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Mapping the FEN1 interaction domain with hTERT

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ABSTRACT

The activity of telomerase in cancer cells is tightly regulated by numerous proteins including DNA replication factors. However, it is unclear how replication proteins regulate telomerase action in higher eukaryotic cells. Previously we have demonstrated that the multifunctional DNA replication and repair protein flap endonuclease 1 (FEN1) is in complex with telomerase and may regulate telomerase activity in mammalian cells. In this study, we further analyzed the nature of this association. Our results show that FEN1 and telomerase association occurs throughout the S phase, with the maximum association in the mid S phase. We further mapped the physical domains in FEN1 required for this association and found that the C-terminus and the nuclease domain of FEN1 are involved in this interaction, whereas the PCNA binding ability of FEN1 is dispensable for the interaction. These results provide insights into the nature of possible protein–protein associations that telomerase participates in for maintaining functional telomeres.

1. Introduction

Telomeres are the nucleo-protein complexes that protect the eukaryotic chromosomal ends from inappropriate nucleolytic degradation and recombination. Telomeres shorten after every round of cell division in normal human somatic cells [1]. In the majority of tumor cells, this shortening is counteracted by telomerase, a special cellular reverse transcriptase [2]. It renders telomeres functional by replenishing the telomeric repeats on the 3′ end. Thus, the telomere regulation mechanism is a key for survival of cancer cells.

Telomerase maintains the telomere length homeostasis by adding tandem hexameric $(TTAGGG)_n$ repeats at the 3′ end of chromosomes. The activity of telomerase is tightly regulated by multiple complicated mechanisms to achieve telomere length homeostasis. Its expression is regulated at a transcriptional level by various factors such as p53, Rb, myc, and wilm's tumor 1 (WT1) [3]. Trafficking and assembly of the telomerase subunits into a functional complex has also been shown to regulate telomerase action [4,5]. In addition, telomerase activity can be regulated by post-translational modifications such as phosphorylation and ubiquitinylation [6–9]. Moreover, a number of telomere binding proteins regulate telomerase extension of telomeres either positively or negatively [10].

Studies from lower eukaryotes have indicated that telomerase action is regulated by components of the conventional DNA replication machinery. In budding yeast, defect in the DNA replication machinery such as polymerase α/primase (Pol α) and polymerase δ (Pol δ) abolishes the *de novo* addition of telomeric DNA [11]. Temperature sensitive mutations in Pol α and replication factor C display uncontrolled telomerase mediated telomere elongation [12]. Consistently, budding yeast Pol α physically interacts with Cdc13p, which in turn interacts with Est1 (yeast telomerase subunit) and regulates the telomerase action [12–14]. In fission yeast, mutations in Pol α/primase and Pol δ show abnormal lengthening of telomeres and Pol α interacts with telomerase catalytic subunit Trt1 [15]. In ciliates *Euplotes crassus*, telomerase physically interacts with primase [16] and inhibition of Pol α and Pol δ by aphidicolin causes C-strand and G-strand heterogeneity [17]. However, in higher eukaryotes the detailed mechanistic events of telomerase action and its regulation are poorly understood [18].

FEN1 is a conserved, structure specific multifunctional nuclease involved in various DNA metabolic pathways including DNA replication and repair, probably due to its ability to participate in multiple protein–protein interactions [19–22]. To date FEN1 is known to interact with more than 30 proteins [22]. Three distinct nuclease activities have been identified in FEN1. The 5′ → 3′ flap endonuclease activity (FEN) is required in the RNA primer removal during lagging stand replication and base excision repair pathways

Abbreviations: hTERT, human telomerase reverse transcriptase; FEN1, flap endonuclease 1; EXO, exonuclease; GEN, gap-dependent nuclease; PCNA, proliferating nuclear antigen; RFC, replication factor C; WRN, Werner syndrome protein; BLM, Bloom syndrome protein; Rb, retinoblastoma.

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[19,23]. The 5′ → 3′ exonuclease (EXO) activity [24] and gap-dependent endonuclease (GEN) activities are implicated in the resolution of trinucleotide repeat derived secondary structures, hairpin loops, and stalled replication forks [25–27]. Being a multifunctional factor, defect in FEN1 has been implicated in causing genome instability. A recent report shows that groups of FEN1 mutations in cancer specimens which abrogated two of the three nuclease activities lead to cancer initiation and progression [28]. FEN1 haploinsufficiency leads to tumor progression in mice [29]. FEN1 depleted null mice showed embryonic lethality and the blastocysts displayed proliferation failure and increased sensitivity to gamma radiation [30]. Thus, FEN1 is an important guardian of genome stability.

Recently FEN1 has emerged as a major player in telomere maintenance. It is recruited to telomeres in a cell cycle regulated manner and interacts with TRF2, a telomere binding protein [18,31]. Depletion of FEN1 leads to telomere shortening in telomerase positive cancer cells [32] and selectively loses lagging strand telomeres in telomerase negative cells [33]. Furthermore, FEN1 physically associates with telomerase, probably through the catalytic subunit of telomerase, hTERT [32]. Due to FEN1's ability to participate in multiple DNA pathways, we speculate that FEN1 may influence telomerase action and helps to maintain telomere length homeostasis through interacting with telomerase. Here, we report that FEN1 forms a complex with telomerase in a cell cycle dependent manner. To better understand the nature of this association, we attempted to dissect the functional domains in FEN1 that had an impact on the FEN1/telomerase complex formation. Analysis of FEN1 mutants indicates that the C-terminus and the nuclease domain of FEN1 are involved in FEN1/hTERT association, whereas FEN1's ability of binding to PCNA is dispensable for the complex formation.

