The human telomerase RNA component, hTR, activates the DNA-dependent protein kinase to phosphorylate heterogeneous nuclear ribonucleoprotein A1

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ABSTRACT

Telomere integrity in human cells is maintained by the dynamic interplay between telomerase, telomere associated proteins, and DNA repair proteins. These interactions are vital to suppress DNA damage responses and unfavorable changes in chromosome dynamics. The DNA-dependent protein kinase (DNA-PK) is critical for this process. Cells deficient for functional DNA-PKcs show increased rates of telomere loss, accompanied by chromosomal fusions and translocations. Treatment of cells with specific DNA-PK kinase inhibitors leads to similar phenotypes. These observations indicate that the kinase activity of DNA-PK is required for its function at telomeres possibly through phosphorylation of essential proteins needed for telomere length maintenance. Here we show that the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) is a direct substrate for DNA-PK in vitro. Phosphorylation of hnRNP A1 is stimulated not only by the presence of DNA but also by the telomerase RNA component, hTR. Furthermore, we show that hnRNP A1 is phosphorylated in vivo in a DNA-PK-dependent manner and that this phosphorylation is greatly reduced in cell lines which lack hTR. These data are the first to report that hTR stimulates the kinase activity of DNA-PK toward a known telomere-associated protein, and may provide further insights into the function of DNA-PK at telomeres.

INTRODUCTION

Telomeres are tandem repeats of short DNA sequences (TTAGGG in humans) at the ends of linear chromosomes. In humans, the telomerase holoenzyme, minimally composed of the reverse transcriptase, hTERT, and an RNA component, hTR, is responsible for synthesizing telomeric repeats during DNA replication. hTERT utilizes hTR as a template to add the repeats onto the 3’ ends of chromosomes (1–3). In addition to its role as a polymerase, telomerase cooperates with members of the Shelterin protein complex to establish a protective nucleoprotein ‘cap’ for chromosome termini (4). Maintenance of this cap is necessary to protect telomeres from cellular DNA damage responses that can disrupt chromosome dynamics; this in turn can result in aneuploidy and/or aberrant fusion events that may result in cellular transformation. Paradoxically, many of the proteins found at the telomere are also critical for the repair of DNA double-strand breaks (DSBs). One such protein is the DNA-dependent protein kinase (DNA-PK).

DNA-PK is composed of a DNA-binding subunit, Ku70/80 and a catalytic subunit (DNA-PKcs). This holoenzyme is required for the repair of DSBs via the non-homologous end-joining (NHEJ) pathway (5,6). Current models for NHEJ propose that the Ku heterodimer binds to exposed ends of double stranded (ds)DNA and serves as the signal to recruit DNA-PKcs to establish the active DNA-bound DNA-PK complex. DNA-PK is a serine-threonine protein kinase that phosphorylates its substrates predominantly on serines or threonines that are followed by glutamine (SQ/TQ motifs) (5,7).

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In vitro DNA-PK substrates include p53, RPA, XRCC4, Ku and DNA-PKcs itself (5). Cells deficient for functional DNA-PKcs show high levels of chromosome end-to-end fusions due to chromosome uncapping and telomere dysfunction (8–10). Moreover, mouse cells deficient for both DNA-PKcs and Terc (mouse TR) exhibit accelerated rates of telomere shortening compared to cells solely deficient for Terc, suggesting a functional interaction between DNA-PKcs and telomerase in sustaining telomere length and function (11). Similarly, mouse cells lacking wild type Ku70 or Ku80 display a higher rate of chromosomal end-to-end fusions due to loss of telomere end-capping function, without any significant decrease in telomere length (8–10). However, in mouse cells that lack both DNA-PKcs and the telomerase RNA, both telomere shortening and telomere dysfunction are observed, in contrast to cells which lack only DNA-PKcs where telomere length is sustained (11). Additionally, in mouse cells, Ku70/80 associates with telomeric DNA (12), and interacts with the core proteins of the Shelterin complex, namely TRF1 and TRF2 (13,14). Moreover, Ku has been reported to associate with hTERT (15). Interestingly, treatment of mouse cells with specific DNA-PK inhibitors also leads to increased rates of end-to-end fusions (16). Together, these data support a role for DNA-PK in telomere function, both in end-capping and in maintenance of telomere length. However, the precise biochemical function and mechanism of action of DNA-PK at telomeres remains unknown (9,10).

We have previously shown that human Ku70/80 interacts with the human telomerase RNA component, hTR (17). Moreover, the interaction between Ku70/80 and telomerase RNA (TLC1) is also observed in yeast (18). Specifically, Saccharomyces cerevisiae strains harboring either TLC1 that lacks the stem loop region for yKu binding (Tcl1A48), or Ku alleles that do not interact with TLC1 (yKu80-135i), show decreased rates of telomere synthesis (18). The yKu70/80/TLC1 interaction is required for proper recruitment of yeast telomerase to the chromosome end for telomere synthesis in late S-phase (19). Yeast, however, do not contain DNA-PKcs, suggesting that the interaction and regulation of telomerase with DNA-PK in human cells is more complex.

