The Biochemical Role of the Heat Shock Protein 90 Chaperone Complex in Establishing Human Telomerase Activity*⑤

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Telomerase is a ribonucleoprotein complex that synthesizes the G-rich DNA found at the 3′-ends of linear chromosomes. Human telomerase consists minimally of a catalytic protein (hTERT) and a template-containing RNA (hTR), although other proteins are involved in regulating telomerase activity *in vivo*. Several chaperone proteins, including hsp90 and p23, have demonstrable roles in establishing telomerase activity both *in vitro* and *in vivo*, and previous reports indicate that hsp90 and p23 are required for the reconstitution of telomerase activity from recombinant hTERT and hTR. Here we report that hTERT and hTR associate in the absence of a functional hsp90-p23 heterocomplex. We also report that hsp90 inhibitors geldanamycin and novobiocin inhibit recombinant telomerase even after telomerase is assembled. Inhibition by geldanamycin could be overcome by allowing telomerase to first bind its primer, suggesting a role for hsp90 in loading telomerase onto the telomere. Inhibition by novobiocin could not similarly be overcome but instead resulted in destabilization of the hTERT polypeptide. We propose that the hsp90-p23 complex fine tunes and stabilizes a functional telomerase structure, allowing primer loading and extension.

Telomerase is an RNA-dependent DNA polymerase that extends the 3′-ends of linear chromosomes, allowing replicating cells to overcome the end replication problem (1). The complete subunit composition of the human telomerase ribonucleoprotein (RNP)[4] complex has yet to be fully elucidated, but the minimally active enzyme requires the reverse transcriptase catalytic subunit (hTERT) and the RNA subunit (hTR), which contains the template (2–5). Recent findings revealed that chaperone proteins, such as hsp90, p23, hsp70, p60, and hsp40/ydj, are also part of the telomerase RNP, at least during part of the cell cycle (6, 7). In fact, these chaperones are required to obtain a functionally active telomerase RNP *in vitro*, although their precise role in telomerase assembly remains enigmatic (6, 7).

hsp90 is a highly conserved and abundant protein found in all eukaryotic cells (8, 9). hsp90 functions as part of a "foldosome" complex that together with other chaperones facilitates the accurate arrangement of numerous proteins (10, 11). This activity is dependent on a number of previously identified functional domains. Important for pharmacological concerns are the N-terminal ATP-binding site (12, 13) and a putative C-terminal ATP-binding site (14, 15). These sites are essential for hsp90 function, because ATP binding and hydrolysis are crucial in the conformational regulation of hsp90 and therefore its effects on client proteins (16). It has been suggested that the two ATP-binding sites act cooperatively, allowing cross-talk mediated by a central charged domain between the two termini (15). hsp90 is a functional homodimer with one homodimerization domain in the C terminus (17) and a second located within the N terminus (16). There are also a number of co-chaperone binding domains, including one for the acidic phosphoprotein p23, which binds to the amino-terminal and central region of hsp90 in an ATP-dependent manner (13, 18–21) and one for other co-chaperones (15, 22–25).

The best characterized domain of hsp90 is the highly conserved, N-terminal, nucleotide-binding pocket (12, 13, 26), which also serves as the binding site for the hsp90 inhibitor geldanamycin (GA) (13, 27). GA, a benzoquinone ansamycin antibiotic, exerts its inhibitory effect by blocking the ATP-dependent binding of p23 to hsp90 (12, 13). Another hsp90 inhibitor, the coumarin-type antibiotic novobiocin (NB), binds to the C terminus of hsp90 and hinders chaperone complex formation by blocking its association with both hsp70 and p23 (15). Due to their antagonizing effects on hsp90 function, both GA and NB are also inhibitors of proper telomerase assembly and therefore telomerase activity (6, 28).

The hsp90 client list, which is constantly being updated, includes various transcription factors, polymerases, signaling protein kinases, steroid receptors, and other proteins (29). Besides telomerase, hsp90 has been implicated in activating another reverse transcriptase (RT), duck hepatitis B virus (DHBV) RT (30). Interestingly, Hu and Seeger (30) demon-

strated that the role of hsp90 in DHBV RT activity is to maintain the RT in a "protein-priming" conformation capable of binding to the viral pregenomic RNA, which serves as the template for reverse transcription.

In this paper, we further elucidate the role of hsp90 in human telomerase RNP assembly and function. We show that the addition of GA or NB to a direct telomerase assay inhibits telomerase activity independent of the order of inhibitor addition (*i.e.* before or after telomerase assembly). We also report that hTERT and hTR are capable of interacting with each other in the absence of a functional hsp90-p23 complex, although such a complex is inactive. In these experiments, GA inhibition could only be overcome if telomerase was preincubated with its primer. This leads us to propose a model in which the mature assembly of human telomerase RNP into its final "primer-accepting" conformation requires hsp90 and its co-chaperones. hsp90 may therefore play a role in human telomerase RNP maturation and ligand binding similar to its role in establishing active DHBV RT (30) and steroid receptors (31).

EXPERIMENTAL PROCEDURES

Antibodies and Chemical Reagents—The anti-hsp90 mouse monoclonal antibody (H90–10) was a generous gift from Dr. David O. Toft (Mayo Clinic, Rochester, MN) (32). GA was purchased from Alexis Biochemicals, and NB was purchased from MP Biomedicals, Inc. Before use, GA and NB were dissolved in sterile Me_2SO and H_2O, respectively.

Reconstitution of Human Telomerase—Wild-type hTR was *in vitro* transcribed and purified as previously described (33) except that the Ampliscribe T7 Transcription Kit was used (Epicenter Technologies). T7-tagged hTERT was transcribed and translated from pET-28c-hTERT and the TNT coupled reticulocyte lysate systems kit (Promega) as previously described (33). Experiments requiring the visualization of hTERT utilized protein that was translated in the presence of [^{35}S]methionine (1175 Ci/mmol, 10 μCi/μl; PerkinElmer Life Sciences). Translation reactions using pET-28c (empty vector control) were used as a negative control in certain experiments. Preassembled telomerase was prepared by adding 4 μg of *in vitro* transcribed hTR to a 400-μl reticulocyte lysate reaction during hTERT translation.

