Host cell factor requirement for hepatitis C virus enzyme maturation

Lloyd Waxman*, Michael Whitney†, Brian A. Pollok†, Lawrence C. Kuo*, and Paul L. Darke*‡

*Department of Structural Biology, Merck Research Laboratories, West Point, PA 19486; and †Aurora Biosciences, 11010 Torreyana Road, San Diego, CA 92121

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The cellular chaperone, HSP90, is identified here as an essential factor for the activity of NS2/3 protease of hepatitis C virus. The cleavage activity of NS2/3 protease synthesized in reticulocyte lysate is ATP-dependent, as evidenced by ATP depletion experiments and inhibition with nonhydrolyzable ATP analogs. Geldanamycin and radicicol, ATP-competitive inhibitors of the chaperone HSP90, also inhibit the cleavage of *in vitro*-synthesized NS2/3. Furthermore, these HSP90 inhibitors prevent NS2/3 cleavage when the protease is expressed in mammalian cells. The physical association of NS2/3 with HSP90 is demonstrated by immunoprecipitation. Thus, by way of a chaperone/folding activity, an HSP90-containing complex is required for maturation of the polyprotein that encodes the enzymes essential for hepatitis C virus replication.

An estimated 170 million people are infected with hepatitis C virus (HCV) worldwide. The infection is usually persistent, and after an asymptomatic period often lasting years, many patients develop chronic liver disease, including cirrhosis and hepatocellular carcinoma (1, 2). The preferred existing treatment with IFN-α is successful in only a small fraction of cases, so an urgent need exists for the development of therapeutically effective inhibitors of HCV replication. With the precedent of numerous successful antiviral therapies based on viral enzyme inhibition, the enzymes encoded by HCV present particularly attractive targets. The recombinant HCV enzymes, NS5B RNA polymerase, NS3 protease, and NS3 helicase have been purified, their enzymatic properties are being defined *in vitro*, and their potential for inhibition is being explored by many laboratories (3–7).

In contrast, the HCV protease activity responsible for cleavage between NS2 and the NS3 proteins, known as NS2/3 protease, has been reported only in cell lysate translation systems and transfection experiments, despite having been described initially several years ago (8–10). Thus, NS2/3 cleavage as a target for inhibition has been particularly refractory to detailed analysis, so that the definition of the critical components of this activity is needed. Based on mutational analyses, the protein region essential for NS2/3 cleavage activity has been approximately mapped to amino acids 898-1207 of the HCV ORF. This region of the HCV polyprotein includes the N-terminal portion of NS3 that is known to be sufficient for NS3 protease activity, residues 1027–1207. Because of the overlap of sequence essential for the two distinct protease activities, it is worthwhile to emphasize that the NS3 protease activity can be totally eliminated through mutagenesis of the NS3 active site Ser-1165 without affecting NS2/3 cleavage activity (10).

Additional features of NS2/3 cleavage activity known at this time include identification of Cys-993 and His-952 as essential residues (8–13). The only known cleavage site sequence, Arg-Leu-Leu $\downarrow$ Ala-Pro-Ile, is conserved across HCV strains, and the catalytic mechanism of NS2/3 cleavage is unknown, but the enzyme is speculated to be either a metalloprotease (10) or cysteine protease (14). NS2, the N-terminal product of NS2/3 cleavage, has no known enzymatic function and has been characterized to be a transmembrane polypeptide (11). The C-terminal cleavage product of NS2/3 protease action is the NS3 protein, containing both protease and RNA helicase activities, catalytic functions that are thought to be independent of the NS2/3 cleavage activity. The conservation of the HCV NS2/3 cleavage site sequence in HCV samples from patients, and the conservation of the NS2/3 residues critical for cleavage activity in HCV suggest an essential function of NS2/3 in clinically significant HCV replication (8–13, 15).

