Stable Association of hsp90 and p23, but Not hsp70, with Active Human Telomerase*

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The ribonucleoprotein telomerase holoenzyme is minimally composed of a catalytic subunit, hTERT, and its associated template RNA component, hTR. We have previously found two additional components of the telomerase holoenzyme, the chaperones p23 and heat shock protein (hsp) 90, both of which are required for efficient telomerase assembly in vitro and in vivo. Both hsp90 and p23 bind specifically to hTERT and influence its proper assembly with the template RNA, hTR. We report here that the hsp70 chaperone also associates with hTERT in the absence of hTR and dissociates when telomerase is folded into its active state, similar to what occurs with other chaperone targets. Our data also indicate that hsp90 and p23 remain associated with functional telomerase complexes, which differs from other hsp90-folded enzymes that require only a transient hsp90-p23 binding. Our data suggest that components of the hsp90 chaperone complex, while required for telomerase assembly, remain associated with active enzyme, which may ultimately provide critical insight into the biochemical properties of telomerase assembly.

Vertebrate telomeres are composed of the repeated sequence TTAGGG and are responsible for maintaining chromosomal stability and integrity (1). Conventional DNA polymerases are incapable of replicating to the end of a linear molecule (the end replication problem), resulting in loss of telomeric DNA during cellular proliferation of normal somatic cells (2, 3). The specialized reverse transcriptase, telomerase, compensates for this loss of telomere sequence and is responsible for maintenance and preservation of telomere ends in germ cells and immortal and cancer cells (4, 5). The reverse transcriptase subunit of telomerase, hTERT, contains the catalytic activity of the enzyme, whereas the associated RNA component, hTR, serves as the template for synthesis of telomeric sequences (6–8). As direct evidence that telomere erosion plays a major role in cellular senescence, hTERT was ectopically expressed in normal human cells, which endogenously express hTR, resulting in activation of telomerase, stabilization of telomere lengths, and extension of cellular life span (9, 10).

Expression of the hTERT and hTR components in heterologous systems has allowed for increased understanding of the biochemical features of the telomerase enzyme. Reconstitution of human telomerase activity has been accomplished in a variety of in vitro systems including yeast, baculovirus, rabbit reticulocyte, wheat germ, and human (6, 7, 11, 12), and each has verified the essential role of hTERT and hTR in active telomerase. Recently, we have demonstrated that the hsp901 chaperone complex is required for assembly of human telomerase both in vitro and in vivo in a rabbit reticulocyte system and in human cells (13). We observe that hsp90 and p23 associate with hTERT in the absence of hTR and that the minimal components necessary for active telomerase assembly are hTERT, hTR, hsp90, p23, hsp70, p60, and hsp40/ydj. In addition, we demonstrate that geldanamycin, a potent inhibitor of hsp90 function, is capable of blocking the assembly of human telomerase in the rabbit reticulocyte system, as well as in cultured cells. Recently, additional groups reported on the importance of factor(s) in the reticulocyte lysate system for telomerase assembly (11, 12, 14), without specifically identifying the components critical for telomerase assembly.

To date, the hsp90-related chaperone complex is the only set of proteins that has been shown to functionally associate with human telomerase and affect its assembly. Here, we report that hsp90 and p23 appear to remain associated with functional telomerase and that hsp70 also associates with hTERT in a transient fashion. Our results demonstrate that the chaperone/telomerase interaction is critical for ribonucleoprotein assembly and the formation of active telomerase enzyme.

EXPERIMENTAL PROCEDURES

Plasmids and Gene Expression—For in vitro expression, the human hTERT cDNA was inserted into pCMN3/HisC (Invitrogen) in frame with tandem copies of a C-terminal hemagglutinin epitope (HA). hTERT was synthesized in the rabbit reticulocyte lysate system (TnT; Promega) as previously described (6) in the presence of [35S]methionine.

pTRC3 was described previously (6). hTR was produced using pTRC3 (8) with the Megascript T7 in vitro transcription system (Ambion). Antibodies and Purified Proteins—Monoclonal anti-HA antibody (12CA5) was purchased from Roche Molecular Biochemicals. Monoclonal anti-p23 antibody (JJ3), monoclonal anti-hsp90 antibody (H9010), and monoclonal anti-hsp70 antibody were described previously (15, 16). Monoclonal TCP-1 was purchased from Stressgen (13). Human hsp90β and human hsp70 (hsp70A) were purified from baculovirus preparations, and human p23 was expressed and purified from Escherichia coli as before (13).