It is becoming clear that phosphorylation regulates the function of many proteins that are involved in the regulation of telomere length. In yeast, phosphorylation of the single strand telomere binding protein Cdc13 by Tel1 and Mec1 (the yeast homologues of human ATM and ATR, respectively) modulates its interaction with Est1p and facilitates telomerase recruitment (20). In human cells, TRF2 is phosphorylated by ATM in response to DNA damage and the phosphorylated form of the protein does not interact with telomeric DNA (21). Similarly, TRF1 is phosphorylated by ATM in an Nbs1-dependent manner, promoting its release from the telomere (22). These data indicate that protein phosphorylation is a critical regulatory mechanism for proteins involved in telomere length maintenance. Since inhibition of the kinase activity of DNA-PK results in telomere dysfunction (16), we speculate that DNA-PK phosphorylates specific telomere or telomerase-associated proteins, thereby regulating their function at the telomere.

One interesting protein that has been shown to function in telomere length maintenance is hnRNP A1. hnRNP A1 is a member of the hnRNP A/B family. Members of the hnRNP family (including hnRNP A1) are involved in a variety of RNA-related processes such as alternative splicing, mRNA maturation/turnover, mRNA transport and telomere and telomerase regulation (23). The hnRNP A/B family includes hnRNP A1, A2 and A3, each of which is alternatively spliced. These proteins contain two N-terminal RNA recognition motifs (RRMs) and a glycine rich domain at the C-terminus (23). There is strong evidence that hnRNP A1 plays a critical role in telomere biogenesis. First, hnRNP A1 and its proteolytic fragment Unwinding Protein 1 (U1P) (24), bind telomeric DNA in a sequence specific manner in vitro (25). Second, a murine erythroleukemia cell line deficient for hnRNP A1 has shortened telomeres, and reintroduction of hnRNP A1 into these cells overcomes this phenotype (25). Third, a U1P containing complex assembles on telomeric DNA oligonucleotides in nuclear extracts and recombinant U1P interacts with telomerase in mammalian cell extracts (26). Fourth, the first RRM motif of hnRNP A1 interacts with telomeric DNA in vitro (26), while simultaneously the second RRM motif interacts with the telomerase RNA (27). And finally, it has been proposed that hnRNP A1 contributes to telomere elongation by unwinding G-quadruplexes that form during telomere elongation (28). This evidence suggests a critical function for hnRNP A1 in telomere length maintenance, possibly by facilitating recruitment of telomerase to chromosome ends or modulating higher order telomere structures.

hnRNP A1 is known to undergo a number of post-translational modifications, including sumoylation (29), methylation (30–33) and phosphorylation (34–37), and each modification is reported to affect its nucleic acid binding properties. Although phosphorylation of hnRNP A1 has been shown to affect the splicing activities of hnRNP A1, little is known about whether post-translational modifications are required for its role in telomere maintenance.

To further elucidate the biological significance of our previous observation that Ku interacts with hTR in human cells (17), we asked whether the interaction of Ku with hTR can establish an active DNA-PK holoenzyme. Here, we show that like DNA, hTR promotes phosphorylation of hnRNP A1 by DNA-PK in vitro. Moreover, hnRNP A1 interacts with Ku in a cellular context, and we have identified a novel phosphorylation site on hnRNP A1 that is targeted by DNA-PK in vitro. Furthermore, inhibition of DNA-PK reduced phosphorylation of hnRNP A1 in vivo and the phosphorylation of hnRNP A1 was greatly decreased in cells lacking hTR. To our knowledge, this is the first report indicating that hnRNP A1 is a direct DNA-PK substrate, and that the telomerase RNA, a specific structured and biologically active RNA molecule, can stimulate the protein kinase activity of DNA-PK.
MATERIALS AND METHODS