Synthesis of ^{32}P-Labeled RNAs—RNA fragments were generated using a modification of previously described protocols (33, 34). Briefly, the DNA template required to transcribe the pseudoknot domain (hTR nucleotides 46–209) and the CR4-CR5 domain (hTR nucleotides 243–328) were generated by PCR. Transcription reactions contained 1× Ampliscribe T7 reaction buffer, 7.5 mM CTP, 7.5 mM GTP, 7.5 mM UTP, 5.8 mM ATP, ~0.7 μM [α-^{32}P]ATP (3000 Ci/mmol, 10 μCi/μl; PerkinElmer Life Sciences), 10 mM dithiothreitol, Ampliscribe T7 enzyme solution, and the required linear template. Reactions were incubated at 37 °C for 4 h. For full length hTR, hammerhead ribozyme cleavage was initiated as described (33). Reactions were treated with 5 μl of RNase-free DNase 1 (1 unit/μl; Epicenter), incubated at 37 °C for 20 min, extracted with phenol/chloroform/isoamyl alcohol, and ethanol-precipitated in the presence of 0.3 M NaOAc. RNA was resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, and unincorporated

[α-^{32}P]ATP was removed using a Microspin G-25 column (Amersham Biosciences).

Synthesis of Biotin-labeled RNAs—A 100-μl Ampliscribe T7 transcription reaction contained 1× Ampliscribe T7 reaction buffer, 7.5 mM GTP, 7.5 mM UTP, 7.5 mM ATP, 6.25 mM CTP, 1.25 mM bio-11-CTP (ENZO Life Sciences), 10 mM dithiothreitol, 10 μl of Ampliscribe T7 enzyme solution, and the appropriate DNA template. Reactions were incubated for 4 h at 37 °C, treated with DNase, and purified as described above (33).

Telomerase Assays—Telomerase activity was quantified using a previously described primer extension assay (33). In postassembly assays, hTR and hTERT were assembled in the absence of hsp90 inhibitors, and the primer-extension reaction included GA or NB. In preassembly assays, hTR and hTERT were assembled in the presence of GA or NB so that the concentration of inhibitor was maintained throughout both the assembly and primer extension reactions. For each series of assays, seven data points were used, with GA or NB concentrations ranging from 0 to 5 mM. IC_{50} values were calculated as previously described (33).

Association of hTR and hTERT in the Presence of GA and NB—The ability of hTERT to associate with hTR in the presence of hsp90 inhibitors was determined using a coimmunoprecipitation assay as described previously with minor modifications (33). Briefly, 75-μl reactions contained T7-tagged [^{35}S]hTERT (250 fmol) and ~80–800 pmol of ^{32}P-labeled pseudoknot or CR4-CR5 domain or full-length hTR (2.7–3.1 × 10^6 cpm/reaction). Inhibition studies also included GA (300 μM) or NB (1 mM). Reactions were incubated at 30 °C for 90 min and immunoprecipitated using 25 μl of preblocked anti-T7 antibody-agarose beads (Novagen). Samples were resolved on a 4–12% bis-Tris SDS gel (Invitrogen) and visualized by phosphorimaging. The RNA band intensities were normalized to the [^{35}S]hTERT protein bands and compared with the positive control.

Association of hTR and hTERT When GA or NB Was Present during Translation of hTERT—The association of hTERT with hTR when hsp90 was inhibited during translation of hTERT was assayed by affinity purification of full-length hTR using a modification of a previously described protocol for purification of human telomerase (35). [^{35}S]hTERT was synthesized in 50-μl reactions as described above in the presence of 5 pmol of hTR and 100 μM GA or 1 mM NB. Following a 90-min incubation at 30 °C, the reactions were combined with 50 μl of Ultra-Link® Immobilized NeutrAvidin™ Plus beads (Pierce) and 50 μl of buffer A (20 mM Hepes-KOH, pH 7.9, 1 mM EDTA, 300 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol) supplemented with 0.5% Triton X-100, 5 μg of yeast RNA (50 μg/ml final concentration), and 100 pmol of hTRbait, an affinity oligonucleotide complementary to the template of hTR (5′-biotin-CTAGACCTGTCATCAmGm-UmUmAmGmGmGmUmAmG-3′ where m represents 2′-O-methyl ribose). Before use, the beads were washed once with 300 μl of buffer A, blocked twice with 250 μl of blocking buffer (buffer A supplemented with 0.5 mg/ml bovine serum albumin) for 15 min at 4 °C, and then washed with 300 μl of buffer A. The beads were precipitated by centrifugation at 2500 × g for 1.5 min. The reactions were then incubated for 10 min at room temperature, followed by 2 h at 4 °C on a rotary

platform. The resulting bead complexes were washed three times with 300 μl of buffer A supplemented with 0.5% Triton X-100, once with 300 μl of buffer A supplemented with 300 m$_M$ KCl, and twice with buffer A alone. Samples were resolved by SDS-PAGE and visualized by phosphorimaging. [^{35}S]hTERT was normalized to inputs and compared with the positive control. Controls containing Me$_2$SO and H$_2$O were conducted for comparison with GA and NB, respectively. The association of the CR4-CR5 and pseudoknot domains of hTR with hTERT was determined using biotin-labeled RNA. [^{35}S]hTERT was synthesized in 50-μl reactions as described above in the presence of ~60 pmol of the biotinylated pseudoknot domain or biotinylated CR4-CR5 domain and 100 μM GA or Me$_2$SO carrier. Each reaction was then affinity-purified with 45 μl of preblocked MPG® Streptavidin beads (Pure Biotech, LLC). Beads were washed four times with 400 μl of wash buffer 1 and blocked twice with 250 μl of blocking buffer for 15 min at 4 °C before use. The beads were precipitated by centrifugation at 2500 × g for 2 min between each step. Each 50-μl reaction was then mixed with 50 μl of blocking buffer and centrifuged at 17,000 × g for 10 min at 4 °C in order to remove any precipitates. The supernatant was added to the blocked beads, and the samples were incubated for 45 min at room temperature on a rotary platform. The resulting bead complexes were washed three times with 350 μl of buffer A and once with 350 μl of TMG. The precipitated bead samples were analyzed by SDS-PAGE as described above.