In Vitro Telomerase Assembly—To assemble active telomerase, 0.2 μl of in vitro-transcribed and -translated hTERT and 0.5 μg of hTR were mixed together in a 4-μl assembly assay with or without additional fresh RRL and incubated at 30 °C for 90 min. For some experiments, hTERT and hTR were coexpressed in the TnT system, followed by immunoprecipitation (see below) or reconstitution of activity. Addition

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The abbreviations used are: hsp, heat shock protein; RRL, rabbit reticulocyte lysate; HA, hemagglutinin; TnT, transcription and translation; TRAP, telomeric repeat amplification protocol; IP, immunoprecipitation; bp, base pair; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
FIG. 1. In vitro reconstitution of telomerase activity using hTERT and hTERT/HA3. A, SDS-PAGE of hTERT and hTERT/HA3 expressed in the TNT system. The hTERT/HA3 construct with the additional HA epitopes is slightly larger than the hTERT without a C-terminal tag. B, telomerase activity was reconstituted using either hTERT or hTERT/HA3, and subjected to the TRAP assay. Shown are dilutions of the reconstituted material, with volumes corresponding to the amount of actual hTERT or hTERT/HA3 TNT mix used in the assay. C, quantitation of relative telomerase activity for the assay of hTERT or hTERT/HA3. Relative activity is calculated by taking the ratio of the telomerase-specific ladder to the internal control, and all quantitation was normalized to 0.5 μl of sample (calculated as 1). The data shown represents the average of three independent experiments.

of purified chaperones (hsp90 and p23) were in the amounts of 750 and 500 ng, respectively.

Telomerase Activity Assays—Telomerase activity in all samples was determined by TRAP as previously described (6) with minor modifications. The TRAP-eze telomerase detection kit (Intergen, Gaithersburg, MD), which includes a 36-36 internal standard to allow quantitation of activity, was used (17). After telomerase extension for 30 min at room temperature, extended products were amplified by a two-step PCR (94 °C for 30 s, 60 °C for 30 s) for 27 cycles. These PCR-amplified products were separated by 10% polyacrylamide gel electrophoresis and exposed to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA). Quantitative estimates of telomerase activity were calculated using ImageQuant software by determining the ratio of the 36-36 internal standard to the 6-bp telomerase-specific ladder observed on the gel (17).

Immunoprecipitations—For immunoprecipitation from in vitro assembly reactions, antibodies were added to a final concentration of 0.5 μg/ml and incubated for 1 h on ice. Protein G-agarose (Roche Molecular Biochemicals) was added, and the mix was incubated at 4 °C for an additional hour with constant agitation. Agarose pellets were subsequently pelleted and washed three times with 20 mM HEPES (pH 7.6), 155 mM NaCl, 20 mM glycerol, 0.2 mM EGTA, 1 mM MgCl2, 0.1% Nonidet P-40, and 0.1% bovine serum albumin. To detect proteins, washed pellets were heated to 80 °C for 10 min and electrophoresed by SDS-PAGE (7.5%); dried gels were exposed to a PhosphorImager screen (Molecular Dynamics) for 24–48 h. For TRAP assays following immunoprecipitations, protein G-agarose pellets were resuspended in a final volume of 5 μl with wash buffer, and 2 μl were removed for TRAP assay as described. The “double immunoprecipitation” was done as above except that 4 μl of the slurry was taken out for traditional TRAP, and the remainder was subjected to extension of a 32P-labeled primer (from the TRAP-eze kit) for 60 min at room temperature under typical TRAP extension conditions. The samples were then reprecipitated using the residual antibody-protein G-agarose complex, washed extensively as above, and subjected immediately to the TRAP-eze assay without telomerase-mediated primer extension.

