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DNA-Protein Kinase Catalytic Subunit-interacting Protein KIP Binds Telomerase by Interacting with Human Telomerase Reverse Transcriptase*

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Telomere homeostasis, a process that is essential for continued cell proliferation and genomic stability, is regulated by endogenous telomerase and a collection of associated proteins. In this paper, a protein called KIP (previously reported as a protein that binds specifically to DNA-dependent protein kinase), has been identified as a telomerase-regulating activity based on the following pieces of evidence. First, complexes between KIP and the catalytic subunit of telomerase (hTERT) were identified using the yeast two-hybrid technique. Second, antibodies specific to KIP immunoprecipitate human telomerase in cell-free extracts. Third, immunolocalization experiments demonstrate that KIP is a nuclear protein that co-localizes with hTERT in cells. Fourth, KIP binds to hTERT both in vitro and in vivo in the absence of human telomerase RNA or telomeric DNA, thus defining the catalytic subunit of telomerase as the site of physical interaction. Fifth, co-immunoprecipitation experiments suggest that KIP-hTERT complexes form readily in cells and that overexpression of KIP in telomerase-positive cells increases endogenous telomerase activity. Finally, continued overexpression of KIP (60 population doublings) resulted in cells with elongated telomeres; thus, KIP directly or indirectly stimulates telomerase activity through hTERT and contributes to telomere lengthening. The collective data in this paper suggest that KIP plays a positive role in telomere length maintenance and/or regulation and may represent a novel target for anti-cancer drug development.

Telomeres are essential and functional components of the physical ends of eukaryotic chromosomes. They are composed of a repetitive DNA sequence (TTAGGG in vertebrates) and associated proteins (1–3). In the absence of functional telomere maintenance pathways, dividing cells show a progressive loss of telomeric DNA during successive rounds of replication due to a DNA end replication problem (4); thus, telomere shortening has been proposed as a ticketing mechanism that controls the replicative capacity of cells and cellular senescence (5). Cells with extended replicative life spans have mechanisms to counteract the loss of telomeric DNA. In some immortalized cells, for example, telomere shortening is alleviated by telomerase, a ribonucleoprotein enzyme that adds short repetitive telomeric sequences to the 3′-chromosomal ends by reverse transcription (6, 7).

Telomerase activity is expressed in a majority of human tumors but not in normal somatic cells (8). The introduction of the telomerase catalytic subunit gene into normal somatic cells prevents telomere erosion and senescence and extends their proliferative life span (9). Conversely, the inhibition of the telomerase activity results in telomere shortening and subsequent growth arrest of cancer cells followed by senescence or cell death (10, 11). Genetic disruption of the telomerase RNA component also causes telomere erosion and chromosomal aberrations, resulting in functional defects in organs containing highly proliferative cells (12, 13). These observations indicate that telomerase activity is necessary for the proliferation of primary and transformed cells and that the activation of telomerase may be a pivotal step in human carcinogenesis.

The human telomerase complex contains a catalytic subunit of reverse transcriptase (hTERT)1 and an integral RNA (hTR). Both hTERT and hTR are essential for assembly of functional telomerase activity in vitro and in vivo (6, 7). In human cells, several proteins have been identified that associate with telomerase. The chaperone proteins p23 and Hsp90 associate with hTERT and are involved in the assembly of telomerase activity (14). Telomerase-associated protein 1 interacts with both hTERT and hTR but is apparently not required for telomerase activity (15–17). The 14-3-3 signaling protein is required for efficient accumulation of hTERT in the nucleus (18). PinX1, identified as the Pin2-terminal restriction fragment 1 (TRF1)-interacting partner, is a potent catalytic inhibitor of telomerase and binds directly to hTERT (19). Recently, the Ku complex was shown to associate with telomerase through interaction with hTERT and this interaction may regulate telomerase access to telomeric DNA ends (20). Because native human telomerase has an estimated mass of 1,000 kDa, other as yet unidentified hTERT-associated proteins are likely to be present and physiologically relevant to telomere transactions.

In a search for proteins capable of interacting with human telomerase, we identified KIP (a DNA-PKcs-interacting protein) as an hTERT-interacting protein using the yeast two-

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1 The abbreviations used are: hTERT, human telomerase reverse transcriptase; DNA-PKcs, DNA protein kinase catalytic subunit; hTR, human telomerase RNA; KIP, DNA-PKcs-interacting protein; TRAP, telomeric repeat amplification protocol; TRF, terminal restriction fragment; GST, glutathione S-transferase; HA, hemagglutinin.
hybrid screening assay. GST pull-down and immunoprecipitation analyses reveal that KIP interacts with telomerase by binding to the catalytic subunit hTERT both in vitro and in vivo. KIP appears to function as a positive modulator in telomere homeostasis.