Immunoprecipitation with an hsp90 Antibody—Preassembled telomerase complexes were immunoprecipitated using a previously described protocol (6). Briefly, 75 μl of telomerase preassembled with [^{35}S]hTERT was incubated at 30 °C in the presence of 1 μg of human telomeric primer, no telomeric primer, or 1 μg of nontelomeric primer. After 90 min, anti-hsp90 mouse monoclonal antibody (H90−10) was added to a final concentration of ~0.5 μg/ml, and the samples were placed on ice for 1 h. Each reaction was immunoprecipitated by the addition of 22.5 μl of prewashed Protein G-agarose beads (Roche Applied Sciences) and was incubated at 4 °C for 1 h with constant rotation. The beads were then washed three times with 400 μl of Wash Buffer (20 m$_M$ Hepes, pH 7.6, 20% glycerol, 100 m$_M$ NaCl, 0.2 m$_M$ EGTA, 1 m$_M$ MgCl$_2$, 0.1% Nonidet P-40, 0.1% bovine serum albumin). The precipitated bead samples were analyzed by SDS-PAGE as described above.

Lys-C Proteolysis of hTERT—The stability of hTERT was determined by Lys-C proteolysis using a modification of a previously described protocol (36). ~700 fmol of T7-tagged [^{35}S]hTERT and 3 μg of hTR were allowed to assemble either in the presence or absence of 100 μM GA or 1 m$_M$ NB at 30 °C for 90 min. Each reaction was then immunoprecipitated using 30 μl of preblocked anti-T7 antibody-agarose beads (Novagen), as previously described (33). Following immunoprecipitation, the beads were washed with and resuspended in 80 μl of digestion buffer (25 m$_M$ Tris-HCl, pH 8.5, 1 m$_M$ EDTA). Each sample was then treated with ~90 ng of endoproteinase Lys-C (sequencing grade; Roche Applied Science) and was incubated at 30 °C. Aliquots of each reaction were removed at various time points and quenched with an equal volume of Laemmli sample buffer (125 m$_M$ Tris-Cl, pH 6.8, 4% SDS, 0.005% bromphenol blue, 20%
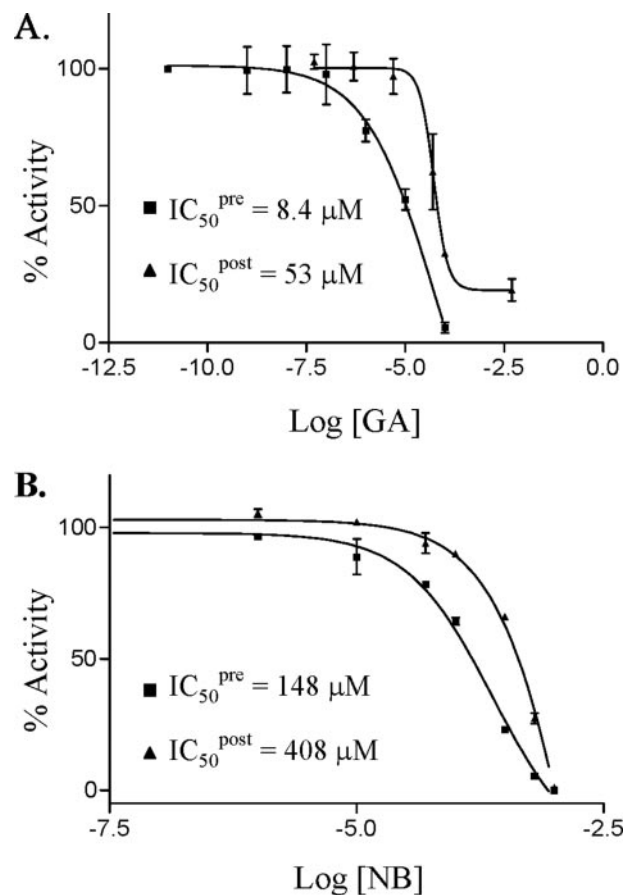

FIGURE 1. **GA and NB inhibit telomerase when added before or after assembly.** *A*, concentration dependence of GA on telomerase activity in an assembly assay (preassembly; ■) and a direct assay (postassembly; ▲). *B*, concentration dependence of NB on telomerase activity in an assembly assay and a direct assay. *Error bars*, S.D. ($n = 4$).

glycerol, 0.72 $_M$ β-mercaptoethanol) followed by heating at 95 °C for 5 min. Samples were resolved on a 4–12% bis-Tris SDS gel (Invitrogen) and visualized by phosphorimaging.

RESULTS

hsp90 Inhibitors Affect in Vitro Reconstituted Human Telomerase Activity both Before and After Assembly—To examine the role of hsp90 in telomerase activation, we used two hsp90 inhibitors: GA, which binds to the N-terminal ATP-binding site, and NB, which binds to the putative C-terminal ATP-binding site. Despite their separate binding sites, both compounds have been reported to inhibit human telomerase (6, 28). In our hands, GA inhibited the *in vitro* reconstitution of telomerase activity when added before assembly with an IC_{50}^{pre} value of 8.4 ± 3.7 μM, and NB inhibited with an IC_{50}^{pre} of 148 ± 18 μM (Fig. 1). Notably, the concentrations of GA and NB were maintained throughout the assays, including both the assembly and the primer extension reactions. These findings are consistent with the hypothesis that hsp90 inhibitors exert their effect by preventing hsp90-dependent association between hTR and hTERT. We further tested the role of hsp90 by adding the hsp90 inhibitors to telomerase that was preassembled. Surprisingly, we found that both GA and NB inhibited telomerase activity and found IC_{50}^{post} values of 53 ± 7 and 408 ± 50 μM, respectively,

for these drugs (Fig. 1). Although the IC_{50} values for GA and NB were slightly higher when added postassembly as compared with their addition preassembly, both drugs were full antagonists of assembled telomerase. We found that the addition of p23 partially suppressed inhibition by GA, so its effects are unlikely to be a direct effect on telomerase (Fig. 2).

Telomerase Maintains hTR-hTERT Interactions in the Presence of Geldanamycin and Novobiocin after hTERT Translation—hsp90 was previously shown to be required for reconstituting active recombinant telomerase and to be involved in maintaining telomerase activity in cultured human cells (6, 37). The precise role

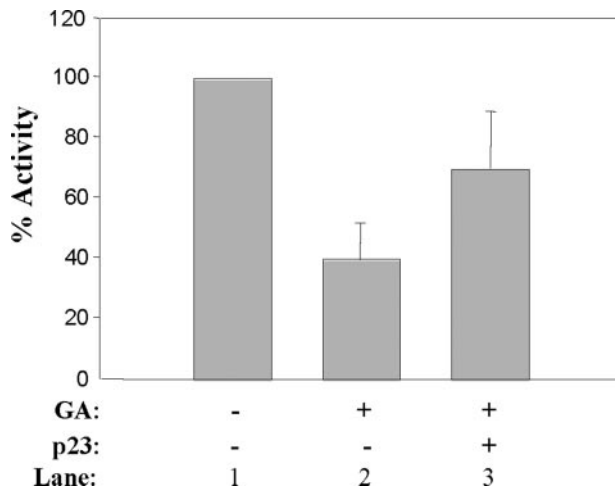FIGURE 2. **p23 partially rescues GA-induced telomerase inhibition.** Telomerase activity was inhibited in an assembly assay by the addition of 75 μM GA (*lanes 2* and *3*). p23 was added to the reaction prior to assembly at a final concentration of 20 μM (*lane 3*). Error bars, S.D. (*n* = 6).