The NS region of HCV RNA has been replicated in a cell-based system by using antibiotic selected transfections with bicistronic RNA (16). This system does not include NS2 or NS2/3 cleavage and does not support viral particle production. Because the HCV NS2 has no well-conserved counterpart in other viral families (2), understanding the cleavage reaction and its role in replication through use of analogous viruses capable of undergoing complete replication in cell culture is yet to be accomplished. Given these limitations, and the need to identify the factors essential for NS2/3 processing, we initiated a study using NS2/3 protein translated in rabbit reticulocyte lysate (RRL), a system previously used to define the protein regions essential for cleavage (8). The cleavage reaction study was extended through the use of a cell-based NS2/3 assay system. As a result, the chaperone HSP90 is identified here as a participant in NS2/3 cleavage in these systems and a potential point of regulation for the generation of mature HCV NS proteins.

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Materials and Methods

***In Vitro* Translations.** HCV residues 810-1615 of the BK strain, which includes all of NS2 and most of NS3 (termed 810-1615BK) was produced from the plasmid pCITE 810-1615BK (a gift from Nicola La Monica, Merck Research Laboratories, Rome) and has been described (13). After plasmid linearization with BLP1, RNA was transcribed with T7 RNA polymerase and purified. Protein translation was in RRL (Promega) at 30°C for 40 min using ^{35}S-methionine as a label. Translation was then blocked by the addition of cycloheximide (250 μM final), and the samples were immediately frozen on dry ice. Before processing experiments, translated NS2/3 (810–1615BK) was thawed on ice, and aliquots were equilibrated at room temperature. A 10% (wt/vol) stock solution of Triton X-100 was used to initiate autoprocessing of the NS2/3 (810–1615BK) and was added at a final concentration of 1% (13). After a typical 30-min incubation, SDS gel sample buffer was added, the samples were heated to 95°C for 5 min, and the proteins were separated on SDS/14% polyacrylamide gels. The distribution of ^{35}S-labeled proteins on dried gels was determined with a PhosphorImager (Molecular Dynamics), as has been described (17). Product bands were quantified and expressed as a proportion of total signal in the gel lane so that variations in gel lane loading were normalized. The band corresponding to the product NS2 from 810-1615 BK was used to generate data shown for inhibitor IC_{50} calculations,

Abbreviations: RRL, rabbit reticulocyte lysate; HCV, hepatitis C virus; BLA, β-lactamase.

‡To whom reprint requests should be addressed. E-mail: paul_darke@merck.com.

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because of its migration on gels in a region with less background than the higher molecular weight NS3 fragment.

The solution termed "10-kDa filtrate" is a low molecular weight fraction prepared by filtration of RRL through Amicon Microcon-10 filters. Low molecular weight components of commercial RRL include cellular components as well as supplemental DTT, potassium acetate, GTP, and creatine phosphate.

The plasmid pM3A, derived from pCDNA (Invitrogen), encodes a fusion protein termed Ubi-849-1207J-β-LA (BLA) that contains ubiquitin at its N terminus, followed by NS2/3 residues 849-1207 (J strain) linked to bacterial TEM-1 BLA at the C terminus. The NS3 protease domain in this construct has the inactivating mutation S1165A, which does not affect NS2/3 processing activity (8, 10). RNA synthesis for this construct is driven by the T7 promoter, and RNA was separately prepared for translation. On translation in RRL, the ubiquitin is immediately cleaved from the protein by cellular ubiquitin hydrolases (18). The cleavable linkage has the sequence, ubiquitin $\downarrow$ Arg-His-Gly-Ser-Glu-Phe-NS2/3. Translation of this construct inevitably produced some processed NS2 and NS3 products, because NS2/3 processing for the J strain does not require detergent or membranes as does NS2/3 from the BK strain (13). Translations were limited to 30 min for that reason. Quantification of processing at room temperature was by comparison of samples prepared immediately after addition of cycloheximide with later time samples.

