Characterization of Interactions between PinX1 and Human Telomerase Subunits hTERT and hTR*

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The addition of telomeric repeats to chromosome ends by the enzyme telomerase is a highly orchestrated process. Although much is known regarding telomerase catalytic activity in vitro, less is known about how this activity is regulated in vivo to ensure proper telomere elongation. One protein that appears to be involved in negatively regulating telomerase function in vitro is PinX1 because overexpression of PinX1 inhibits telomerase activity and causes telomere shortening. To understand the nature of this repression, we characterized the interactions among PinX1 and the core components of telomerase, the human telomerase reverse transcriptase (hTERT) and associated human telomerase RNA (hTR). We now show that in vitro PinX1 binds directly to the hTERT protein subunit, primarily to the hTR-binding domain, as well as to the hTR subunit. However, in a cellular context, the association of PinX1 with hTR is dependent on the presence of hTERT. Taken together, we suggest that PinX1 represses telomerase activity in vivo by binding to the assembled hTERT-hTR complex.

Given the linear organization of eukaryotic chromosomes and the inherent inability of DNA polymerases to replicate completely the 3′ end of a parental strand of DNA (1, 2), most eukaryotes use the specialized enzyme telomerase to restore repetitive sequences to terminal telomere regions that define chromosome ends (3). Telomerase is a RNA-directed DNA polymerase, which carries its own template for the synthesis of telomeric repeats (4). In humans, the catalytic subunit hTERT is a reverse transcriptase (RT)† that copies the accompanying hTR RNA template sequence onto chromosome ends to maintain telomeres. The regulation of telomerase has now been completely the 3′ and the inherent inability of DNA polymerases to replicate in vivo to ensure proper telomere elongation. One protein that appears to be involved in negatively regulating telomerase function in vitro is PinX1 because overexpression of PinX1 inhibits telomerase activity and causes telomere shortening. To understand the nature of this repression, we characterized the interactions among PinX1 and the core components of telomerase, the human telomerase reverse transcriptase (hTERT) and associated human telomerase RNA (hTR). We now show that in vitro PinX1 binds directly to the hTERT protein subunit, primarily to the hTR-binding domain, as well as to the hTR subunit. However, in a cellular context, the association of PinX1 with hTR is dependent on the presence of hTERT. Taken together, we suggest that PinX1 represses telomerase activity in vivo by binding to the assembled hTERT-hTR complex.

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EXPERIMENTAL PROCEDURES

Plasmids—The constructs used to make proteins in vitro were as follows: FLAG-hTERT-FLAG in pCI-neo and fragments FLAG-hTERT (+1–183) in pCDNA3, FLAG-hTERT (+170–546) in pCDNA3, FLAG-hTERT (+523–924) in pCDNA3, myc-hTERT (+915–1132) in pCI-neo, FLAG-hTERT (+1–325) in pCI-neo, FLAG-hTERT (+326–620) in pCI-neo, and FLAG-hTERT (+621–1132) in pCI-neo. FLAG-HDAC1 in pCMV (a gift from T.-S. Yao) was used as a negative control. All open reading frames were downstream of a T7 promoter. For GST fusions, PinX1 (a gift from K. P. Lu) was cloned into pGEX-4T3 with a linker of ~20 amino acids upstream of the initiating methionine for PinX1. FLAG-PinX1 (+2–252) and FLAG-PinX1 (+253–328) were cloned in-frame
PinX1 Interacts with Human Telomerase Subunits hTERT and hTR

51746

with GST in the pGEX-4T1 vector. pGEX-4T1 was used as a negative control for GST expression. FLAG-PinX1 and FLAG-b74PARG (a gift from G. Poirier) were cloned into pBabePuro for retroviral use.