EXPERIMENTAL PROCEDURES

Expression Vectors—The KIP-V5 expression vector was constructed by inserting the EcoRI and XhoI fragments from the full-length KIP cDNA (generated by PCR with the appropriate synthetic primers) into pCDNA3.1/V5-His (Invitrogen). The hTERT-HA expression vector (generously provided by H. Seimiya, Japanese Foundation for Cancer Research) was constructed by cloning the full-length hTERT cDNA into pCR3 vector (18). The expression vector for GST-KIP was constructed by cloning the EcoRI and XhoI fragments from the full-length KIP cDNA into pGEX-5X-1 (Amersham Biosciences). The GST fusion protein was expressed and purified by glutathione-Sepharose column chromatography according to the manufacturer’s instructions.

Yeast Two-hybrid Screening—Yeast two-hybrid screening was performed as described previously (21). The yeast strain EGY48 harboring pLexA-hTERT and pSH18-34 was transformed by the lithium acetate method with a HeLa cDNA library fused to the activation domain vector pB42AD (Clontech).

Cell Lines and Culture Conditions—The human lung carcinoma cell line H1299 and the human breast cancer cell line MCF7 were cultured in RPMI 1640 medium, and the human fibrovascular cell line HT1080 and human embryonic kidney cell line 293T were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO2 at 37 °C.

To establish cell lines stably expressing KIP, the KIP-V5 construct was transfected into MCF7 cells. As a control, MCF7 cells were also stably transfected with an empty vector. Multiple independent single clones were isolated and checked for protein expression by immunoblotting analysis with anti-KIP or anti-V5 antibodies.

Production of Antibodies—To raise antibodies against KIP, rabbits were immunized with recombinant KIP prepared by cleaving GST-KIP with Factor Xa followed by the removal of the cleaved GST and uncleaved GST-KIP with glutathione-Sepharose. Anti-KIP antibodies were affinity-purified on KIP coupled to CNBr-activated Sepharose 4B (Amersham Biosciences).

GST Precipitation, Immunoprecipitation, and Immunoblotting Analyses—The KIP-V5 and hTERT-HA expression vectors were transfected into H1299 cell line using LipofectAMINE (Invitrogen) according to the manufacturer’s protocol. Cells were suspended in lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 50 mM KCl, 0.01% Nonidet P-40, 20% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol), incubated on ice for 30 min, and sonicated at 50 W for 3 s pulses. Lysates were then centrifuged at 12,000 rpm for 30 min to remove insoluble material. For GST pull-down experiments, lysates were precleared with glutathione-Sepharose 4B and incubated with glutathione-Sepharose beads containing GST-KIP or control GST for 2 h at 4 °C. For immunoprecipitation, lysates were preincubated with protein G-Sepharose and incubated with primary antibodies precooled to 4 °C. The precipitated proteins were washed extensively and subjected to immunoblot analysis. Immunoprecipitation and immunoblot analyses were performed using anti-HA (Santa Cruz Biotechnology), anti-V5 (Invitrogen), anti-DNA-PKcs (NeoMarkers), and anti-KIP antibodies. Immunoprecipitation for telomerase repeat amplification protocol (TRAP) assays was performed using anti-KIP, anti-V5, anti-p53 (Santa Cruz Biotechnology), anti-TRF1 (Clontech), and anti-BTIP1 (rice telomere-binding protein) antibodies as primary antibodies.

Telomerase Assays—The TRAP was used as previously described (10). Immunoprecipitated proteins were added to telomerase extension reactions and incubated for 20 min at 37 °C. Reactions were stopped by heating at 94 °C for 90 s and placed on ice. PCR was performed using the TS primer and the ACX primer for 30 cycles (denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s). An internal telomerase assay standard, NT and TSNT primers were added to the PCR mixture as previously described (22). Telomerase products were resolved by electrophoresis on a 10% nondenaturing polyacrylamide gel. Bands were then visualized by staining with SYBR Green (Molecular Probes). The signal intensity was quantified with a LAS-1000 Plus Image analyzer (Fuji Film).

TRF Analysis—To measure the telomere length, genomic DNA was digested with RsaI and HinfI and separated on 0.7% agarose gel. DNA samples were transferred to a nylon membrane (Hybond N°, Amersham Biosciences) and hybridized with a 32P-labeled probe, (TTAGGG)15. Signals were quantified by phosphorimaging.