Inhibitors were dissolved in DMSO and protected from light. Dilutions were in DMSO, such that the final concentration of DMSO was 2% for *in vitro* experiments and 1% for cell-based assays. The IC_{50} values were determined by first expressing the product level found as a fraction of the no-inhibitor control product level, then fitting the equation

$$\textit{Fractional activity} = a + \frac{b}{(1 + x/c)^d}$$

to the data, where a is the minimal level of fractional activity (tending to 0), $a + b$ is the maximal level (tending to 1), x is the concentration of inhibitor, c is the IC_{50}, and d is a slope coefficient.

Immunoprecipitations. The monoclonal IgM antibodies, 3G3 (anti-HSP90, Affinity Bioreagents) and TEPC-183 (control, Sigma), have been described for use in immunoprecipitation of HSP90 (19). Luciferase RNA was obtained from Promega. Immunoaffinity beads were prepared by binding the primary antibody to a solid support by means of a bridging antibody. Thus, protein G-agarose (Boehringer Mannheim) was used to immobilize goat antimouse IgM (5 mg/ml gel) overnight at 4°C. The monoclonal anti-HSP90 antibody 3G3 or an equal concentration of control mouse IgM antibody TEPC-183 was then combined with the immobilized antimouse IgM. To immunoprecipitate HSP90 and any associated proteins, lysate containing translated ^{35}S-labeled NS2/3 was incubated with the beads essentially as described (19). After binding for 2 h at 4°C, the beads were washed, suspended in SDS sample buffer, and heated to 95°C. Immunoprecipitated proteins were resolved on SDS/14% polyacrylamide gels.

Cell-Based Assay of NS2/3 Cleavage. A plasmid conferring neomycin (G418) resistance, pUbBla3X NS2/3–3A, was transfected into Jurkat cells. The cytomegalovirus promoter-driven ORF of the plasmid encodes a 91-kDa protein, ubiquitin-ubiquitin-ubiquitin-NS2/3-BLA, with the C termini of the three ubiquitin domains rendered noncleaveable to ubiquitin C-terminal hydrolases (40). The ubiquitin C-terminal sequence here is Arg-Leu-Arg-Gly-Val. The NS2/3 region includes HCV residues 849-1207. The BLA domain is TEM-1 from *Escherichia coli*. Expression of the BLA moiety is readily detected with the fluorogenic cell-permeant substrate, CCF2 (20). Transfectants were sorted by fluorescence-activated cell sorting by using treatment with CCF2 to indicate BLA expression and individual clones were grown under G418 selection. The full fusion protein expressed is highly unstable to ubiquitin-directed proteosomal degradation because of its ubiquitin N-terminal tag, but the C-terminal product of NS2/3 cleavage in this polyprotein, NS3-BLA, is stable for hours. Thus, buildup in the cells of BLA activity, as indicated by CCF2 hydrolysis (high 460-nm/530-nm ratio) is indicative of successful NS2/3 cleavage, and suppression of BLA activity indicates NS2/3 inhibition. Within this context, the NS2/3 mutation C993A, which is incapable of processing (8, 10, 11), reduces BLA activity in the cells $\approx$8-fold. Full details of this cell-based protease assay are being presented elsewhere.

Results and Discussion

NS2/3 from the BK strain of HCV requires detergent or membranes to initiate processing (13), so that addition of Triton X-100 to translated 810-1615BK was used here to separate protein synthesis from the processing reaction. NS2/3 (810–1615BK) was synthesized with ^{35}S-methionine labeling in RRL and passed through a P-6 spin column by centrifugation to remove species of molecular weight $<$6,000. This preparation of NS2/3 that lacks low molecular weight components of RRL does not process on incubation with added Triton X-100, either alone or when diluted into buffer. In contrast, dilution of this fraction 10-fold into a RRL filtrate containing solutes $<$10,000 Da (10-kDa filtrate) supported the cleavage reaction to an extent similar to that observed in undiluted lysate, as described (17). The necessity of soluble, low molecular weight factors for NS2/3 processing has been noted (13).