of hsp90 in establishing active telomerase, however, is not clear from these studies. To determine if hsp90 is involved in establishing the association of hTR and hTERT during telomerase assembly, we performed co-immunoprecipitation assays using T7-tagged [^{35}S]hTERT and [^{32}P]RNA in the presence of 300 μM GA or 1 mM NB. We tested the ability of GA and NB to inhibit hTERT binding to full-length hTR as well as the CR4-CR5 and pseudoknot domains, which are two regions of hTR previously shown to interact independently with hTERT (33, 34, 38–40). The CR4-CR5 domain is a structurally conserved domain found in vertebrate telomerase RNAs. In humans, the CR4-CR5 domain is well defined and contains a stem-loop termed p6.1, whose direct interaction with hTERT is required for telomerase activity (34, 38–40). The pseudoknot domain, which contains the template, also independently binds hTERT (39, 40). We found that hsp90 inhibition does not affect the association of hTERT with hTR or the two domains of hTR we tested (Fig. 3*A*). This suggests that hsp90 does not facilitate the association of hTR and hTERT. Clearly, however, the telomerase complex that is assembled when hsp90 is inhibited is dysfunctional.

hsp90 and other chaperones function in part to stabilize peptides during translation to prevent improper folding (41). In the previous experiments, GA and NB were added after hTERT had been translated, while hsp90 was presumably active. To determine if hsp90 activity is required to allow the nascent hTERT transcript to assume an hTR binding-competent state, we inhibited hsp90 during hTERT translation. Because inhibition of hsp90 during translation appeared to affect immunoprecipitation efficiency in preliminary studies, we used affinity purification of the RNA subunit to assess hTR-hTERT association. To analyze the association of full-length hTR with hTERT, telomerase complexes were affinity-purified using a biotinylated oligonucleotide that was complementary to the RNA template (35). To analyze the association of the CR4-CR5 and pseudoknot domains with hTERT, [^{35}S]hTERT was translated in the presence of biotin-labeled RNAs and GA (33). Interestingly, only the interaction of the CR4-CR5 domain was dramatically affected by the presence of GA during hTERT translation (Fig. 3*B*, *lanes 3* and *4*), although there was a small decrease in the amount of pseudoknot domain that co-purified with hTERT (Fig. 3*B*, *lanes 1* and *2*). The interaction between hTERT and full-length hTR was not affected by the presence of GA during translation (Fig. 3*B*, *lanes 5* and *6*). NB, on the other hand, was found to prevent over 60% of the full-length hTR/hTERT interaction when present during translation (Fig. 3*B*, *lanes 7* and *8*).

Primer Binding Overcomes GA, but Not NB, Inhibition of Human Telomerase—If GA does not affect telomerase by preventing the asso-
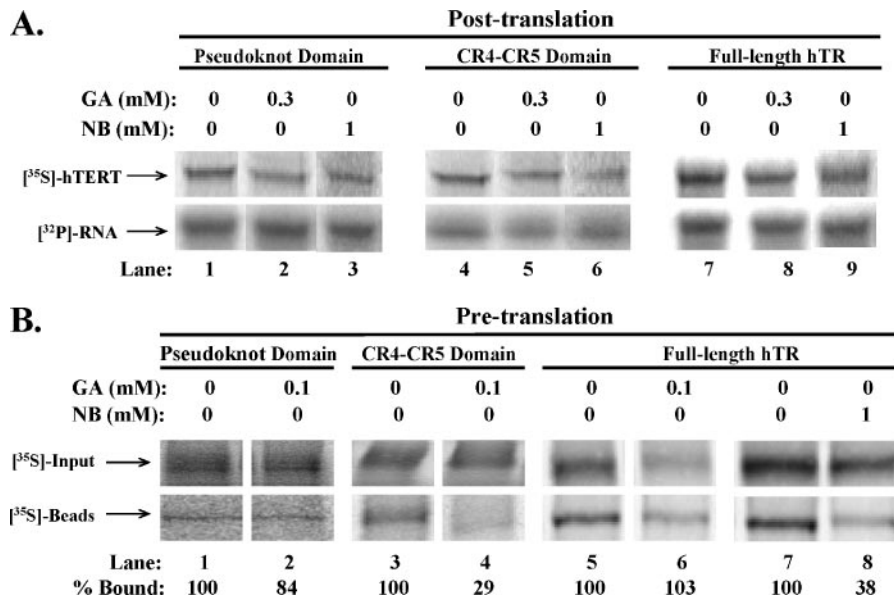