Kinase assays

DNA-PKcs and Ku70/80 were purified from HeLa cells as previously described (38). GST-hnRNP A1 and GST-hnRNP A2 were expressed in Escherichia coli BL21 strain and purified using glutathione-sepharose beads as previously described (27). GST-XRCC4 and GST-Artemis were expressed in bacteria and purified as described previously (38,39). All kinase reactions were performed with 0.1 μg of DNA-PKcs, 0.05 μg of Ku and 1.0 μg of protein substrate to be tested in the presence of 0.5 μg calf thymus (CT) DNA (or 0.5 μg of hTR or the indicated nucleic acids), 100 mM NaCl, 25 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA and 0.25 mM ATP containing 1 μCi ³²P-γ-ATP. Final reaction volumes were 20 μL. The reactions were incubated at 30°C for 10 min, stopped with SDS sample buffer and fractionated on SDS-PAGE. The gels were stained with Coomassie blue, dried and analyzed by autoradiography.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSA) studies with full-length hTR (FL-hTR) or an hTR fragment spanning nucleotides 404–451 were carried out as previously described (17). The amount of protein used in each experiment is described in the figure legends. For western blot analysis of the EMSA reactions, the non-denaturing gels were transferred onto PVDF membrane under the previously described conditions (17). The blots were analyzed for immunoprecipitation experiments (40). Approximately 13,000 rpm for 20 min at 4°C. The subsequent immune complexes were washed 3 times with CHAPS lysis buffer [100 mM NaCl, 10 mM Tris pH 7.5, 25% (w/v) glycerol, 1% CHAPS, 200 mM NaCl, 100 mM MgCl₂, 1 mM DTT, CompleteMini EDTA free protease inhibitors (Boehringer Mannheim), 1 mM NaF, 100 μM sodium orthovanadate, 20 μM β-glycerophosphate] for 30 min on ice. The extracts were then centrifuged at 13,000 rpm for 20 min at 4°C, to obtain whole cell extracts for immunoprecipitation experiments (40). Approximately 1 μg of each antibody was pre-coupled to 20 μl of a 50% slurry of protein G sepharose beads by incubating for 1 h at 4°C on a rotator. Whole cell extracts were pre-cleared with protein G sepharose and then added to the antibody-coated beads; the mixtures were then subjected to constant rotation for 4 h at 4°C. The subsequent immune complexes were washed 3 times with CHAPS lysis buffer and fractionated on SDS–PAGE for western analysis using the aforementioned antibodies.

In vivo phosphorylation

For in vivo labeling experiments in Figure 8A, HeLa cells cultured in DMEM containing 5% fetal calf serum (FCS) were incubated in phosphate-free DMEM (Invitrogen) containing 5% FCS for 1.5 h. Protein kinase inhibitors (suspended in DMSO) were added directly into the media, and the cells were further incubated for an additional 1 h before the addition of 400 μ Ci ³²P inorganic phosphate (10 μ Ci/ml, Perkin Elmer) per 2 ml of media. The cells were further cultured for 4 h and then harvested for immunoprecipitation for hnRNP A1 as described above. Protein concentrations were determined by standard Bradford assays (BioRad) using BSA as standard to ensure equal amounts of total protein were used for each immunoprecipitation reaction. Following the western transfer, the PVDF membrane was exposed to film to determine ³²P incorporation into hnRNP A1, before being analyzed by western blotting.

For experiments in Figure 8B, HeLa and WI38-VA13 cells were mock transfected or transfected with control plasmid, pU3-500, or plasmid expressing hTR, pU3-hTR-500 (a kind gift from Kathleen Collins) (41), using Fugene 6 according to the manufacturer’s guide. Transfected cells were grown for 48 h, and then subjected to in vivo labeling in the presence of DMSO or the DNA-PK inhibitor NU4771 as described above. Extracts were prepared for immunoprecipitation for hnRNP A1. To determine hTR levels, cell cultures treated the same way were grown in parallel, but not exposed to ³²P-inorganic phosphate. The cells were then isolated and RNA extracts were prepared for RT–PCR analysis for hTR as previously described (17).

Generation of hnRNP A1 clones

Vectors expressing GST-hnRNP A1 and GST-hnRNP A2 (in pGEX-4ET) were kind gifts from Dr Benoit Chabot (University of Sherbrooke). The phosphorylation mutants for GST-hnRNP A1 S95A and S95/192A were created by a standard PCR strategy in the pGEX vector by using the following primers. The GST-hnRNP A1 S95A mutant was created first with the primers: RNP-N: 5′-CGGGATCCGAGAGGTCGC-3′; RNP-R: 5′-GCGGATGAAGCAC-3′; S95A-N: 5′-CTCAAGAGAAGATGCTCAGCGACCAGGTG-3′; S95A-C: 5′-GCGACCTCTCTCGAGACCGACCCAGGTC-3′. The GST-hnRNP A1 S95/192A mutant was created in the GST-hnRNP A1 S95A mutant with the primers RNP-N: 5′-CGGGATCCGAGAGGTCGC-3′; S95A-C: 5′-GCGACCTCTCTCGAGACCGACCCAGGTC-3′. The GST-hnRNP A1 S95/192A mutant with the primers RNP-N, RNP-R, and the following primers: S192A-N: 5′-GTGCTTCATCCTGGCTCAAGAGGTCGC-3′; S192A-C: 5′-GCGACCTCTCTCGAGACCGACCCAGGTC-3′.