Recombination experiments were conducted to identify essential factors for processing, wherein reconstitution with the 10-kDa filtrate was taken as full, or 100%, reconstitution of activity, as shown by the leftmost bar of the Fig. 1*A* histogram. The components of the 10-kDa filtrate that support processing are stable to boiling or to treatment with trypsin suggesting a nonproteinaceous nature (data not shown). Small molecular weight compounds known to be present in RRL, including ATP, Mg^{2+}, K^+, and DTT, were examined individually and in combination for their ability to support processing. Although no component alone or in pairs efficiently supported processing, a solution containing ATP and a combination of salts enabled processing at a level comparable to that achieved with the 10-kDa filtrate, as shown in Fig. 1*A*. The necessity of an ionic strength approximating intracellular was apparent from processing reactions that included either sodium or potassium acetate, illustrated in Fig. 1*A* for the case of potassium, which was slightly more effective than sodium. [The requirement of physiological ionic strength for optimal activity of HSP90 has been reported for *in vitro* ATPase reactions with the pure HSP90, with stimulation of activity obtained for both potassium and sodium cations (39).] Particularly noteworthy is that substitution of ATP with ATP-γ-S in this combination caused a loss of processing, suggesting that the ATP contribution requires hydrolysis to ADP (Fig. 1*A*). The possible importance of ATP for NS2/3 processing has been suggested (13).

To complement the reconstitution experiments indicating a role for ATP in NS2/3 processing, RRL containing translated ^{35}S-labeled NS2/3 (810–1615BK) was depleted of ATP by treatment with glucose plus hexokinase before processing was initiated by the addition of Triton X-100 to 1%. Although neither glucose nor hexokinase alone had a significant effect, the combination of the two, which consumes ATP in the phosphorylation of glucose, inhibited processing by 60% (Fig. 1*B*). Alternatively, inhibition was observed with the addition of either ATP-γ-S or adenosine 5′-[$\beta,\gamma-$imido]triphosphate (AMP-