**GST-PinX1-hTERT Co-immunoprecipitation**—GST protein lysates were prepared in a lysis buffer consisting of phosphate-buffered saline supplemented with 1% Triton X-100, 1.5 mM dithiothreitol, 0.1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. For immunoprecipitation, approximately 1 mg of bacterial protein lysate was incubated with 15 μl of GammaBind G-Sepharose (Amersham Biosciences) and 1.5 μg of Z-5 anti-GST antibody (Santa Cruz Biotechnology) in lysis buffer (described above) supplemented with 0.1 mM phenylmethylsulfonyl fluoride and incubated overnight at 4 °C. Proteins captured on beads were then washed with 1 ml of lysis buffer and incubated for 1 h at room temperature with [35S]methionine-labeled proteins produced using the T7 quick-coupled Tnt system (Promega) in the presence of lysis buffer supplemented with blocking agents (100 ng/ml bovine serum albumin, 100 ng/ml casein, 100 ng/ml tRNA, and 100 ng/ml glycogen). Amounts of TnT proteins were adjusted in some cases to approximately normalize the amounts of specifically labeled protein that were added. Beads were washed three times with 1 ml of chilled lysis buffer and heated in SDS buffer. Proteins were resolved by SDS-PAGE and visualized by autoradiography.

**GST-PinX1-hTR Pulldowns**—RNAs were produced in vitro and 32P-labeled with the T7-coupled Maxiscript kit (Ambion) using 1 μg of linearized pBluescriptSK-hTR or 1 μg of linearized pBluescriptSK-U6 (a gift from M. Garcia-Blanco). Unincorporated nucleotides were removed using a G-25 minispin column (Amersham Biosciences). RNA was incubated with ~1 mg of bacterial protein lysate in lysis buffer supplemented with blocking agents (described above) in addition to 200 units of RNasin (Promega) and 15 μl of glutathione-Sepharose (Amersham Biosciences) for 1 h at room temperature. Beads were washed and prepared as described above. RNA and proteins were resolved by SDS-PAGE. Proteins were visualized by Coomassie Blue stain, and RNA was visualized by autoradiography.

**Immunoprecipitation RT-PCR**—Using protocols described previously (7, 27), HT-1080 or HA5 cells were infected with retroviruses derived from pBabePuro constructs to stably express FLAG-PinX1 and FLAG-PARG alone or in combination with hTERT, which was integrated stably by the use of retroviral infection using pBabeHygro. Cell lysates were prepared in a phosphate-buffered saline buffer containing 5 mM EDTA, 10% glycerol, 2% Nonidet P-40, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM benzamidine, 0.1 mM phenylmethylsulfox-
PinX1 Interacts with Human Telomerase Subunits hTERT and hTR

RESULTS AND DISCUSSION

PinX1 Interacts with an N-terminal Region of hTERT—To determine the functional region of hTERT involved in binding PinX1, we analyzed interactions between PinX1 and domains of hTERT in vitro. hTERT or regions thereof were produced in rabbit reticulocyte lysate (RRL) and tested for interactions with GST-PinX1 from a bacterial lysate. As a control, we incubated PinX1 with GST alone, although the best interaction occurred with the RNA-binding domain fragment (Fig. 2C), as determined by the intensity of the co-immunoprecipitated signal in comparison with the input. This interaction did not rely on hTR or the ability of hTERT to bind hTR because the absence of this subunit or a mutation to the hTR-binding domain of hTERT did not abrogate PinX1 binding. In fact, hTERT was immunoprecipitated readily with hTERT in the presence of PinX1, suggesting that PinX1 does not impede this interaction (although the hTR detected could also be the result of an association with PinX1, which interacts with hTERT (data not shown) (see below)). These data are in agreement with recent studies (24) of the yeast PinX1 protein Gnp1p, which was found to bind the homologous regions CP, QFP, and T of the yeast catalytic subunit that analogously are known to interact with the TLC-1 telomerase RNA subunit.