RESULTS

DNA-PK phosphorylates hnRNP A1 in an hTR- and DNA-dependent manner

Linear dsDNA with exposed termini is the most effective activator of DNA-PK activity in vitro. Association of Ku with ends of dsDNA recruits DNA-PKcs to form the active kinase holoenzyme, which can then undergo autophosphorylation or target other protein or peptide substrates (5). Although most studies suggest that the
major activator of DNA-PK is dsDNA, it has also been reported that DNA-PK activity can be stimulated by a variety of nucleic acids in vitro, including single stranded DNA, and nicked closed circular DNA (42,43). Recently, it has been reported that poly(G) RNA can stimulate DNA-PK activity towards purified recombinant DNA helicase II (NHD II) and hnRNPs C and D (44). Immune complexes that contain both hnRNPs C and hnRNPs A1 were phosphorylated with exogenously added DNA-PK and treatment of this reaction with RNase A reduced phosphorylation, suggesting that this phosphorylation event was RNA dependent (44). We previously reported that Ku70/80 interacts with hTR both in vivo and in vitro (17), and therefore, we wanted to test whether this interaction could stimulate the kinase activity of DNA-PK. We first examined the ability of hTR to stimulate autophosphorylation of the components of the DNA-PK holoenzyme (DNA-PKcs, Ku70 and Ku80) as well as a well-established DNA-PK peptide substrate containing the SQE motif (7). Incubation of purified DNA-PKcs and Ku70/80 with hTR did not support autophosphorylation of the complex, whereas, as expected, the addition of CT DNA did (Figure 1A). Similarly, hTR did not support phosphorylation of the SQE peptide (data not shown).

Since both hnRNPs A1 and DNA-PK are involved in telomere function, and members of the hnRNP family are DNA-PK substrates, we sought to determine whether hnRNPs A1 was a direct substrate for DNA-PK. hnRNPs A1 contains two potential DNA-PK phosphorylation sites at Serine 95 and Serine 192 (Figure 2). We also tested, whether or not the closely related protein hnRNPs A2 was a DNA-PK substrate. hnRNPs A2 is redundant with hnRNPs A1 with respect to its splicing function, however, unlike hnRNPs A1, hnRNPs A2 does not function in telomere length maintenance (25). Furthermore, hnRNPs A2 lacks the two SQ sites found in hnRNPs A1 (Figure 2). In vitro DNA-PK assays using recombinant GST-hnRNPs A1 or A2 purified from bacteria were performed in the presence of either CT-DNA or hTR. As shown in Figure 1B (lanes 5 and 6), DNA-PK phosphorylated recombinant GST-hnRNPs A1 in the presence of either CT DNA or hTR, but not TE buffer alone (lane 4). However, the closely related protein, hnRNPs A2 (GST fusion protein) was not phosphorylated under either condition (Figure 1B, lanes 8 and 9). Treatment of the kinase reaction with RNase A abolished hTR-dependent phosphorylation of hnRNPs A1, but not its DNA-dependent phosphorylation (Figure 1C, compare lanes 5 to 6). Similarly, RNase A had no effect on the DNA-dependent phosphorylation of hnRNPs A1 by DNA-PK (Figure 1C, lane 3). These observations reveal a novel in vitro property of DNA-PK: namely that it can be activated by hTR to phosphorylate hnRNPs A1.

**Specificity of hnRNPs A1 phosphorylation by DNA-PK**

In vitro, DNA-PK phosphorylates a number of different proteins that are involved in NHEJ. Two of these substrates are XRCC4 and Artemis (38,39). To test whether hTR-stimulated phosphorylation was also observed with these proteins, we repeated the in vitro kinase assays with recombinant GST-Artemis or GST-XRCC4 purified from bacteria. As can be seen in Figure 3A, these proteins were not efficiently phosphorylated by DNA-PK in the presence of hTR, however, as expected, they were robustly phosphorylated in the presence of CT-DNA (compare lanes 1 to 2 and 3 to 4, right panel). These data demonstrate that hTR-dependent phosphorylation by DNA-PK is specific for hnRNPs A1.

**Nucleic acid specificity for hnRNPs A1 phosphorylation**

As mentioned earlier, poly(G) RNA can stimulate DNA-PK activity in vitro, although the physiological relevance of this observation remains unknown (44). To compare the specificity of hTR-stimulated phosphorylation by DNA-PK with that of other nucleic acids, purified DNA-PKcs, Ku70/80 and GST-hnRNPs A1 were incubated under kinase reaction conditions with either oligo(dT), poly(AU), poly(G) or tRNA. As seen in Figure 3B, only hTR and CT DNA supported DNA-PK-dependent phosphorylation of hnRNPs A1. To further confirm that the hTR- and DNA-dependent phosphorylation of hnRNPs A1 was indeed due to DNA-PK, a specific DNA-PK inhibitor, NU4771 (45), was added to the kinase reactions. As shown in Figure 3C, NU4771 inhibited phosphorylation of GST-hnRNPs A1 by DNA-PK in vitro (Figure 3C).