RESULTS

In Vitro Properties of C-terminal HA-tagged hTERT—hTERT was expressed using the RRL system with 2 constructs, pcDNA3.1-hTERT and pcDNA3.1-hTERT/HA3. For efficient immunoprecipitation and detection purposes, the latter has a triple hemagglutinin epitope tag at the C terminus of the expressed protein, which does not have the ability to elongate telomeres and extend the life span of normal human fibroblasts (18). To determine whether there are differences in expression and functionality in our in vitro reconstitution system, we tested the C-terminal tagged hTERT against hTERT without a C-terminal tag and found that both are expressed at consistent levels and have similar amounts of reconstituted telomerase activity (Fig. 1). In addition, both hTERT molecules appear to associate with the molecular chaperones hsp90 and p23 (data not shown) as previously described (13). Consistent with our prior experiments where reconstitution with additional RRL was used as a source of chaperone proteins, we observe an enhanced activity after adding RRL using the HA-tagged hTERT (Fig. 2), similar to what was observed for other hTERT molecules (13). Interestingly, our previous data suggested that we needed all five chaperone proteins (hsp90, p23, hsp70, p60, and hsp40/9d) to establish sufficiency for enhanced reconstructed activity (13). Fig. 2 shows that addition of only hsp90 and p23 to the minimal reconstitution mix results in an increase in activity, suggesting that these chaperones may be limiting in the overall assembly reaction. Thus, because C-terminally tagged hTERT has nearly identical properties in our in vitro assembly system to the hTERT without a C-terminal tag, the hTERT/HA3 protein was used for all subsequent experiments.

Association of hsp70 with hTERT—Other reverse transcriptase activities that associate with chaperone proteins do so in a transient fashion (19, 20); that is, hsp90/p23/hsp70 are transient, addition of hTR to the reconstitution system (data not shown). Consistent with our prior experiments where reconstitution with additional RRL was used as a source of chaperone proteins, we observe an enhanced activity after adding RRL using the HA-tagged hTERT (Fig. 2), similar to what was observed for other hTERT molecules (13). Interestingly, our previous data suggested that we needed all five chaperone proteins (hsp90, p23, hsp70, p60, and hsp40/9d) to establish sufficiency for enhanced reconstructed activity (13). Fig. 2 shows that addition of only hsp90 and p23 to the minimal reconstitution mix results in an increase in activity, suggesting that these chaperones may be limiting in the overall assembly reaction. Thus, because C-terminally tagged hTERT has nearly identical properties in our in vitro assembly system to the hTERT without a C-terminal tag, the hTERT/HA3 protein was used for all subsequent experiments.

In the absence of hormone, steroid hormone receptors associate with hsp90, p23, and hsp70 (16). We have previously established sufficiency for telomerase assembly components in that the hsp90 chaperone machinery can provide the same enhanced telomerase assembly/activity that addition of RRL does in our enhanced reconstitution assay (13). The five hsp90-related proteins that functionally associate with each human telomerase are hsp90, p23, hsp70, p60, and hsp40/9d. With this knowledge in hand, our goal was to determine the nature of the hsp70 requirement in telomerase assembly. We found that hsp70 bound specifically to hTERT in the absence of hTR.
but that the hsp70 binding is reduced once hTR is complexed with hTERT, suggesting more of a transient hsp70 association with telomerase (Fig. 3B).

