Published in final edited form as:

Biochem Biophys Res Commun. 2011 April 1; 407(1): 34–38. doi:10.1016/j.bbrc.2011.02.087.

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.

Keywords

FEN1; telomerase; telomere

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 tumour 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

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phosphorylation and ubiquitinylation [6,7,8,9]. 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 [12,13,14]. 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 multi-functional nuclease involved in various DNA metabolic pathways including DNA replication and repair, probably due to its ability to participate in multiple protein-protein interactions [19,20,21,22]. 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 strand replication and base excision repair pathways [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 [25,26,27]. 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 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% CO₂ 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 , $\Delta P\Delta C$, $\Delta P\Delta C + NLS$, FFAA, and E160D mutants using the following primers. For ΔC : 5'-GCTAAGCG CAAGGAGCCAGAATAAGGGCGGCCGCTTCCCTTTAG-3' and 5'-CTAAAGGGAAGCG GCCGCCCTTATTCTGGCTCCTTGCGCTTAGC-3'; for $\Delta P\Delta C$: 5'-GAGCCGCCAAGGCA GCACCTAAGGGCGGCCGCTTCCCTTTAG-3' and 5'-CTAAAGGGAAGCGGCCGCCCTT AGGTGCTGCCTTGCGGCTC-3'; for ΔP : 5'-AGAGCCGCCAAGGCAGCACCAAGGTGA CCG GCTACTCTCT-3' and 5'-AGAGAGTGAGCCGGTCACCTTGGTGCTGCCTTGCGG CTCT-3'; for FFAA: 5'-CAGGGCCGCTGGATGATGCCGCCAAGGTGACCGGC-3' and 5'-GCCGGTCACCTTGGCGGCATCATCCAGGCGGCCCTG-3'; for E160D: 5'-AGTGAGG CAGACGCCAGCTGTGCTGCCCTG-3' and 5'-CAGGGCAGCACAGCTGGCGTCTGCCTC ACT-3'. To generate $\Delta P\Delta C+NLS$ mutant, ΔC was used as the template for mutagenesis using the following primers: 5'-AGAGCCGCCAAGGCAGCACCAAGGTGACCGGCTCACTCTCT-3' and 5'-AGAGAGTGAGCCGGTCACCTTGGTGCTGCCTTGCGGCTCT-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 ug/ul), stained with propidium iodide (50 ug/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, 3ug 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 resuspended with 40 μ l lysis buffer, and 2 μ l 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 μ g of plasmid DNA (5 μ g of pCR3-Flag-hTERT plus 5 μ g 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 hrs 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/watts, three times of 4 sec pulses), and centrifuged at 13,000 rpm for 20 min at 4 °C. Supernatants were then pre-cleared by incubating with 20 μ l 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 μ g 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 1.5hr 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 hrs). We conclude that FEN1 associates with telomerase during the S phase, corresponding to the period when the majority of telomeres are replicating [36].

3.2 Involvement of the C terminus of FEN1 in association with telomerase

It has been shown that the C-terminus of FEN1 is involved in interacting with multiple proteins that are functional in different metabolic pathways such as WRN, BLM, the Rad9/Rad1/Hus1 complex, and TRF2 etc [31,37,38]. This is attributed to its less conserved and structurally flexible nature of C terminus. Since previously we have shown that FEN1 interacts with the catalytic subunit of telomerase, hTERT [32], we speculated that the C terminus might be a plausible domain for its telomerase interaction. Since there are no reports documented on purified recombinant hTERT protein, we used co-IP as an approach to determine whether the C-terminus of FEN1 was involved in interacting with hTERT. We observed that deletion of the final 21 amino acids drastically reduced FEN1's ability to interact with telomerase, suggesting the residues deleted were critical (Fig. 2B, lane 2). However, since the association was not completely abolished and residual interaction was still retained, it is likely that amino acids at other positions may be involved in the interaction between the two.