**DNA-PK and hTR requirements for hnRNPs A1 phosphorylation**

We have previously shown that Ku preferentially associates with the region of hTR spanning nucleotides 404–451 (17). We next tested different regions of hTR for their ability to stimulate DNA-PK phosphorylation of hnRNPs A1. As seen in Figure 4A, only FL-hTR supported DNA-PK phosphorylation of GST-hnRNPs A1 (compare lanes 4–7 to lane 3). We next tested whether or not different regions of hTR could recapitulate the FL-hTR requirement for DNA-PK phosphorylation of hnRNPs A1 when the sequences were expressed on two different RNA molecules. As shown in Figure 4B, equimolar amounts of two different regions of hTR that reconstituted FL-hTR, did not complement each other to support hnRNPs A1 phosphorylation by DNA-PK (lanes 5 and 6). These data suggest that the active DNA-PK holoenzyme may form a complex with hnRNPs A1 in cis on the same FL-hTR molecule (to be discussed further, see Figure 6). Furthermore, FL-hTR in the native confirmation was required for phosphorylation, since heat-denatured FL-hTR did not stimulate DNA-PK activity for hnRNPs A1 (Figure 4C). In combination, these data suggest that intact, FL-hTR that maintains its secondary structure is required for stimulating the phosphorylation of hnRNPs A1 by DNA-PK.

**Interaction of Ku and hnRNPs A1**

The data shown in Figure 4 suggests that both hnRNPs A1 and Ku may bind to the same FL-hTR molecule and form a nucleoprotein complex that is competent for phosphorylation by DNA-PKcs. To determine whether Ku
and hnRNP A1 both interact with FL-hTR, we performed EMSAs with radiolabeled FL-hTR and purified Ku and/or GST-hnRNP A1. As seen in Figure 5A, upper panel, hnRNP A1 or Ku alone formed nucleoprotein complexes with FL-hTR that migrate at a different position on the non-denaturing gel (Figure 5, top panel, compare complex ‘a’ to ‘b’ in lanes 2 and 3 versus lanes 4 and 5). In the presence of both Ku and hnRNP A1, a new slower ribonucleoprotein complex was formed (indicated by complex ‘c’), suggesting that Ku and hnRNP A1 may bind to the same FL-hTR molecule. Previous studies have shown that hnRNP A1 interacts with the first 208 nucleotides of hTR (27), and Ku preferentially associates with the 3' end of hTR constituting nucleotides 404–451 (17). Consistently, in EMSA studies with the 3' end of hTR, spanning nucleotides 404–451, only a Ku–hTR complex was observed, but no hnRNP A1–hTR complex was formed (Figure 5A, lower panel).
Moreover, the ribonucleoprotein complex ‘c’ that formed in the presence of Ku, hnRNP A1 and hTR was not seen with the hTR (404–451) probe.

To confirm the presence of Ku and hnRNP A1 in complex ‘c’ with FL-hTR, the EMSA gel was transferred onto PVDF membrane, and analyzed by western blotting. As seen in Figure 5B, monoclonal antibodies to Ku and hnRNPA1 indicated the presence of Ku (right panel, lanes 5 and 6) and hnRNPA1 (left panel, lanes 5 and 6), respectively. Collectively, these data suggest that hnRNPA1 and Ku bind the same hTR molecule, possibly at the 5' and 3' ends, respectively.

To extend these studies to a cellular context, we performed immunoprecipitation studies with extracts prepared from either HeLa cells, which are telomerase positive and express hTR, or VA-13 cells which are SV40 transformed human lung fibroblasts that lack detectable hTR and hTERT and maintain their telomeres by the ALT pathway (46). Western blots of complexes from immunoprecipitation with a monoclonal antibody to Ku indicated the presence of both Ku and hnRNPA1 in both cell lines. Immunoprecipitates from reciprocal immunoprecipitation assays using a monoclonal antibody to hnRNPA1 also contained Ku and hnRNPA1 (Figure 6). Therefore, these data suggest that Ku interacts with hnRNPA1 in cells independent of hTR. This interaction may be direct or mediated by addition factors in cells. It appears however, that the interaction between Ku and hnRNPA1 is not sufficient to support efficient phosphorylation of hnRNPA1 in the absence of hTR. Moreover, although Ku70/80 and hnRNPA1 can interact in the absence of hTR, we speculate that the binding of each protein with the telomerase RNA is necessary to activate DNA-PKcs for the phosphorylation of hnRNPA1 (see below).

Identification of in vitro and in vivo DNA-PK phosphorylation sites on hnRNPA1

DNA-PK preferentially targets Ser/Thr followed by a Gln residue (S/T-Q motifs) (5). To date, hnRNPA1 has been shown to be phosphorylated on Ser 192 and Ser 310–312 by MAP Kinase interacting kinases (MNKs) during the activation of T-cells (47). As an initial step toward elucidating the importance of hnRNPA1 phosphorylation, we wanted to identify which amino acid residues are targeted by DNA-PK. Examination of the amino acid sequence of hnRNPA1 revealed two DNA-PK consensus phosphorylation sites at Ser 95 and Ser 192 (Figure 2).