Stable Association of hsp90 and p23, but Not hsp70, with Functional Telomerase Enzyme—The experiments in Fig. 3 suggest that hsp90 and p23 are present in telomerase complexes and that hsp70 is only transiently associated with hTERT rather than the assembled telomerase complex. However, this does not address whether hsp90 and p23 interact with active enzyme, only that they are associated with the hTERT-hTR complex. To more directly address this issue, we have devised a protocol for specific immunoprecipitation of proteins associated with functionally active telomerase by precipitating telomerase-extended products, a method we call double immunoprecipitation (Fig. 4A). This procedure involves the reconstitution of telomerase using our in vitro reconstitution system and standard immunoprecipitation of activity using antibodies directed against hsp90, p23, and hsp70, as well as control antibodies (the chaperonin TCP-1) (13). A portion of that precipitation is then assayed for telomerase activity using the TRAP assay, and the remainder of the reaction is subjected to telomerase-mediated extension of a telomerase substrate primer under normal TRAP assay conditions. These extended products are then reprecipitated and subjected to the PCR portion of the TRAP assay without additional extension time (Fig. 4A). In other words, the precipitated telomerase is allowed to extend, and the telomerase-extended products are reprecipitated using chaperone antibodies to determine which, if any, are associated with active enzyme.

As expected, p23 and hsp90 antibodies are capable of precipitating active telomerase, whereas the hsp70 antibody and the control antibodies, TCP-1 and an antibody specific for rat hsp90, do not pull down any telomerase activity (Fig. 4B, left panel). This confirms that although hsp70 associates with hTERT in the absence of hTR, the association is transient, as hsp70 antibodies are incapable of precipitating active telomerase.

Pretreatment of telomerase activity using the single immunoprecipitation approach alone does not prove that p23 and hsp90 remain associated with active telomerase, as assembly could occur during the extension step prior to the PCR amplification of telomerase-extended products. To rule out that possibility, the double immunoprecipitation shows that hsp90 and p23 appear to remain associated with functional enzyme in that they are capable of precipitating telomerase-extended products in our in vitro reconstitution system (Fig. 4B, right panel). Again, neither control nor hsp70 antibodies are able to precipitate active telomerase, a result that was expected based on the single immunoprecipitation. Interestingly, we consistently observe less precipitated telomerase activity using the

DISCUSSION

The hsp90 chaperone complex is currently the only known set of proteins that functionally interacts with telomerase to induce proper assembly of the enzyme. In addition to a direct interaction of hsp90 and p23 with telomerase, we show here that hsp70 specifically interacts with the catalytic subunit, hTERT, in the absence of template RNA, hTR, and that the hsp70/telomerase interaction appears transient. Other reverse transcriptases from viral origins associate with hsp70, hsp90, and p23 in a transient fashion, as well, although there is some evidence that the viral particle contains hsp90 and p23 (19, 20). However, what makes the interaction of the molecular chaperones with the telomerase reverse transcriptase more unique is the apparent stable association of hsp90 and p23 with functioning telomerase. Our working model suggests that although hsp90, p23, hsp70, and perhaps other chaperone proteins are associated with hTERT in its unassembled or inactive form, the assembled, fully functional telomerase molecule contains both p23 and hsp90 (Fig. 5). Although the exact stoichiometry is currently unknown, hsp90 is known to bind as a dimer to other chaperone targets (21).

The question remains: why would these chaperones be associated with already assembled telomerase? The answer may lie in the reverse transcription process of the telomerase enzyme. Telomerase utilizes its associated RNA component as a template for synthesis of telomeric DNA, and this replication elongates the 3′ overhang at the telomere, generally in 6-base increments, for conventional DNA polymerases to replicate further out on the linear chromosome. Thus, telomerase recognizes and elongates the telomere through association with the hTERT template region and then translocates to the next available position for hTR binding. It is this translocation step that may require additional “tweaking” of conformation of the assembled complex, which would be provided by the stably associated hsp90 and p23. One interesting caveat is the cellular

Fig. 3. hsp70 associates with hTERT in a transient fashion, whereas hsp90 and p23 appear stably associated. A, hTERT/HA3, was expressed in the Tnt system and immunoprecipitated with HA (positive control; αHA), hsp90 (αhsp90), or p23 (αp23) antibodies in the absence (−hTR) or presence (+hTR) of hTR. hTERT/HA3 protein was separated by SDS-PAGE and exposed to the PhosphorImager screen. B, similar to part A, hTERT/HA3 was immunoprecipitated in the absence or presence of hTR with αHA, p23 antibody (αp23), or hsp70 antibody (αhsp70) followed by SDS-PAGE.