PinX1 Binds Directly to hTR—Because the RNA-binding domain of hTERT mediated interactions with the telomerase inhibitory protein PinX1 in vitro, we speculated that the mechanism of PinX1 action might involve the RNA component of telomerase. To determine whether PinX1 could interact with hTR, GST-PinX1 was incubated with in vitro transcribed 32P-labeled hTR and purified by glutathione-Sepharose beads. Complexes were resolved using SDS-PAGE to detect radiolabeled RNA by autoradiography. We found that PinX1 was capable of binding to hTR, thereby defining a novel activity for PinX1. To determine the selectivity of this interaction, nonspecific binding was assessed with the irrelevant structural RNA involved in catalysis, consistent with the direct inhibitory effect of PinX1 on telomerase catalytic activity.

The two hTERT fragments found to interact with PinX1 overlapped with the hTERT RNA-binding domain composed of N-terminal domains II and III and the T motif (amino acid fragment 326–620). To determine whether the RNA-binding region of hTERT bound PinX1, fragment 326–620 and flanking region fragments 1–325 and 621–1132 were assayed for PinX1 binding. All three fragments bound preferentially to GST-PinX1 in comparison with GST alone, although the best interaction occurred with the RNA-binding domain fragment (Fig. 2C), as determined by the intensity of the co-immunoprecipitated signal in comparison with the input. This interaction did not rely on hTR or the ability of hTERT to bind hTR because the absence of this subunit or a mutation to the hTR-binding domain of hTERT did not abrogate PinX1 binding. In fact, hTERT was immunoprecipitated readily with hTERT in the presence of PinX1, suggesting that PinX1 does not impede this interaction (although the hTR detected could also be the result of an association with PinX1, which interacts with hTERT (data not shown) (see below)). These data are in agreement with recent studies (24) of the yeast PinX1 protein Gnp1p, which was found to bind the homologous regions CP, QFP, and T of the yeast catalytic subunit that analogously are known to interact with the TLC-1 telomerase RNA subunit.

PinX1 Interacts with Human Telomerase Subunits hTERT and hTR

Fig. 3. PinX1 binds selectively to the hTR RNA template subunit of telomerase in vitro. GST-PinX1 (GST-PinX1 pulldown) and N- (N-term) and C-terminal (C-term) portions thereof were assayed for the ability to bind hTR. Bacterially produced proteins were incubated with in vitro transcribed 32P-labeled hTR or the nonspecific U6 RNA. Protein-RNA complexes were subjected to GST pulldown and resolved by SDS-PAGE. Proteins and RNA were visualized by Coomassie Blue stain followed by autoradiography. Input RNA corresponding to 1/1000 of the input is shown in the left panel at an increased exposure relative to the pulldown products. Of note, RNA complexed with proteins demonstrated a decreased mobility that is indicated by an upward shift in some experiments.

Fig. 4. PinX1 binds hTR in a cellular context. Extracts from telomerase-positive (+) HT-1080 cells, telomerase-negative (−) HA5 human embryonic kidney cells, and HA5 cells ectopically co-expressing hTERT and FLAG-PinX1 or as a control FLAG-PARG were immunoprecipitated (IP) using an anti-FLAG antibody. Immunoprecipitated products were visualized by Western blot or analyzed for hTR and nonspecific GAPDH RNA transcript by RT-PCR. As a positive control for RNA transcripts, 2 μg of total RNA from HT-1080 cells was used for first strand synthesis. In the case of the hTR-positive control, one-sixth of the PCR product was loaded with respect to experimental samples.
U6 and the GST protein alone. The selectivity of this interaction was demonstrated by the inability of hTR to interact with GST and the lack of interaction between GST-PinX1 and the structural RNA U6 (Fig. 3).