Figure 3. Specificity of hnRNPA1 phosphorylation by DNA-PK. (A) hTR does not support DNA-PK-mediated phosphorylation of XRCC4 or Artemis. DNA-PK kinase assays were performed with purified recombinant GST-XRCC4 (1.0 μg, lanes 1 and 2) or GST-Artemis (1.0 μg, lanes 3 and 4) in the presence of CT-DNA (lanes 1 and 3) or hTR (lanes 2 and 4). The Coomassie stained gel is shown on the left panel (Gel), and the corresponding autoradiogram is shown in the right panel (Autorad). The migrating position of GST-XRCC4 and GST-Artemis are indicated by ‘−’ and ‘+’, respectively. (B) Nuclease acid requirements for hnRNPA1 phosphorylation by DNA-PK. Kinase reactions with DNA-PK and GST-hnRNPA1 were performed in the presence of TE (lane 1) or 0.5 μg each of CT-DNA (lane 2), hTR (lanes 3), Oligo(dT) (lane 4), poly(AU) (lane 5), poly(G) (lane 6), or tRNA (lane 7). The upper panel represents the X-ray film (Autorad) showing the phosphorylated GST-hnRNPA1 and the bottom panel is the corresponding region of the Coomassie stained gel for GST-hnRNPA1 (Gel). (C) DNA- and hTR-dependent phosphorylation of hnRNPA1 by DNA-PK is inhibited by the DNA-PK inhibitor NU7441. DNA-PK kinase reactions with GST-hnRNPA1 and CT DNA (lanes 1–4) or hTR (lanes 5–8) were carried out with increasing concentrations (lanes 1–4) and lanes 5–8; 0, 0.02, 0.05, 0.1 μM, respectively) of the specific DNA-PK kinase inhibitor NU4771. The autoradiogram is featured in the upper panel (Autorad), and the corresponding Coomassie stained gel for GST-hnRNPA1 is shown in the lower panel (Gel).

These residues are highly conserved between mouse and human. Moreover, in the closely related protein hnRNPA2, which is not a substrate for DNA-PK (Figure 1B), these Ser residues are not followed by Gln. We therefore reasoned that these two sites were excellent candidates for...
DNA-PK phosphorylation. Based on these observations, we generated GST hnRNP A1 proteins harboring either a single Ser to Ala mutation at Ser 95 (S95A) and Ser 192 (S192A) or the double mutation at both Ser 95 and Ser 192 (S95/192A). These mutant proteins were expressed as GST-recombinant proteins, purified and tested in DNA-PK assays in vitro in the presence of CT DNA or hTR (Figure 7). In the presence of either CT DNA or hTR, when compared to the WT, phosphorylation was reduced by 39% in the single S95A mutant (compare lanes 5 and 6 to lanes 2 and 3); however, phosphorylation was not significantly reduced the S192A mutant (data not shown). In contrast, DNA-PK phosphorylation was reduced by 69% in the double S95/192A mutant hnRNP A1 (compare lanes 8 and 9 to lanes 2 and 3, Figure 7). We speculate that phosphorylation of Ser-95 and Ser-192 by
DNA-PK might be coordinately regulated, such that elimination of Ser-192 might enhance phosphorylation at Ser-95. Alternatively, mutation of both Ser-95 and Ser-192 might induce a conformational change that reduces the ability of DNA-PK to target hnRNP A1. Regardless, these data are the first to report the phosphorylation of hnRNP A1 at Ser 95, and also suggests that DNA-PK may target Ser-192.

**hnRNP A1 is phosphorylated in a DNA-PK-dependent manner in vivo**

In order to determine if hnRNP A1 is an in vivo substrate for DNA-PK, we cultured HeLa cells with 32P-inorganic phosphate in the presence or absence of DNA-PK inhibitors (wortmannin or NU4771) for 4 h and prepared cell extracts for immunoprecipitation with anti-hnRNP A1 antibodies. As seen in Figure 8A, 32P incorporation into hnRNP A1 was reduced in hnRNP

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**Figure 6.** hnRNP A1 and Ku coimmunoprecipitate from 293T and VA13 cells. Cell extracts were prepared as described in ‘Materials and Methods’ section, and antibodies used for immunoprecipitations are as indicated. The western blots were initially probed with monoclonal antibodies to Ku70 and Ku80 (top panel); the blots were stripped and reprobed with monoclonal antibodies against hnRNP A1 (bottom panel). Lanes 1 and 2 contain 1% of the amount of extract used for each immunoprecipitation reaction.