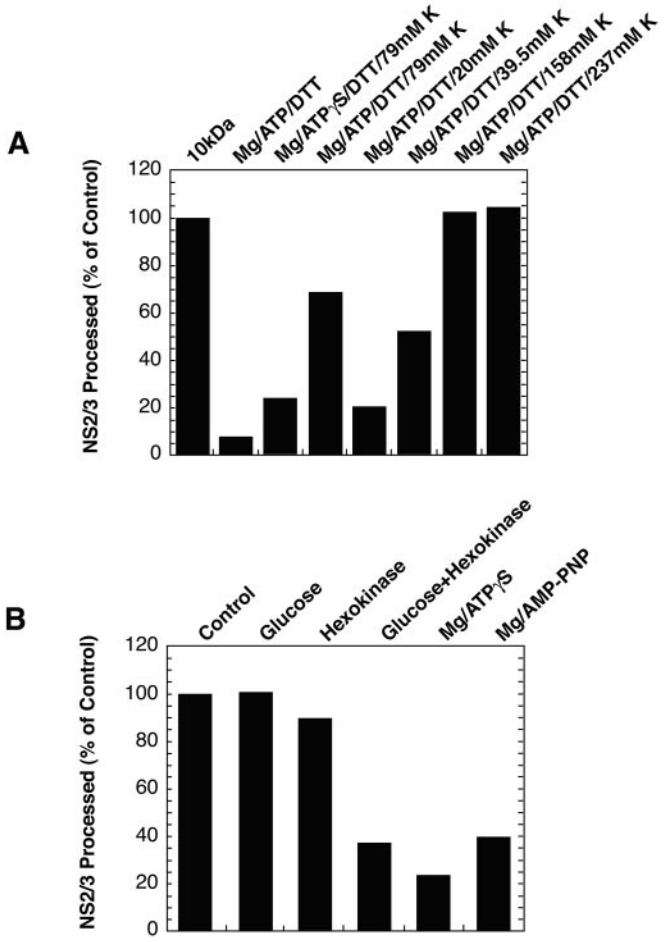


Fig. 1. HCV NS2/3 processing is supported by low molecular weight components of reticulocyte lysate. (*A*) NS2/3 protease was synthesized *in vitro* with ^{35}S-methionine labeling, desalted, and recombined with selected low molecular weight components of RRL. Activity is expressed as a percent of the amount of NS2/3 cleavage achieved by recombination with the 10-kDa filtrate of RRL. Lysate containing translated, ^{35}S-methionine-labeled NS2/3 (810–1615BK) was centrifuged through a spin column containing P-6 polyacrylamide gel (Bio-Rad; exclusion limit 6,000 Da) equilibrated in 20 mM Tris·HCl buffer, pH 7.5. The filtered lysate was diluted 10-fold into the column buffer or into buffer containing combinations of 1 mM ATP, 1.5 mM $Mg(OAc)_2$, 2 mM DTT, and KOAc. In some samples, the amount of KOAc was varied (20–237 mM) or ATP-γ-S was substituted for ATP. Analyses were performed on SDS gels and quantified as described in *Materials and Methods*. (*B*) ^{35}S-methionine-labeled NS2/3 (810–1615BK) was synthesized in RRL and 5-μl aliquots were directly combined with 1 μl of 500 mM glucose, 0.5 unit yeast hexokinase, or glucose plus hexokinase at the aforementioned concentrations, and incubated for 30 min at room temperature. Similarly, lysate containing NS2/3 was incubated with Mg/ATP-γ-S or Mg/adenosine 5′-[$\beta,\gamma-$imido]triphosphate, for final concentrations of the nucleoside analogs of 5 mM. The data shown are typical of additional experiments in which numerous other solutes were tested either alone or in combination.