RESULTS

Identification of KIP as an hTERT-interacting Partner—To identify hTERT-interacting factors, we screened a HeLa cell cDNA library using the yeast two-hybrid system. The cDNA library was cloned into a vector (pB42AD) containing a transcriptional activation domain. We constructed a plasmid vector (pLexA/hTERT-(762–855)) that expressed a fusion protein with the DNA-binding domain of the LexA protein and hTERT reverse transcriptase motifs (amino acids 762–855, see Fig. 1A) (23, 24). Transformation of pLexA/hTERT-(762–855) alone (the “bait”) into yeast strain EGY48 did not activate lacZ transcription. Of ~3.5 × 109 colonies screened, 21 positive clones were obtained and sequenced, resulting in an isolation of eight independent clones encoding a gene subsequently identified as KIP. Full-length KIP did not result in transcriptional activation of the reporter gene when tested against the hTERT region spanning amino acids 856–944 or with a heterologous DNA-binding protein (Fig. 1B, TOP3). KIP was previously identified as a protein that binds specifically to the upstream kinase domain of DNA-PKcs (25). Additionally, KIP was reported as a calcium and integrin-binding protein (26). KIP displays significant homology to calcineurin B and calmodulin, which contain two EF-hand motifs that correspond to the calcium-binding domains. The mouse KIP cDNA encodes a protein 95% identical to the human protein (27).

To map the region in KIP that interacts with hTERT, yeast strain EGY48 was co-transformed with pLexA/hTERT-(762–855) and pB42AD plasmids containing various regions of KIP (Fig. 2A). Only full-length KIP was found to associate with hTERT based on yeast two-hybrid as an end point assay (Fig. 2B).

hTERT-KIP Binding in Vitro and in Vivo—To confirm the direct interaction between hTERT and KIP, we analyzed the binding of hTERT and KIP fused to glutathione S-transferase (GST). When H1299 cells were transfected with hTERT-HA expression construct and subjected to GST pull-down experiments, GST-KIP but not the control GST precipitated hTERT-HA from cell extracts (Fig. 3A). These results indicate that KIP interacts with hTERT in vitro. To further determine whether hTERT and KIP associate in vivo, H1299 cells were...
Fig. 2. Analysis of KIP deletion mutants and hTERT binding. A, schematic representation of KIP mutants compared with wild type full-length KIP (KIP-F). Open boxes represent the regions used in this analysis. Values in parentheses indicate the positions of amino acid residues. Locations of two EF hands are indicated by gray boxes. B, KIP mutants compared with wild type KIP-F were analyzed by the yeast two-hybrid assay. Unrelated topoisomerase III (TOP3) “prey” is used as a negative control. The blue signal and the yeast growth are described in the legend of Fig. 1, A, synthetic; G, galactose; D, glucose; H, histidine (+); W, tryptophan (+); U, uracil, (+); L, leucine (+); X, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

cotransfected with hTERT-HA and KIP-V5 expression constructs (Fig. 3B) and subjected to immunoprecipitation with anti-HA or anti-V5 antibodies followed by immunoblot analysis. KIP-V5 was detected in anti-HA immunoprecipitates only when both hTERT-HA and V5-KIP proteins were expressed. Likewise, hTERT-HA was recovered in anti-V5 immunoprecipitates, indicating that KIP interacts with hTERT in mammalian cells (Fig. 3C). Subcellular distribution of KIP and hTERT was examined in co-transfected H1299 cells by immunofluorescence analysis. Both proteins were clearly localized in the nucleus of the cell as expected. In addition, KIP appears to co-localize with hTERT in distinct subnuclear pockets of fluorescence (Fig. 3D).

KIP Association with Telomerase—Because KIP interacts with hTERT in vitro and in vivo, we next examined KIP binding to catalytically active telomerase. Cell lysates were prepared from 293T, HT1080, or H1299 cells, immunoprecipitated with a KIP-specific antibody, and analyzed for telomerase activity by the TRAP assay. As shown in Fig. 4, the anti-KIP antibody immunoprecipitated telomerase activity from the three telomerase-positive cell lines. We observed no telomerase activity when cell lysates were immunoprecipitated with control antibodies against TRF2, p53, and RTBP1 (28).