**Figure 7.** Identification of phosphorylation sites on hnRNP A1. GST-hnRNP A1 harboring single serine to alanine substitution, S95A, or the double mutation, S95/192A, were generated as described in ‘Materials and Methods’ section. A total of 1.0 μg of each GST-hnRNP A1 wild-type (WT, lanes 1–3), S95A (lanes 4–6), or S95/192A (lanes 7–9) were tested for DNA-PK phosphorylation under standard assay conditions in the presence of TE, CT-DNA, or hTR as indicated. The Coomassie stained gel showing the GST-hnRNP A1 proteins is shown in the bottom panel, and the corresponding autoradiogram is shown in the top panel. The intensity of the 32P incorporation in each of the phosphorylation mutant GST-hnRNP A1 proteins was quantified using Quantity One software (Biorad) and expressed as a percentage compared to phosphorylation of WT (lanes 2 and 3, shown in the middle panel).

**Figure 8.** hnRNP A1 is phosphorylated in vivo and phosphorylation is inhibited by the DNA-PK and PIKK inhibitors, NU4771 and wortmannin. (A) HeLa cells were incubated in phosphate-free medium in the presence of DMSO (lanes 1 and 2), wortmannin (5 μM, lane 3) or the DNA-PK inhibitor NU7441 (10 μM, lane 4) as described in ‘Materials and Methods’ section. Inorganic 32P-phosphate was then added to the media, and the cells were further cultured for 4 h. Extracts were immunoprecipitated with control monoclonal IgG antibodies (lane 1) or antibodies to hnRNP A1 (lanes 2–4). The immune-complexes were fractionated on SDS-PAGE, transferred onto PVDF membrane, and exposed to X-ray film, before western blot analysis. The top panel shows the western blot for hnRNP A1 and the middle panel shows the resulting autoradiogram. The intensity of the 32P incorporation in the immunoprecipitated hnRNP A1 lanes was quantified using Quantity One software (Biorad) and expressed as a percentage compared to the control (lane 2). (B) Cells were mock-transfected (HeLa, lane 1), transfected with control plasmid pU3-500 (HeLa, lane 3 and VA13, lane 4), or transfected with pU3-hTR-500 (VA13, lane 5 and 6) for 48 h as described in ‘Materials and Methods’ section. The cells were then incubated with DMSO (lanes 2–5) or with 10 μM NU4771 (lane 6) and metabolic labeling with 32P-inorganic phosphate was carried out as in panel A. Extracts were made for immunoprecipitations with control monoclonal IgG antibodies (lane 2) or antibodies to hnRNP A1 (lanes 3–6). The immune-complexes were fractionated on SDS-PAGE, and transferred onto PVDF membrane, and exposed to X-ray film, before western blot analysis. Parallel cell cultures treated the same way but grown without 32P-inorganic phosphate were isolated for RNA preparation and RT–PCR analysis as described in ‘Materials and Methods’ section. The contents of each lane are indicated at the top of the panels. The top panel shows the western blot for hnRNP A1, the middle panel shows the resulting autoradiogram, and the bottom panel shows the RT–PCR reaction for hTR. Lane 1 represents non-template PCR reaction control.
A1 immunoprecipitated from cell extracts treated with wortmannin or NU4771 compared to the control cell extracts (bottom panel, compare lanes 3 and 4 to lane 2). Although significantly reduced in the presence of NU4771, hnRNP A1 phosphorylation was not completely abolished, suggesting that other protein kinases may play a role in the phosphorylation of this protein in vivo. Regardless, this observation strongly suggests that hnRNP A1 is a physiological substrate for DNA-PK.

Next, we looked at the phosphorylation of hnRNP A1 in WI38-VA13 cells. hnRNP A1 immunoprecipitated from VA13 cells showed reduced 32P-incorporation compared to hnRNP A1 immunoprecipitated from HeLa cells (Figure 8B, compare lanes 3 to 4, middle panel). Phosphorylation of hnRNP A1 was increased to levels comparable to those seen in HeLa cells when hnRNP A1 was immunoprecipitated from VA13 cells exogenously expressing hTR (Figure 8B, compare lanes 5 to 3). This enhanced phosphorylation of hnRNP A1 in VA13 cells expressing hTR was inhibited by NU4771 (Figure 8B, lane 6), suggesting that this effect was mediated through DNA-PK. Together these data strongly suggest that hnRNP A1 is phosphorylated by DNA-PK in a hTR-dependent manner in vivo.