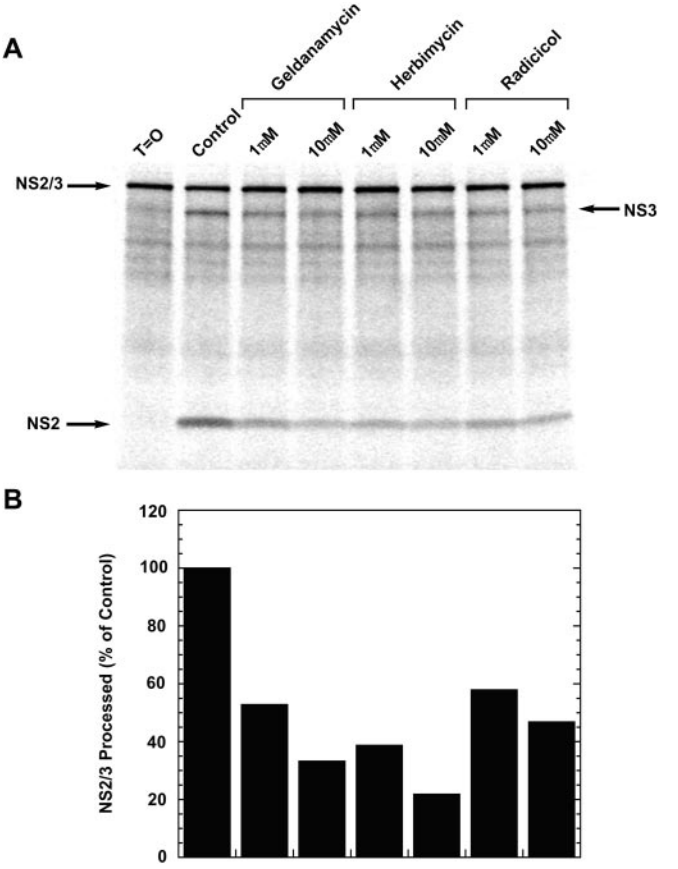


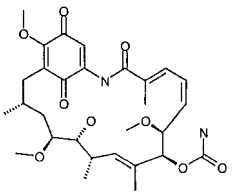
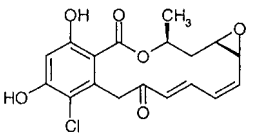
Fig. 2. Inhibition of HCV NS2/3 processing by inhibitors of HSP90 function. (*A*) ^{35}S-methionine-labeled NS2/3 (810–1615BK) was synthesized in reticulocyte lysate in the presence of either 1 or 10 μM geldanamycin, herbimycin A, or radicicol. After blocking further synthesis with cycloheximide, an aliquot was removed and processing was initiated with the addition of Triton X-100 to 1%. After 30 min, the reaction was terminated with SDS sample buffer and analysis was performed as described in *Materials and Methods*. (*B*) Each bar in the graph gives quantification of the NS2 product band in the gel lane directly above it in *A Upper*, expressed as percent processed relative to the control.

PNP), nonhydrolyzable analogs of ATP (Fig. 1*B*). Titration of the inhibition yielded IC_{50} values of 2 mM and 4 mM for ATP-γ-S or AMP-PNP, respectively, with inhibition at the maximum concentration tested (5 mM) of 77% and 60%, respectively. Because residual ATP is also present in these assays ($\approx$1 mM) complete inhibition was not expected. Inhibition by ATP-γ-S was also observed with NS2/3 from the J strain of HCV, Ubi-849-1207J-BLA, expressed as a fusion protein consisting of ubiquitin-NS2/3-BLA (data not shown).

The involvement of ATP is consistent with the participation of ATP-dependent cellular chaperones at a stage in the processing pathway. Geldanamycin and herbimycin A, two related benzoquinone ansamycins, and radicicol, a macrocyclic antibiotic, are compounds that specifically inhibit HSP90 by binding at the ATP site (21). Inhibition of NS2/3 processing was observed with all three of these compounds when added to *in vitro*-synthesized precursor 810-1615BK (up to 50%). Somewhat greater inhibition of processing was observed, in a dose-dependent manner, if these compounds were included during the synthesis phase of the experiment as well as being present during NS2/3 processing (Fig. 2). The compounds had no effect on overall efficiency of protein synthesis and similar potencies of inhibition were observed by using the NS2/3 fusion protein Ubi-849-1207J-BLA. Titration of geldanamycin and radicicol yielded EC_{50} values in the low micromolar range (Table 1), similar to what has been observed in analogous *in vitro* studies where the involvement of HSP90 has been demonstrated (22–24).

Evidence for a physical association of *in vitro*-translated NS2/3 with HSP90 was obtained by immunoprecipitation (19). Immunoprecipitation of HSP90 with a monoclonal IgM antibody coimmunoprecipitated NS2/3 derived from either BK or J strains, 810-1615BK and Ubi-849-1207J-BLA, as shown in Fig. 3. A control IgM antibody, TEPC-183 immunoprecipitated only minimal amounts of the proteins of interest. Association with HSP90 was not observed, however, with a control protein,

Table 1. Inhibition of NS2/3 by HSP90 inhibitors

Compound name	Structure	Maximum inhibition, %	EC_{50}, μM
Geldanamycin		91	1.8 ± 0.5
Radicicol		51	0.22 ± 0.12

NS2/3 processing reactions were performed with 810–1615BK. The inhibitory effects of geldanamycin and radicicol were titrated as described previously for peptide inhibition titrations (17). For both inhibitors, inhibition leveled out at the maximum extent indicated in the table so that an effective concentration (EC_{50}) is used to describe the relative potency (see *Materials and Methods*). Values given are the average of two determinations.