FIGURE 3. **hTR/hTERT interactions are unaffected by the presence of GA and NB after translation of hTERT, but hTR/hTERT interactions are affected by the presence of GA and NB during translation.** *A*, ^{32}P-labeled pseudoknot domain (*lanes 1–3*), ^{32}P-labeled CR4-CR5 domain (*lanes 4–6*), or ^{32}P-labeled full-length hTR (*lanes 7–9*) was incubated with T7-tagged [^{35}S]hTERT either in the presence or absence of 300 μM GA or 1 mM NB. *B*, pseudoknot domain (*lanes 1* and *2*), CR4-CR5 domain (*lanes 3* and *4*), or full-length hTR (*lanes 5–8*) were incubated with T7-tagged [^{35}S]hTERT and 100 μM GA or 1 mM NB. The complexes were affinity-purified and resolved on an SDS gel. % *Bound* values indicate the amount of protein bound to the RNA when compared with control reactions with no GA or NB. Gels shown are representative of multiple (2–4) experiments. Independent experiments revealed no significant nonspecific binding (<5%).
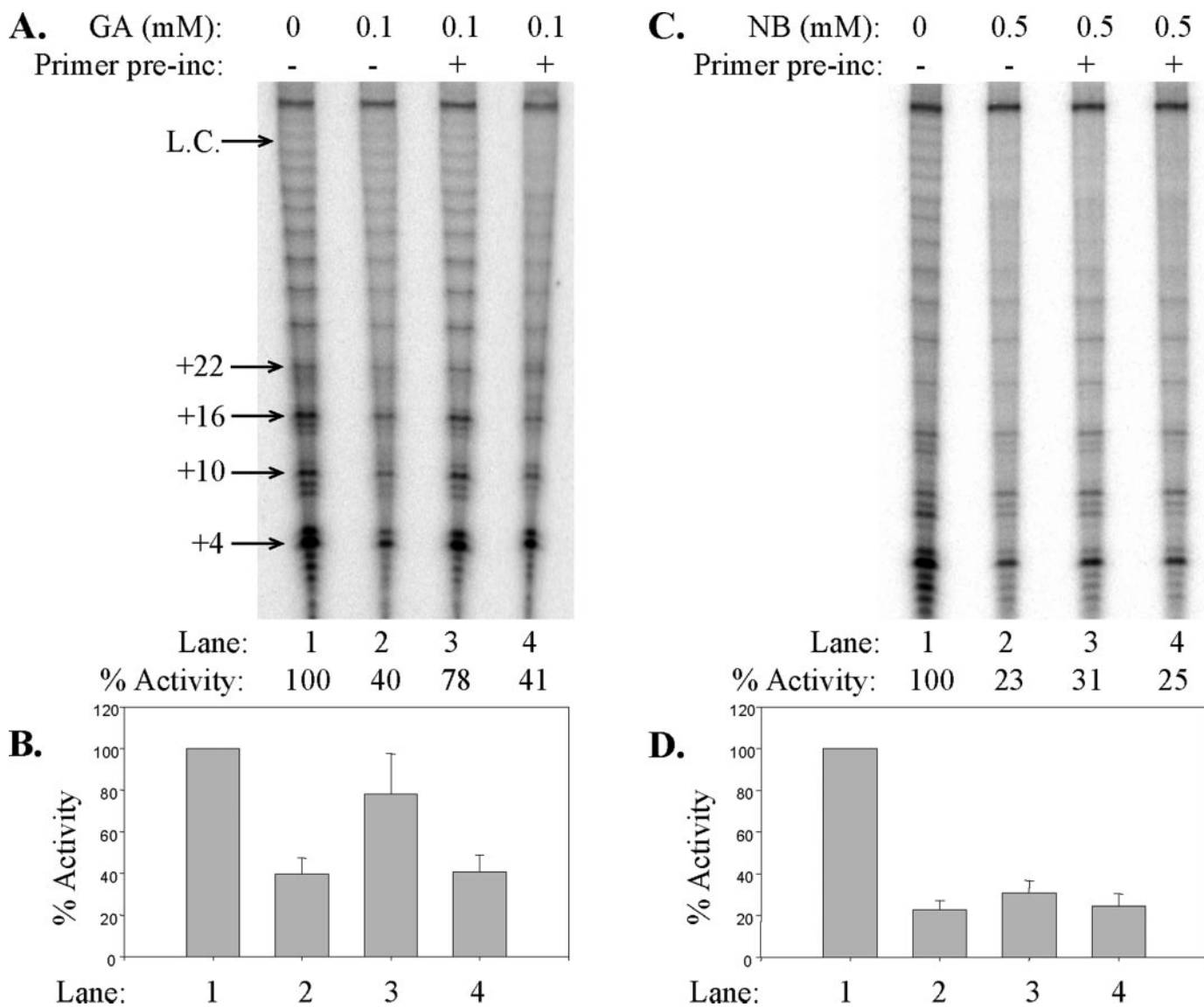
FIGURE 4. **Preincubation with primer rescues GA-induced but not NB-induced telomerase inhibition.** Preassembled telomerase was assayed for activity in the absence (*lane 1*) or presence (*lanes 2–4*) of GA (*A*) or NB (*C*). Preassembled telomerase was preincubated with primer (*Primer pre-inc*), followed by the addition of GA or NB (*A* and *C*, *lanes 3* and *4*). *Lane 3* included a telomeric primer, whereas *lane 4* included a nontelomeric primer. Quantified telomerase activity is displayed in *B* and *D*. *% Activity* is the amount of residual telomerase activity when compared with the positive control (*lane 1*). The ^{32}P loading control (*L.C.*) and the human telomere primer-extension products (+4, +10, +16, +22, etc. nucleotides added) are indicated. The *lane numbers* for *B* and *D* correlate with *A* and *C*, respectively. *Error bars*, S.D ($n = 3$).

ciation of hTR with properly translated hTERT, then how does it inhibit its enzymatic activity? One possibility is that hsp90 is involved in loading telomerase onto the chromosome end (*i.e.* hsp90 might be involved in telomerase-primer docking). Hu and Seeger (30) found that hsp90 maintains the DHBV RT in a "protein-priming" conformation capable of binding the RNA template used for reverse transcription. Similarly, hsp90 promotes binding of steroid receptor complexes to their ligands (31, 42–44). Thus, it seems that hsp90 can participate in preparing its clients for substrate and ligand binding. Based on this precedent, we determined if hsp90 plays a direct role in telomerase-primer loading.

We performed direct extension assays in the presence of either GA or NB and telomerase or telomerase preincubated with a telomeric primer. We found that preincubation of a telomeric primer with telomerase overcame GA-induced inhibi-

tion (Fig. 4, *A* and *B*). This effect was dependent on a telomeric primer, since a nontelomeric primer did not elicit the rescue effect (Fig. 4, *A* and *B*, *lane 4*). Because NB, like GA, is an hsp90 inhibitor, we would expect to see a similar rescue effect. Surprisingly, preincubation of a telomeric primer with telomerase did not overcome NB inhibition (Fig. 3, *C* and *D*). This suggests that the two drug-binding sites separate two different telomerase-specific functions of hsp90.