To explore the possibility that anti-KIP antibody may bind nonspecifically to telomerase, H1299 cells were transfected with the KIP-V5 expression construct (or the empty vector) and immunoprecipitated with anti-V5 antibody. Telomerase activity was detected in the immunoprecipitates from cells expressing KIP-V5 but not from the empty vector-expressing cells (Fig. 4D). Note that the KIP-V5-transfected cells express significantly higher levels of KIP relative to the endogenous KIP gene (see also Fig. 6A); therefore, the amount of telomerase activity recovered by anti-V5 antibody was reproducibly higher relative to that recovered using anti-KIP antibody in H1299 cells expressing the endogenous gene (compare Fig. 4D, lane 3, with Fig. 4C, lane 3). Taken together, these results indicate that KIP associates physically with telomerase through interaction with hTERT in vitro and that this association is probably specific.

Analysis of DNA-PKcs Complexes with KIP and hTERT—As noted above, KIP was originally identified as a DNA-PKcs-interacting protein using a two-hybrid screening (25). To explore the interaction between KIP and DNA-PKcs in mammalian cells, H1299 cells were co-transfected with KIP-V5 and hTERT-HA and analyzed for interaction with DNA-PKcs. Fig. 5A shows a control experiment documenting ectopic expression of KIP-V5 and hTERT-HA in these cells along with endogenous DNA-PKcs expression. We tested whether anti-DNA-PKcs antibody would precipitate KIP-V5 and/or hTERT-HA. KIP-V5 was detected in anti-DNA-PKcs immunoprecipitates from KIP-V5-transfected cells but not from the empty vector-transfected cells (Fig. 5B, top panel, lanes 4 and 5), indicating that KIP interacts with DNA-PKcs in mammalian cells. An analysis of DNA-PKcs immunoprecipitates in cells expressing both KIP-V5 and hTERT-HA revealed the presence of KIP-V5 (Fig. 5B, top panel, lane 2); however, DNA-PKcs pull-down experiments of this type did not recover detectable levels of hTERT-HA (Fig. 5B, bottom panel, lane 2). We also observed that DNA-PKcs immunoprecipitates contained much less KIP-V5 in cells co-transfected with both hTERT-HA and KIP-V5 relative to cells expressing only KIP-V5 (Fig. 5B, top panel, compare lanes 2 and 4). To further examine the binding of hTERT and DNA-PKcs to KIP, we expressed both KIP-V5 and hTERT-HA in H1299 cells and performed immunoprecipitations with anti-V5 or anti-HA antibodies. As shown in Fig. 5C, DNA-PKcs was detected in anti-V5 immunoprecipitates from cells expressing KIP-V5 (lanes 2 and 4). As in Fig. 5B, the amount of DNA-PKcs recovered by immunoprecipitation was reduced in cells expressing both hTERT-HA and KIP-V5 compared with cells expressing only KIP-V5 (Fig. 5C, compare...
lanes 2 and 4). DNA-PKcs was not detected in anti-HA immunoprecipitates from cells expressing hTERT-HA (Fig. 5C, lanes 6 and 7). These data are consistent with the idea that KIP interacts specifically with DNA-PKcs (25); however, DNA-PKcs does not appear to form detectable and/or stable complexes with soluble hTERT.

**Overexpression of KIP in MCF7 Cells Increases Telomerase Activity**—Because KIP associates physically with telomerase in vivo, we examined whether KIP affects telomerase activity. We established MCF7 cell lines stably expressing KIP-V5 (or an empty vector negative control). Levels of endogenous and ectopically expressed KIP proteins were examined by immunoblotting using anti-KIP and anti-V5 antibodies (Fig. 6A). As noted above, the ectopic expression of KIP-V5 was ~10-fold greater than the endogenous KIP gene. Cells expressing KIP-V5 and the empty vector grew normally and exhibited no...
detectable differences in growth rates or morphology over 60 population doublings. After 60 population doubling, we compared telomerase activity in stable cell lines expressing KIP-V5 and the empty vector. Telomerase activity in KIP-V5 cells was increased by roughly 2-fold relative to the control cell lines (Fig. 6, B and C). These results suggest that KIP acts as a positive regulator of telomerase; however, it is not clear whether an increase in telomerase activity by KIP was due to the direct effect of telomerase enzyme per se or to a KIP-related increase in the expression of hTERT or hTR genes. To address this issue, the impact of KIP on gene expression of hTERT and hTR was evaluated (29). We observed no significant differences in steady-state levels of hTERT mRNA or hTR transcripts in cell lines expressing KIP-V5 or the empty vector (Fig. 6D).

Overexpression of KIP in MCF7 Cells Increases Telomere Length—To examine whether a KIP-mediated increase in telomerase activity was caused by changes in telomere lengths, we performed a TRF analysis. Control cells (MCF7 cells with or without vector) exhibited an average telomere length of roughly 5 kb with most fragments spread over a range of ~3–8 kb (Fig. 6E). Telomeres were clearly elongated in the clones expressing KIP-V5 with an increase in TRF length to ~10 kb or more and spanning down to roughly 5 kb. These findings suggest that KIP exerts a positive role in telomere maintenance and/or regulation.