3.3 Deletion of the PCNA-binding domain retains the FEN1/hTERT association

The residual association of ΔC with hTERT indicates that additional domain may be involved in this association. We then deleted a larger region containing the entire C terminus starting from the conserved PCNA binding (P) domain ($\Delta P\Delta C$) (Fig. 2A). This mutant showed a complete elimination of FEN1/hTERT association (Fig. 2B, lane 5). However, this deletion also removed the nucleus localization signal (NLS) which was required for FEN1's ability to localize into the nucleus [39]. We then generated a new truncation to preserve the NLS but still retained the P and C deletions ($\Delta P\Delta C+NLS$) (Fig. 2A). Surprisingly, this mutant retained wild-type association with telomerase (Fig. 2B, lane 7). Repeat of the experiments confirmed the same association by $\Delta P\Delta C+NLS$ (data not shown), suggesting that deletion of P region is able to rescue the diminished FEN1/hTERT association caused by ΔC .

We then questioned the effect of ΔP alone on the association. When a truncation mutant that removes the conserved PCNA-binding domain (345 to 349) was used, it showed similar association as full-length FEN1 (Fig. 2B, lane 6). In addition, a mutant containing substitution of F343 and F344 to alanines (FFAA), which completely abolishes FEN1's ability to bind to PCNA [40], did not show any change in FEN1/hTERT association (Fig. 2B, lane 8). Collectively, our results suggest that the P domain is dispensable for the FEN1/hTERT association.

3.4 The nuclease activity of FEN1 is needed for FEN1/hTERT association

Substitution of glutamic acid at 160 to aspartic acid in the active site of FEN1 (E160D) disrupts FEN1's two of three nuclease activities [28,41]. This mutant retains the ability to process the Okazaki fragments but is deficient in the EXO and GEN activities which are required for resolving secondary structures and stalled replication forks [28]. Previously we showed that mouse embryonic cells harboring this mutant showed chromosomal end to end fusions [32]. In a recent report the GEN activity was shown to be involved in replication fork restart at telomeres [27]. To determine whether the nuclease activity is involved in the formation of the FEN1/hTERT complex, we used E160D mutant in the co-IP experiment. As shown in Fig. 2B lane 10, this mutant showed decreased association with hTERT, suggesting that FEN1/hTERT complex may be under the influence of EXO and GEN activities of FEN1.

4. Discussion

FEN1 is a structure specific nuclease playing a role in DNA replication, repair and genome stability. Its involvement in multiple protein-protein interactions makes it a central player in various DNA metabolic pathways [42]. Our findings on the association between FEN1 and telomerase have prompted us to further analyze this association. In this report we present evidence that FEN1 associates with telomerase in a cell cycle regulated manner, with the maximal complex formation in the mid S phase (Fig. 1C). This is consistent with the time when telomerase and FEN1 are recruited to telomeres and also when the majority of telomeres undergo replication [18,33,36,43]. In human cells telomerase action occurs immediately following the telomere replication on the majority of telomeres in the S phase [36]. FEN1 may associate with telomerase for regulating the latter's activity, for example, by activating telomerase complex. Their association also strengthens the notion that replication and repair proteins may regulate telomerase action in higher eukaryotes.

By analyzing various mutants in FEN1 defective in either nuclease activity, PCNA binding, or substrate binding, we have identified that FEN1's C terminus and nuclease domain as the plausible regions involved in the complex formation. It has been shown that the flexible structural nature of C-terminus of FEN1 makes it an extensive interaction site for various protein-protein interactions [37]. Thus, the C terminus may act as a regulatory domain in coordinating various protein interactions, and FEN1 and hTERT complex may be one of them. On the other hand, this domain is also the binding site of WRN and BLM, both of which have been shown to be able to stimulate FEN1 activity [38,44]. It remains to be determined whether FEN1 stimulation by its interacting partners is required for this complex.