hsp90 Is Associated with hTERT after Telomeric Primer Binding—If hsp90 is involved in primer loading, then it might be expected to release the telomerase complex once docking has occurred, similar to the behavior of hsp90 in facilitating ligand binding to steroid receptors (31). To test this possibility, we examined the association of [^{35}S]hTERT with hsp90 in the presence or absence of a telomeric primer. The holoenzyme complexes were preincubated with a telomeric primer or a non-
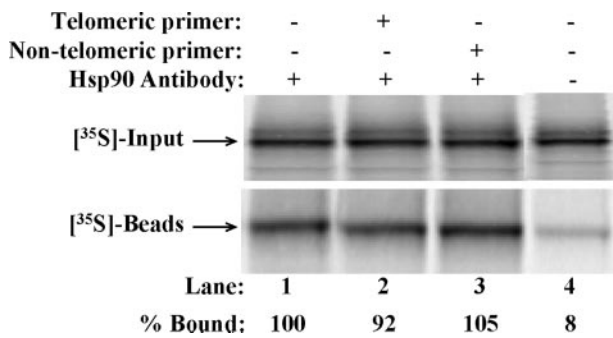
Telomeric primer:	−	+	−	−
Non-telomeric primer:	−	−	+	−
Hsp90 Antibody:	+	+	+	−

| | Lane: | 1 | 2 | 3 | 4 |
| % Bound: | | 100 | 92 | 105 | 8 |

FIGURE 5. **hsp90 remains associated with hTERT after primer loading.** ^{35}S-Labeled telomerase was incubated alone (*lane 1*), in the presence of telomeric primer (*lane 2*), or in the presence of nontelomeric primer (*lane 3*) and immunopurified with an hsp90 antibody. % *Bound* values indicate the amount of ^{35}S-labeled telomerase bound to hsp90 when compared with the positive control (*lane 1*). All immunoprecipitations were performed in duplicate, and representative results are shown.

telomeric primer or in the absence of added DNA. The resulting complexes were immunoprecipitated with an anti-hsp90 antibody. We found that the presence of the telomeric primer had no significant effect on the interaction between hsp90 and hTERT when compared with the positive control without primer (Fig. 5). Similarly, a nontelomeric primer, which served as a control for specificity, exhibited little effect (Fig. 5, *lane 3*). This result is consistent with a previous report that demonstrated the association of hsp90 with active human telomerase (6). A negative control with no primer or antibody yielded ≤8% nonspecific binding of [^{35}S]hTERT to the beads (Fig. 5, *lane 4*).

hsp90 Inhibition Alters hTERT Stability—One possible explanation for the effects of GA and NB on telomerase is that hsp90 is involved in stabilizing hTERT and therefore the telomerase complex. For example, hTERT stability or its conformation could depend on hsp90 activity. We used partial proteolysis to examine the global structure of hTERT. Partial proteolysis is a powerful technique used to identify substructures and functional domains in various proteins (45) and has been used to examine the global structure of *Tetrahymena thermophila* TERT mutants (36). To determine if GA and NB affect hTERT stability, we treated hsp90-inhibited assembly reactions with Lys-C, an endoproteinase that cleaves at K–X bonds, and determined the rate of proteolysis in the absence and presence of hsp90 inhibitors. In the absence of hsp90 inhibitors, hTERT degraded with a half-life of ~15 min (Fig. 6). Further, several prominent hTERT digestion products were observed. In the presence of GA, the hTERT half-life was similar, and the same degradation banding pattern was observed. In contrast, we found that NB significantly affected the rate of full-length hTERT proteolysis by decreasing the half-life to ~5 min. The most obvious difference in hTERT degradation between NB and its control is seen at the earliest time point, at which there is ~100% hTERT remaining in the control reaction but only ~50% left when NB is present (Fig. 5, compare *lane 10* with *lane 14*). Furthermore, when comparing the amounts of truncated proteolysis products at the 60-min time point, the NB-treated sample has a significantly decreased level of products when compared with both the H_2O control and the GA-treated sample (Fig. 6, compare *lane 16* with *lanes 8* and *12*). This presumably resulted from increased Lys-C digestion of the already

truncated proteolysis products due to decreased hTERT stability. Further experiments are needed to determine precisely what domains of hTERT have become vulnerable to proteolysis. The increase in Lys-C degradation in the presence of NB, but not GA, may explain why primer binding does not overcome NB inhibition.

A possible consequence of decreased hTERT stability is a commensurate decrease in telomerase complex stability. Using a pulse-chase experiment, we determined if telomerase stability was affected by GA or NB by examining the dissociation rate of the CR4-CR5 and pseudoknot domains from hTERT. We did not detect any change in the dissociation rate of the RNA-protein complexes in our assay (Fig. S1). However, we consistently observed a time-dependent decrease in the ability to immunoprecipitate hTERT in the presence of NB as compared with the positive control. GA also had a slight effect on immunoprecipitation, although the effect was much less severe than the NB effect. These results are consistent with the conclusion that NB destabilizes hTERT.

hsp90 Is Associated with hTERT following Geldanamycin or Novobiocin Treatment—Yun *et al.* (46) showed that NB binding to hsp90 induces a conformational change that induces dissociation from client proteins. They specifically showed that this "client release" conformation promotes the dissociation of hsp90 from the heme-regulated eIF2α kinase. A similar phenomenon would explain our finding that NB caused increased Lys-C proteolysis of hTERT and explain why preloading primer does not overcome NB inhibition. Previously, Holt *et al.* (6) showed that GA has no effect on hsp90/hTERT interaction. Using an anti-hsp90 antibody to co-immunoprecipitate [^{35}S]hTERT, we confirm that GA does not affect the hsp90/hTERT interaction and report that NB caused no decrease in the co-immunoprecipitation efficiency of hTERT with hsp90, even at a concentration that caused 80% inhibition in telomerase activity (Fig. S2). Apparently, NB-induced dissociation of hsp90 from its clients is dependent on the particular client, and hTERT remains bound to hsp90 even in the presence of NB.

DISCUSSION

Human telomerase must be properly assembled to produce an active complex. The biogenesis of telomerase has received much attention and appears to be a complex process that requires specific pathways for maturation of its RNA subunit (47, 48) as well as assembly of the holoenzyme complex (6, 7, 49). It was previously revealed that the hsp90 chaperone complex interacts with hTERT and that hsp90 activity is required for establishing active telomerase in both *in vitro* reconstitution assays and cultured human cells. In this work, we set out to further expand our understanding of the biochemical role of hsp90 in maintaining telomerase activity, and we have established that the role of hsp90 in activating telomerase is more complex than previously thought.