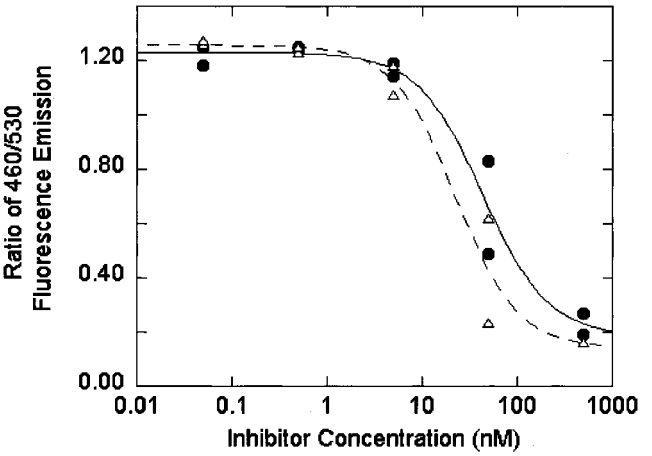


Fig. 4. HSP90 inhibitors inhibit NS2/3 cleavage in a cell-based assay. Cloned Jurkat cells expressing a fusion protein of NS2/3 in which BLA activity is the indicator of successful NS2/3 cleavage were treated with either geldanamycin or radicicol (see *Materials and Methods*). Inhibitor treatment was for 5 h, followed by addition of cycloheximide to stop protein synthesis (30 min) and subsequent addition of the BLA substrate, CCF2 (20). After 2 h, BLA activity was quantified by fluorescence readings (460-nm/530-nm ratio). Lower 460-nm/530-nm ratios indicate inhibition of NS2/3 cleavage. The geldanamycin (●) IC_{50} is 40 nM and the radicicol (△) IC_{50} is 13 nM.

translated firefly luciferase (Fig. 3*A*). The results indicate that *de novo*-synthesized NS2/3 forms a stable complex with HSP90 in solution.

The ability of geldanamycin to interfere with the immunoprecipitation of HSP90 with NS2/3 was examined. ^{35}S-labeled NS2/3 810-1615BK was synthesized in RRL in the absence or presence of 10 μM geldanamycin. In the presence of geldanamycin the amount of NS2/3 coimmunoprecipitated with the anti-HSP90 mAb was decreased by 60% (Fig. 3*B*). Thus, some inhibition of NS2/3 processing by geldanamycin may be due to the prevention of HSP90 association with NS2/3 during or immediately after translation, and the effect observed here is similar to observations in the Raf-1 system (25).

Validation of the concept that HSP90 is critical for NS2/3 processing in living cells was obtained by treating cells expressing NS2/3 with HSP90 inhibitors. Through the use of a neomycin-selectable transfection vector, stable expression of NS2/3 in Jurkat cells was obtained. The plasmid pUbBla3X-NS2/3–3A expresses a fusion form of the NS2/3 protein, with three ubiquitin domains appended to the N terminus and BLA at the C terminus. The complete, uncleaved protein has an *in vivo* half-life estimated to be 5–10 min (M.W. and B.P., unpublished observations). The NS2/3 cleavage reaction separates the destabilizing ubiquitin degradation signal at the N terminus from the NS3-BLA C-terminal product, thus stabilizing the BLA activity within the cell. By using this system, the inhibitory potency of HSP90 inhibitors toward NS2/3 processing in mammalian cells was measured, as shown in Fig. 4. Geldanamycin and radicicol are potent inhibitors of NS2/3 cleavage in this context, with IC_{50} values of 40 nM and 13 nM, respectively. In addition, inhibition is nearly complete at the highest concentrations tested (Fig. 4). The results are comparable to what others have noted, in that the concentration of geldanamycin required to inhibit HSP90 in cells is much lower than required *in vitro* (22, 26). Given that geldanamycin specifically interacts with HSP90 in cells, as demonstrated by affinity labeling (27) and affinity chromatography (24, 28, 29), the inhibition observed here for NS2/3 cleavage is HSP90-mediated.