The PCNA binding motif in FEN1 is a conserved region required for its association with PCNA [24]. Binding of FEN1 to PCNA is critical for its function involving DNA substrates during DNA replication and repair [24,45]. Disruption of FEN1/PCNA leads to replication defects that are believed to be the cause for pulmonary hypoplasia, pancytopenia, and embryonic lethality [40]. Despite the importance of this domain, we found it dispensable for the formation of FEN1/hTERT complex, as neither the FFAA mutation nor ΔP affected the association (Fig. 2B). Surprisingly, our result showed that the removal of the P domain was able to rescue the weakened association caused by ΔC mutant (Fig. 2B), suggesting that the PCNA binding motif may be inhibitory to FEN1/telomerase formation. The significance of this region with respect to telomeric pathways is open for exploration.

Another mutant E160D showed a decrease in its association with hTERT, suggesting its influence over the complex. The E160D mutant was earlier shown to retain its ability to bind to DNA and is also partially catalytic in its function [41]. This mutant directs us to a notion

that the reduced association is probably due to the influence of EXO and GEN activities, for example, resolving the structural difficulties encountered during telomere replication.

Although we have mapped the regions in FEN1 involved in the formation of complex with hTERT, the functional significance of this association at telomeres remains to be determined. The possible functions of this complex are summarized in Fig. 3. One function may be to regulate the action of telomerase in extending telomeres. Studies in lower eukaryotes suggest that telomerase action is tightly regulated by the components of the lagging strand machinery [17]. In higher eukaryotes, more complex molecular mechanisms of telomerase action may take place. These may include assembly of higher order protein consortium comprising of telomere binding proteins, DNA replication and repair proteins and telomerase. Alternatively, FEN1 and telomerase complex in S phase may be required for switching from an inactive state to an active state, allowing telomerase to become functional immediately following replication. Finally, since FEN1 is a guardian of genome stability that plays an important role in faithful DNA replication and effective DNA repair, this complex could just be a part of a major complex coordinating crucial roles to maintain telomere stability. Collectively we suppose that FEN1 and hTERT complex could be participating in crucial events at telomeres. Understanding the role of this complex can pave way for telomerase based cancer therapeutics.

Acknowledgments

We thank L. Harrington at the Wellcome Trust Center for Cell Biology at the University of Edinburgh for FLAG-hTERT construct. This work is supported by NIH grant R15CA132090 to W.C.

Abbreviations used

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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Figure 1.

FEN1 associates with telomerase in a cell-cycle specific manner. **(A)** FACS analysis to determine the DNA content in HeLa cells synchronized at the G1/S boundary and then released into the S phase. Cells were collected at indicated time points for FACS analysis. **(B)** Representative image of FEN1 co-IP TRAP. FEN1 immunoprecipitates from synchronized HeLa cell lysates were subjected to nonradioactive TRAP assay for detection of telomerase activity. Aliquots equivalent to 25,000 cells were used in TRAP assay. ITAS represents the 36-bp internal TRAP assay standard. **(C)** Relative telomerase activity from five independent co-IP-TRAP assays was quantitated and the average was plotted. Error bars: s.d.

Figure 2.

(A) FEN1 mutations used in this study. All contain myc-tag at the N-terminus. FL: full length Myc FEN1. ΔC, ΔP, ΔPΔC and ΔPΔC+NLS represent deletions in the C terminus and PCNA binding regions. E160D and FFAA (F343A/F344A) represents single amino acid substitution mutants. (B) Myc-FEN1 along with FLAG-hTERT were co-transfected to 293T cells and co-IP was performed with anti-Myc antibody followed by anti-FLAG western blotting for detecting FLAG-hTERT. As negative controls single transfection of either FLAG-hTERT or pCI-neo empty vector (top panel lanes 3 and 4) were used. IP Input for both FLAG-hTERT and Myc-FEN1 (bottom panels) represents 20% of the total protein used in immunoprecipitation. Representative images from at least 4 independent experiments are shown.

Figure 3.
Possible roles of FEN1/hTERT association. See text for explanation.