Fig. 4. Stable association of hsp90 and p23 with active telomerase enzyme. A, a schematic representation of the single immunoprecipitation (left side) and the double immunoprecipitation (right side). The single precipitation of telomerase activity using chaperone antibodies involves standard conditions, followed by telomerase extension and the TRAP PCR from precipitated material. The double IP requires the initial precipitation, the telomerase-mediated extension while the precipitated material is on the beads, followed by a second precipitation using the chaperone antibodies of telomerase-extended products. The products precipitated in the second round are immediately subjected to the TRAP PCR without telomerase extension. B, immunoprecipitations of telomerase activity using chaperone antibodies. The single IP (left panel; IP only) and the double IP (right panel; IP, extension, IP) were done using p23 (αp23), hsp90 (αhsp90), hsp90 (specific for rat hsp90; αhsp90-rat), hsp70 (αhsp70), and TCP-1 (αTCP-1) antibodies. Precipitated products were PCR-amplified using the TRAP assay and electrophoresed on 10% gels. The arrow indicates a 36-bp internal control band.
The model represented indicates the stable versus transient association of the molecular chaperones associated with telomerase. In the absence of template RNA (hTR), the catalytic subunit of telomerase (hTERT) is complexed to a variety of proteins, including hsp90, p23, and hsp70, as well as other as yet unidentified proteins. hTR has an 11-nucleotide template region (boxed) that serves to associate with the telomere. The model suggests that the hsp90 chaperone complex serves to recruit hTR to hTERT to form an active enzyme. Our data indicate that hsp90 and p23 remain associated with the active telomerase, whereas hsp70 is only associated with hTERT in its inactive form (in the absence of hTR).

Clearly, there are additional steps in the association process that occur prior to the inactive form shown in Fig. 5 and may include an ordered assembly with certain chaperone components. Many other chaperone targets, including steroid hormone receptors, require the ability of hsp70 to provide energy to the hsp90:p23 portion of the reaction (16, 22, 23), a process that occurs prior to the hsp90:p23 association and requires ATP hydrolysis. Our in vitro assembly/reconstitution system will be useful in defining the kinetics and order of chaperone-mediated telomerase assembly, which is critical for understanding telomerase function.

Because telomerase is associated with the vast majority of human malignancies (24), inhibition of telomerase activity may be an attractive molecular target for specific cancer therapy. In fact, telomerase activity has been inhibited by a variety of molecular methods, and the result of that inhibition has been either a delayed senescence phenotype or programmed cell death (apoptosis). Use of dominant-negative hTERT mutants, which contain a mutation in one of the reverse transcriptase motifs rendering the expressed protein devoid of activity, has shown an immediate down-regulation of telomerase activity in tumor-derived cells, followed closely by apoptosis (25, 26). Given that the dominant-negative hTERT mutant is overexpressed in these eukaryotic systems, one of the mechanisms for telomerase inhibition may be the preferential association of molecular chaperones to the overexpressed mutant rather than the wild-type hTERT, mainly because of the increased number of mutant molecules. This inhibition would prevent the assembly and function of wild-type telomerase, leading to a reduction in activity and ultimately senescence or apoptosis.

Understanding the molecular events leading to telomerase assembly and function are important in designing effective therapeutic strategies directed against telomerase and its interfering/modulating proteins. The hsp90 chaperone complex has been touted as a potential anti-cancer target, with certain ansamycin compounds (geldanamycin, radicicol, and herbimycin A) capable of interacting with hsp90 to reduce the association with ATP and p23, thereby blocking its function (21). One obvious problem with this approach is the nonspecific toxicity associated with such therapy, as hsp90 function is required for both cancer and normal cell function. Recently, there have been additional geldanamycin analogs that have shown promise in selectively inhibiting growth of breast cancer cells (27). Using the knowledge of the chaperone/telomerase interaction may allow for directed therapy against the assembly of functional telomerase, which may be useful as a potential adjuvant chemotherapy in the treatment of many types of human cancers.

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