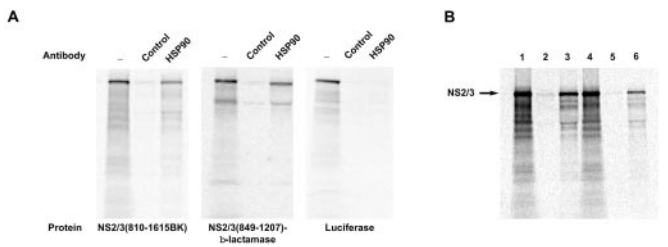


Fig. 3. Physical association of NS2/3 with HSP90. (*A*) Coimmunoprecipitation of NS2/3 with HSP90-specific antibody. ^{35}S-methionine-labeled NS2/3 (810–1615BK), Ubi-849-1207J-BLA, or firefly luciferase synthesized in RRL were immunoprecipitated with anti-HSP90 mAb 3G3 or the control IgM TEPC-183 (19). (*B*) Geldanamycin interferes with the association of NS2/3 and HSP90. ^{35}S-methionine-labeled NS2/3 synthesized in RRL in the absence (lane 1) or presence (lane 4) of 10 μM geldanamycin was immunoprecipitated with the anti-HSP90 mAb 3G3 (lanes 3 and 6) or the control IgM TEPC-183 (lanes 2 and 5).

Conclusions

Taken together, the *in vitro* and cell-based data presented support a role for the HSP90 chaperone in promoting the NS2/3 cleavage event and resemble data obtained in established cases of HSP90 regulation of enzymes and receptors. In the best-studied examples, complexes of chaperones that include accessory proteins are involved in regulating protein activity. For instance, as many as 10 different proteins may participate in the folding/regulation of steroid hormone receptors (30). In light of this, the coimmunoprecipitation of NS2/3 and HSP90 shown here may have included other proteins yet to be identified, allowing for the possibility that HSP90 and NS2/3 are not in direct contact.

Chaperones are believed to have multiple roles within cells, including preventing incorrect interactions within and between nonnative proteins, as well as increasing the yield, but not rate of folding reactions, of many newly synthesized proteins (31). In addition, by mechanisms related to their participation in protein folding, chaperone complexes modulate the activities of a variety of signaling proteins, including tyrosine kinases such as $p60^{src}$ (28), steroid hormone receptors (for review see ref. 32), and nitric oxide synthase (33, 34). The role of HSP90 and potential

cochaperones in the activity of NS2/3 may be simply for folding of nonnative, newly synthesized NS2/3. Alternatively, an HSP90 complex may position the cleavage site relative to catalytic residues in a conformation most appropriate for cleavage, one that is not the most stable conformation for the NS2/3 protein by itself. The absence of any report describing activity of the purified NS2/3 may be attributable to the thermodynamically most stable conformation of isolated NS2/3 being inactive. Because only a single cleavage is required of NS2/3 protease during the complete maturation process of HCV proteins, the cleavage could occur during the folding process, the end result of which is cleaved NS2 and NS3 in conformations appropriate to their subsequent function.

Opportunities now exist for further definition of the details of HSP90 binding to the HCV polyprotein, both in terms of other possible protein participants, and in terms of the sequence of events leading up to and after cleavage. For instance, when does NS2/3 cleavage occur relative to the different stages of HSP90 cycling? The fact that a low level of cleavage occurs in our system on the addition of ATP-γ-S may be due to completion of some partially completed cycles in the HSP90-NS2/3 complex, Indeed, it is known that ATP-γ-S binds HSP90 (36, 37) and can induce complex rearrangements (38).

Our finding of a functional interaction of a cellular chaperone with a viral enzyme has precedent. HSP90 has been shown to be incorporated into hepatitis B virus nucleocapsids, and it is required for formation of a complex between the viral reverse transcriptase and an essential RNA ligand/primer of DNA synthesis (22, 35). It is not currently possible to conduct completely analogous studies with HCV because of the lack of a cell-culture-based HCV replication system. Nonetheless, given that the inhibitor and immunoprecipitation data presented here parallel well-characterized examples of HSP90 modulation of cellular and viral enzyme activities, we find it compelling that host cell chaperones participate in the maturation of HCV enzymes.

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