We confirmed that hsp90 inhibitors GA and NB prevent reconstitution of active human telomerase (Fig. 1). Surprisingly, we found that this inhibition was not the result of preventing the association of hTERT with hTR or specific domains of hTR (Fig. 3A). On the contrary, we found that hTR and hTERT are capable of interacting in the presence of either GA
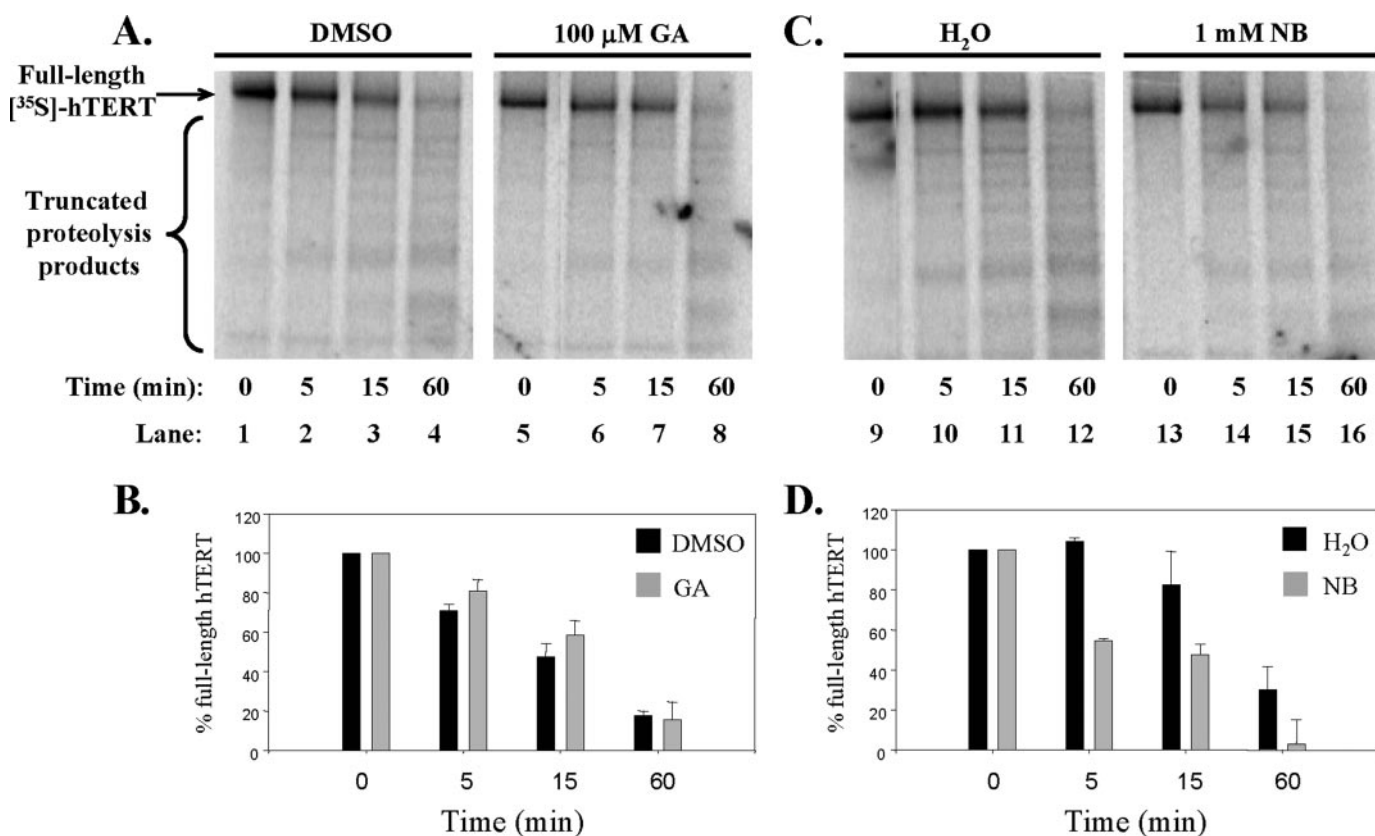
FIGURE 6. **NB, but not GA, increases the proteolysis rate of hTERT.** [^{35}S]hTERT and hTR were assembled in the presence or absence of hsp90 inhibitors. Following co-immunopurification, the complexes were digested with Lys-C, and aliquots were quenched at the indicated time points. [^{35}S]hTERT fragments digested after GA (*A*) or NB (*C*) treatment were resolved by SDS-PAGE. The amount of full-length [^{35}S]hTERT proteolysis is displayed in *B* and *D*. *Error bars*, S.D. (*n* = 2). *DMSO*, dimethyl sulfoxide.

or NB, although the RNP complex that is assembled in the presence of these hsp90 inhibitors is inactive. Interestingly, when added to an assembly reaction prior to translation of the hTERT gene, NB caused a decrease in the amount of hTERT that co-immunoprecipitated with full-length hTR, whereas GA only affected the association of the CR4-CR5 domain with hTERT (Fig. 3*B*). This suggests that the nascent hTERT transcript requires hsp90 activity to achieve a structure that is fully competent in hTR binding and that the NB-targeted C terminus of hsp90 may be more important in maintaining polypeptide stability than the GA-targeted N terminus. In summary, our results indicate that hsp90 does not chaperone the marriage of hTERT to hTR, *per se*, but may instead be involved in ensuring that nascent hTERT folds properly and in fine tuning and maintaining the structure of the assembled telomerase complex.

When GA or NB was added to preassembled telomerase, we observed complete inhibition of telomerase activity, albeit with a higher IC$_{50}$ than when the inhibitors were added before assembly (Fig. 1). There are several possible explanations for this result. During early work on the mechanism of GA action, it was noted that GA is an inhibitor of several nucleotide transferases, including DNA pol α (50), although it is not clear from these studies if the effect is related to hsp90 inhibition or specific polymerase inhibition. More recently, it was found that whereas GA inhibits hepatitis B RT, it does so by binding hsp90 and is not an RT inhibitor, since GA inhibition could be overcome using a synthetic mutant that was hsp90-independent

(30, 51). Although we cannot entirely rule out direct inhibition of telomerase by GA and NB, the history of GA and NB suggests that hsp90 is the more likely candidate in our studies. Several observations are consistent with this conclusion. First, GA and NB have different effects on telomerase and are known to bind two distinct sites on hsp90 that have distinct functions in the hsp90 reaction cycle. Furthermore, the addition of p23, which has an overlapping binding site with GA, partially rescued telomerase activity from GA inhibition (Fig. 2). We also showed that NB affects the stability of hTERT, consistent with an hsp90-dependent activity. Together, the known pharmacological effects of GA and NB on hsp90 and the observations we describe here are more consistent with an effect on hsp90 than a direct inhibitory effect on telomerase.