In yeast, Gnop1 has additional cellular roles in RNA processing, specifically in the maturation of rRNA and small nuclear RNAs. The G-patch motif of this protein, a putative protein-RNA interaction domain, is known to play a key role in this RNA processing function. However, the effect of human PinX1 on telomerase appears to be exclusive of the G-patch region and is mediated instead by the C terminus of the protein (17). This suggests that human PinX1 may have functionally separable cellular effects in which the N terminus is involved in RNA processing via the G-patch, and the C terminus is involved in telomere dynamics. To determine the significance of hTR binding to PinX1 in the context of these two cellular functions, the N terminus of PinX1 and the C-terminal telomerase inhibitory domain of PinX1 characterized previously (17) (consisting of amino acid fragments +2–252 and +235—328, respectively) were constructed as GST fusion proteins and assayed for their ability to bind hTR. Nearly identical PinX1 domains constructed as GST fusions retain the ability to bind hTERT, supporting the notion that these represent functional regions of the protein (17). The C terminus of PinX1 bound hTR at levels comparable with the wild-type protein, whereas the N-terminal region was largely unable to bind this RNA (Fig. 3). This suggests that the interaction with hTR cannot be ascribed to a general RNA binding feature of PinX1 mediated through the G-patch. Instead, these findings demonstrate that hTR binding is correlated with the repressive function of PinX1 on hTERT.

PinX1 Binds hTR in Vivo in Telomerase-positive Cells—To determine whether the PinX1-hTR interaction that was characterized in vitro occurred in a cellular context, we ectopically expressed epitope-tagged PinX1 in HT-1080 cells and performed immunoprecipitations to assess the association with endogenous hTR. We found that PinX1 immunoprecipitates contained the endogenous hTR telomerase RNA as detected by RT-PCR of immunoprecipitated material (Fig. 4). We confirmed that this amplified product was not a result of DNA contamination by performing duplicate reactions that lacked reverse transcriptase (data not shown). Finally, we demonstrated that this association was specific because immunoprecipitates containing hTR did not contain significant levels of nonspecific GAPDH RNA, nor did the nonspecific protein PARG co-immunoprecipitate hTR.

Because PinX1 was shown previously (17) to interact with hTERT in human cells, we wished to determine whether PinX1 binds directly to hTR in a cellular context or whether this immunoprecipitated RNA was a result of an interaction with the telomerase RNA-protein complex. In the transformed HA5 human embryonic kidney cell line, which lacks hTERT but retains hTR expression (30), PinX1 did not immunoprecipitate hTR as it did in the HT-1080 cell line, which contains active telomerase (Fig. 4). Thus, we propose that although PinX1 has the intrinsic ability to bind hTR, it does not interact with free hTR in a cellular environment in the absence of hTERT. Indeed, ectopic expression of hTERT in the same HA5 cell line restored the association of PinX1 and hTR, indicating that in a cellular context PinX1 binds hTR via hTERT.

A Model for PinX1-mediated Telomerase Repression by PinX1—In the budding yeast, the PinX1 orthologue Gnop1 appears to inhibit telomerase biogenesis by sequestering the uncomplexed TERT protein (Est2p) and preventing its association with the telomerase template RNA (TLC1) (24), consistent with the localization of this protein to the nucleolus (25) where telomerase assembly is believed to occur. On the other hand, in humans, PinX1 1) inhibits the activity of presumably already assembled telomerase complexes in vitro (17) and does not decrease the amount of hTR immunoprecipitated with hTERT; 2) binds not only to hTERT, as in yeast, but also to the hTR subunit; and 3) associates with hTR in cells only in the presence of hTERT. Thus, we propose that in higher eukaryotes, PinX1 may inhibit telomerase activity by also binding to an assembled hTERT-hTR complex. Given the fact that PinX1 also associates with telomeres via binding to TRF1, we further speculate that this inhibition could occur even at the telomeres, perhaps as a means to fine-tune telomerase-dependent telomere elongation. Further studies aimed at understanding the nature of PinX1 repression of telomerase in distinct subcellular pools will be instrumental in elucidating the dynamic regulation of the telomere structure in vivo.

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