DISCUSSION

We have previously shown that Ku70/80 interacts with the RNA component of human telomerase, hTR. To elucidate the functional significance of this interaction, we examined whether or not this interaction could activate DNA-PK activity. In this study, we show that both hTR and DNA stimulate DNA-PK kinase activity towards the novel substrate, hnRNP A1. Our EMSA results suggest that hnRNP A1 and Ku70/80 can interact on the same full length, intact hTR molecule. Furthermore, we observed that Ku and hnRNP A1 were found in the same complex in immunoprecipitates from cellular extracts. Treatment of cells with the highly specific DNA-PK inhibitor NU7441 resulted in reduced hnRNP A1 phosphorylation. Furthermore, in VA13 cells that lack hTR, phosphorylation of hnRNP A1 was greatly reduced and was restored by exogenous expression of the telomerase RNA. Together, these data strongly suggest that hnRNP A1 is a physiological substrate for DNA-PK. As an initial step towards understanding the functional significance of this phosphorylation event, we identified Ser 95 and Ser 192 on hnRNP A1 as potential phosphorylation sites for DNA-PK. With respect to its function in splicing, hnRNP A2 is thought to be redundant with hnRNP A1, however, hnRNP A2 cannot overcome the hnRNP A1 deficiency and telomere length defects in erythroleukemic cell lines (25). One possible explanation for the inability of hnRNP A2 to function at telomeres might be that phosphorylation of hnRNP A1 at specific sites by DNA-PK is required for its role in telomere length maintenance. In addition, although Ku and hTR can interact in cells lacking hTR, this interaction is not sufficient to stimulate phosphorylation of hnRNP A1 by DNA-PK since VA13, cells which lack hTR, have significantly reduced levels of hnRNP A1 phosphorylation and this phosphorylation is significantly increased upon the exogenous expression of hTR. Together, these data suggest that in vivo, hTR is required for the phosphorylation of hnRNP A1 by DNA-PK.

It is interesting to note that in vitro, DNA-mediated autophosphorylation of DNA-PKcs is associated with loss of protein kinase activity and dissociation of the DNA-PK complex (5,49,50). Moreover, cells expressing DNA-PKcs that lack multiple in vitro autophosphorylation sites are radiosensitive and defective in DSB repair (51). Together, these studies suggest that upon interaction with DSBs, DNA-PK becomes activated and subsequently undergoes autophosphorylation induced inactivation (5). Our in vitro results showing that DNA-PK can be activated by hTR without undergoing autophosphorylation suggests the possibility that DNA-PK may be regulated differently at telomeres than at DSBs. These data suggest that hTR might stimulate DNA-PK phosphorylation of telomere associated proteins in the absence of a DSB without inactivating its kinase activity and/or releasing DNA-PKcs from the telomere.

In yeast, the association of TLC1 with Ku70/80 is crucial to recruit the telomerase components, Est1p and Est2p to telomeres for telomerase synthesis during
S-phase (19). However, since yeast do not contain DNA-PKcs, the function of Ku at telomeres may be subtly different between humans and yeast. Whether or not the hTR/Ku interaction in human cells serves to recruit telomerase components to telomeres or serves another critical function in cells remains to be explored. Nevertheless, based on the data presented here we speculate that the interaction of Ku and hnRNP A1 with hTR during telomere synthesis could recruit DNA-PKcs to phosphorylate hnRNP A1 and regulate its function during telomere synthesis. We speculate that phosphorylation of hnRNP A1 could influence its ability to recruit telomerase to chromosome ends or modulate higher order telomere structures. Studies to address the function of hnRNP A1 phosphorylation mutants at telomeres are ongoing.

Unlike hTERT and telomerase activity, which are only present in germ cells, stem cells and cells with a high proliferative index, hTR is ubiquitously expressed in somatic cells (52). It is therefore very likely that hTR has other, yet to be discovered roles, in the cell. We suggest that in addition to its critical role in directing telomere addition by telomerase, hTR may act as a co-factor for DNA-PK to phosphorylate proteins that are involved in telomere length maintenance. Consistent with additional roles for hTR in cells, hTR levels increase irrespective of telomerase status in response to low levels of UV radiation (53). Consequently, the elevated levels of hTR attenuate the activity of ATR, a PIKK family member related to DNA-PKcs, and facilitate cellular recovery of cells from UV radiation (53). Interestingly DNA-PK interacts with, and is targeted by ATR in response to UV exposure, implicating DNA-PK in the same damage pathway (54). Together, these observations lead to the intriguing possibility that hTR may be used as a signaling molecule in response to UV-induced DNA damage to regulate the activities of DNA-PK and ATR, in a manner that is independent of its telomere templating function.

In summary, we have shown that DNA-PK phosphorylates hnRNP A1 in an hTR-dependent manner in vitro and that hTR is important for the phosphorylation of hnRNP A1 by DNA-PK in cells. These results reveal a novel property for DNA-PK, namely, the ability of a physiologically relevant RNA molecule, hTR, to activate its kinase activity. Moreover, since the kinase activity of DNA-PK is required for telomere function (16) we speculate that the hTR-dependent phosphorylation of hnRNP A1 might play a critical role in telomere function in vivo.

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