Since hsp90 does not seem to be explicitly required to promote the association of hTERT and hTR, we speculated that it might be involved in primer loading. This hypothesis was based on work with DHBV RT, for which hsp90 removes an autoinhibitory domain away from the RT active site to allow primer loading and RNA binding (30, 51). We found that preincubation of a telomerase primer with preassembled telomerase, prior to the addition of GA, rescued GA-induced telomerase inhibition (Fig. 4, *A* and *B*). However, preincubation with primer did not overcome NB-induced telomerase inhibition (Fig. 4, *C* and *D*). Although it has been suggested that the two termini of hsp90 engage in cooperativity and cross-talk (15), our results indicate that GA and NB dissociate different facets
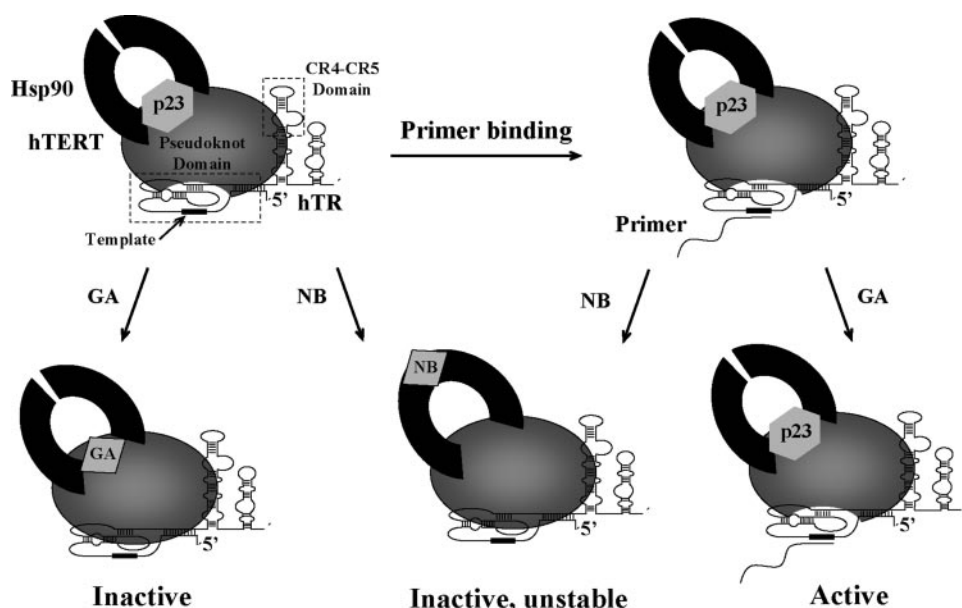
FIGURE 7. **Conceptual model for hsp90-mediated human telomerase maturation and the effects of GA and NB.** hTR and hTERT assemble in the presence of an active hsp90-p23 complex into an active complex that is competent in primer binding. The addition of GA or NB to the assembled complex leads to the formation of an inactive complex, in part by preventing primer binding and in part by destabilizing the hTERT polypeptide. The addition of primer overcomes inhibition by GA, but not NB, because it blocks the role of hsp90 in stabilizing hTERT. Note that the presence of p23 in the primer-telomerase complex and after GA addition is speculative. The CR4-CR5 and pseudoknot domains are indicated by *labeled dotted boxes*. The N terminus of hsp90 is shown as the p23- and GA-binding sites. The hsp90 C terminus is shown as the NB-binding site. Note that the individual proteins and hTR are not drawn to scale.

of hsp90 involvement with telomerase and implicate different functions of the N- and C-terminal hsp90 domains in the telomerase reaction cycle. hsp90 appears to be directly involved in primer loading and in stabilizing the active telomerase complex as shown in the model presented in Fig. 7. In this model, the N-terminal ATP binding site functions to maintain telomerase in a primer-binding state. The role of the putative C-terminal ATP binding site is to confer hsp90-dependent stability to hTERT. This proposal is consistent with the apparent instability of hTERT in the presence of NB and the rescue of GA inhibition by primer. Furthermore, although GA and NB target different ligand-binding sites on hsp90, they both displace p23 from hsp90 (12, 13, 15). This suggests that p23 displacement is not related to primer binding.

Unlike the transient nature of some chaperones and client proteins, it is clear from our studies and those of the Holt laboratory (7) that hsp90 is stably associated with the telomerase holoenzyme both after hTERT/hTR assembly and after primer binding (Fig. 5). To explain this, we propose that hsp90 remains bound to hTERT in order to maintain the holoenzyme in a conformation capable of primer binding and catalysis (Fig. 7). By comparison with the DHBV holoenzyme (51), perhaps one role of hsp90 in telomerase holoenzyme maturation may include the manipulation and offsetting of an autoinhibitory domain to allow primer loading. It is not clear if hTERT contains an autoinhibitory domain, but it is clear that the presence of functional hsp90 does allow for efficient primer binding and thus increased telomerase activity. Further studies using truncated hTERT mutants will address the existence of an hsp90-antagonized autoinhibitory domain on hTERT.

The mechanism of hsp90-targeted inhibition of telomer-ase in cells appears more complex. Antisense oligonucleotides targeting cellular hsp90 have been shown to directly reduce hsp90 mRNA expression and decrease telomerase activity (52). However, hsp90 plays a significant role in regulating many cellular signaling pathways, including that for the serine/threonine kinase Akt. Akt has been found to increase human telomerase activity via phosphorylation of hTERT (53). The association with hsp90 is required to maintain Akt in an active state (54), and GA- and NB-mediated hsp90 inhibition has been found to inhibit the Akt cascade (28, 55), thus leading to decreased telomerase activity. Furthermore, GA-induced hsp90 inhibition in H1299 cells leads to the ubiquitination and proteosome-mediated degradation of hTERT (56). These indirect effects on telomerase activity must therefore be taken into account when elucidating the cellular mechanisms of GA- and NB-induced telomerase inhibition.

In conclusion, we have found that the role of hsp90 in the telomerase reaction cycle is more complex than expected. hsp90 is not specifically involved in allowing hTR to bind hTERT, although it appears to be involved in fine tuning and stabilizing the structure of the telomerase complex. Active hsp90 seems to be required both during translation of the nascent hTERT transcript and for the assembly of active telomerase. Importantly, hsp90 appears to be involved in maintaining telomerase in a conformation that is competent to bind the telomere. Although it remains to be seen if this is true *in vivo*, it is an attractive model for allowing the hsp90-dependent regulation of telomerase and is consistent with the role of hsp90 in facilitating ligand binding to other hsp90 clients.

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