**DISCUSSION**

KIP interacts with hTERT both in vitro and in vivo and is found to associate with the telomerase complex. Overexpression of KIP in telomerase-positive cells stimulates telomerase activity. The possibility that KIP stimulates hTERT or hTR gene expression, thereby leading to elevated levels of endogenous telomerase, was ruled out by showing that steady-state levels of mRNA were not altered by KIP overexpression. A direct or indirect consequence of high level KIP expression is the longer average telomere lengths. Because KIP is a nuclear protein that co-localizes with hTERT, our collective results strongly suggest that KIP positively regulates telomerase and telomere length.

A number of factors have been identified that associate with mammalian telomeres and telomerase. PinX1, identified as a Pn2-TRF1-binding protein, interacts with hTERT and directly inhibits telomerase activity (19). The expression of PinX1 results in progressive telomere shortening following by cellular senescence. It is conceivable that KIP and PinX1 are counteracting proteins that set to regulate an equilibrium level of telomerase activity required for the replicative capacity of cells. Although KIP was first identified as a novel DNA-PKcs-interacting protein (25), little is known regarding the physiological role of this interplay. KIP is expressed ubiquitously in many tissues in the embryo (25, 27), suggesting a role in development. Telomerase activity is necessary for the continuous proliferation of many tumor cells, and because KIP appears to positively influence telomerase and telomere length, this protein may represent a novel drug target in cancer. Moreover, its role in cellular senescence is also relevant to our understanding of aging. For example, improper expression of KIP could elevate or repress telomerase activity in cells. It is also possible that KIP-hTERT interactions could be altered by the presence (and amount) of other KIP-interactive partners, thereby titrating telomerase activity up or down. Because conserved KIP homologs are present in other eukaryotes (25), KIP might represent a general mechanism for regulating telomerase activity.

We demonstrate that overexpression of KIP in telomerase-positive MCF7 cells increases telomerase activity and telomere length. We also expressed the KIP cDNA in an antisense orientation to deplete the endogenous protein. Among the clones that displayed reduced KIP expression, we observed unambiguous decreases in TRF length (data not shown). Unfortunately, these clones were unstable and, for this reason, were not included in the current study; however, these findings underscore the notion that the KIP concentration is important to the cell in maintaining telomere lengths in vivo. The critical question that remains to be answered is how KIP regulates telomerase activity and telomere length in vivo. We do not have any evidence that hTERT binds directly to DNA-PKcs, and we did not detect formation of a ternary complex (KIP/DNA-PKcs/hTERT). Detection of these sorts of soluble complexes should be feasible using our experimental approach; however, the absence of evidence does not mean that such complexes do not exist (or they may exist only in a chromatin context). The data in Fig. 5B further suggest that KIP binding to DNA-PKcs is somehow influenced by the presence of hTERT. It is possible, for example, that KIP can shuttle between the DNA-PK complex and telomerase. Differential binding of KIP in this case suggests a mechanism to regulate the positive action of KIP on telomerase as noted above. Alternatively, KIP may simply play multifunctional roles in diverse or intersecting pathways in the cell, because it binds other proteins as well (26).

It was recently reported that DNA-PKcs deficiency severely disrupts telomere “capping” without affecting telomere length, suggesting that DNA-PKcs plays a critical role in capping (30,
DNA length by KIP and a competition based binding model is highly involved in the regulation of telomerase activity and telomere of telomeres as seen when KIP is overexpressed in cell lines. The prospect that low DNA-PKcs levels promote higher KIP-ently (or not at all) with KIP, thereby leading to alterations in severe combined immunodeficiency mice might interact differently (or not at all) with KIP, suggesting their essential roles in telomere function (33, 39, 40); however, the precise mechanism of their action is unclear. Although this report is the first to show that KIP associates with DNA-PKcs with telomerase and, as noted above, our data do not rule out the possibility that DNA-PKcs associates with telomerase via KIP (but only in a chromatin context, for example). Although this report is the first to show that KIP associates physically with telomerase, many questions remain regarding the physiological role of KIP-telomerase interaction and how such a complex might be regulated if at all. Finally, the subtle details on the possible interplay between KIP and DNA-PKcs in the presence and absence of hTERT should lead to a better understanding of how KIP is involved in telomere biology during development, aging, and cancer.

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