2. Materials and methods

2.1. Cell culture

HeLa and 293T cells were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% cosmic calf serum (Hyclone).

2.2. Antibodies

The following primary antibodies were used: rabbit polyclonal anti-FEN1 (Bethyl), monoclonal anti-Myc (Santa Cruz), monoclonal anti-FLAG (Sigma). Secondary antibody was horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (BD Biosciences).

2.3. Generation of FEN1 mutants

The plasmid pCI-neo carrying the full length Myc-FEN1 [32] was used for QuickChange site-directed mutagenesis (Agilent Technologies) to give rise to ΔC, ΔP, ΔPΔC, ΔPΔC + NLS, FFAA, and E160D mutants using the following primers. For ΔC: 5′-GCTAAGCGCAAGGAGCCAGAATAAGGGCGGCCGCTTCCCTTTAG-3′ and 5′-CTAAAGGGAAGCGGCCGCCCTTATTCTGGCTCCTTGCGCTTAGC-3′; for ΔPΔC: 5′-GAGCCGCCAAGGCAGCACCTAAGGGCGGCCGCTTCCCTTTAG-3′ and 5′-CTAAAGGGAAGCGGCCGCCCTTAGGTGCTGCCTTGGCGGCTC-3′; for ΔP: 5′-AGAGCCGCCAAGGCAGCACCAAGGTGACCGGCTACTCTCT-3′ and 5′-AGAGAGTGAGCCGGTCACCTTGGTGCTGCCTTGGCGGCTCT-3′; for FFAA: 5′-CAGGGCCGCCTGGATGATGCCGCCAAGGTGACCGGC-3′ and 5′-GCCGGTCACCTTGGCGGCATCATCCAGGCGGCCCTG-3′; for E160D: 5′-AGTGAGGCAGACGCCAGCTGTGCTGCCCTG-3′ and 5′-CAGGGCAGCACAGCTGGCGTCTGCCTCACT-3′. To generate ΔPΔC + NLS mutant, ΔC was used as the template for mutagenesis using the following primers: 5′-AGAGCCGCCAAGGCAGCACCAAGGTGACCGGCTCACTCTCT-3′ and 5′-AGAGAGTGAGCCGGTCACCTTGGTGCTGCCTTGGCGGCTCT-3′.

2.4. Cell synchronization

Cell synchronization was carried out as described previously using the double thymidine block [34]. Cells collected at different time points were fixed in 70% ethanol, digested with RNase A (0.02 μg/μl), stained with propidium iodide (50 μg/ml), and the DNA content was analyzed using a Becton-Dickinson FACSCalibur or Beckman Coulter EPICS® XL™ flow cytometer.

2.5. Co-immunoprecipitation-TRAP

Co-IP-TRAP was performed as described previously [32,35]. Briefly, 3 μg of each antibody was coupled to protein A/G+ agarose beads (Santa Cruz Biotechnology) by incubating overnight at 4 °C with constant rotation. Antibodies were used for IP of proteins from cell lysate corresponding to 500,000 cells. After IP, the agarose bead pellets were re-suspended with 40 μl lysis buffer, and 2 μl were used for nonradioactive TRAP analysis to detect telomerase activity.

2.6. Co-immunoprecipitation

HeLa and 293T cells were co-transfected with a total of 10 μg of plasmid DNA (5 μg of pCR3-Flag-hTERT plus 5 μg of pCI-neo vector or full-length and mutant myc-FEN1 plasmid) using FuGENE HD transfection reagent (Roche Applied Science) according to the manufacturer's instructions. The cells were harvested 18 h after transfection, and the cell pellets were washed once with cold phosphate buffered saline, re-suspended in lysis buffer (50 mM Tris–HCl, pH 7.4, 50 mM NaCl, 0.1% Nonidet P-40, 2 mM dithiothreitol) supplemented with EDTA-free protease inhibitor cocktail (Roche Applied Science), sonicated on ice (50 J/W, three times of 4 s pulses), and centrifuged at 13,000 rpm for 20 min at 4 °C. Supernatants were then pre-cleared by incubating with 20 μl of 50% slurry of protein G-agarose beads (Roche) for 1 h at 4 °C with constant rotation. After brief centrifugation, pre-cleared lysates were transferred to new tubes and incubated with antibody-coupled beads in the presence of 50 μg of bovine serum albumin for overnight at 4 °C with constant rotation. The beads were then washed three times with lysis buffer and re-suspended in Laemmli buffer, boiled for 5 min, and used immediately on 6% SDS–PAGE for immunoblotting.

3. Results

3.1. FEN1/telomerase association takes place during the S phase of cell cycle

Previously we have demonstrated that FEN1 and telomerase are in a complex [32]. To better understand this association, we first determined whether this association was cell cycle regulated. HeLa cells were synchronized at the G1/S boundary by double thymidine block and then released into S phase and collected at 1.5 h intervals. Following IP, the precipitates were subjected to TRAP analysis for detecting telomerase activity. As shown in Fig 1B and C, FEN1-shows moderate association with telomerase during the early and late S phase, but the association reaches its peak at mid S phase (4.5 h). We conclude that FEN1 associates with telomerase during the S phase, corresponding to the period when the majority of telomeres are replicating [36].