was replaced with media containing unlabeled methionine for an hour or for the times indicated in text describing the figure. For La Crosse virus, a similar procedure was followed, but cells were labeled 16 hpi for 30 min and chased for 60 min. Cells were lysed in RIPA buffer and proteins were separated by SDS-PAGE. For virion purification, media from infected cells were layered on top of a 15% sucrose solution and spun for some 40,000×g for 45 min. Pelleted virions were resuspended and proteins were separated by SDS-PAGE.

Paramyxovirus experiments

Infections of HeLa cells at high MOI (10) or low MOI (0.05) with SV5 expressing GFP (rSV5-GFP), HPIV-2, HPIV-3, or SV41 and virus growth analyses were carried out as described previously (Parks et al., 2001). Geldanamycin or radicol was added after attachment of virus to cells, and was maintained throughout growth experiments. GFP expressing cells were analyzed with the Nikon Eclipse microscope and a 20× objective. Images were captured using a QImaging digital camera and processed using QCapture software. Exposure times were manually set to be constant between samples. For radiolabeling

experiments, cells were treated with Geldanamycin for 1 h prior to radiolabeling with Tran³⁵S-label (100 uCi/ml), lysed in SDS, and used for immunoprecipitation analysis with polyclonal antibodies specific for the SV5 NP, P, or L proteins as described previously (Parks et al., 2001).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2006.12.026.

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