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Identification of Human Rap1: Implications for Telomere Evolution

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Summary

It has been puzzling that mammalian telomeric proteins, including TRF1, TRF2, tankyrase, and TIN2 have no recognized orthologs in budding yeast. Here, we describe a human protein, hRap1, that is an ortholog of the yeast telomeric protein, scRap1p. hRap1 has three conserved sequence motifs in common with scRap1, is located at telomeres, and affects telomere length. However, while scRap1 binds telomeric DNA directly, hRap1 is recruited to telomeres by TRF2. Extending the comparison of telomeric proteins to fission yeast, we identify *S. pombe* Taz1 as a TRF ortholog, indicating that TRFs are conserved at eukaryotic telomeres. The data suggest that ancestral telomeres, like those of vertebrates, contained a TRF-like protein as well as Rap1. We propose that budding yeast preserved Rap1 at telomeres but lost the TRF component, possibly concomitant with a change in the telomeric repeat sequence.

Introduction

The protection and maintenance of human chromosome ends requires the function of two related factors, TRF1 and TRF2, which bind to the duplex telomeric TTAGGG repeat array (Chong et al., 1995; Bilaud et al., 1997; Broccoli et al., 1997). Inhibition of TRF2 in cultured cells results in immediate deprotection of chromosome ends as evident from the loss of the 3′ telomeric overhang and appearance of covalently fused telomeres (van Steensel et al., 1998; A. Smogorzewska and T. d. L., unpublished data). Compromised TRF2 function also leads to cell cycle arrest, ATM/p53-mediated apoptosis, and, in some cells, a senescence-like phenotype (van Steensel et al., 1998; Karlseder et al., 1999). Truncated forms of TRF1 and its interacting partner, TIN2, induce inappropriate telomere elongation in telomerase expressing cells (van Steensel and de Lange, 1997; Kim et al., 1999; Smogorzewska et al., 2000); overexpression of full-length TRF1 and TRF2 results in gradual telomeric decline (van Steensel and de Lange, 1997; Smogorzewska et al., 2000).

Although certain parallels between mammalian and yeast telomere function can be drawn, it has been puzzling that there is no ortholog of TRF1 and TRF2 at telomeres in budding yeast. Similarly, TIN2 and tankyrase, proteins associated with mammalian telomeres through their interaction with TRF1, have no recognized orthologs in yeast (Smith et al., 1998; Kim et al., 1999). Inversely, orthologs of a number of budding yeast telomeric proteins (for instance, Sir3p, Sir4p, Rif1p, Rif2p, Cdc13p, Stn1p, and Tel2p) have not been identified in mammals, although complete genome information will be required to settle this issue. The telomerase reverse transcriptase, Est2p, represents a striking exception to this theme, being obviously homologous to the human hTERT protein (Lendvay et al., 1996; Lingner et al., 1997; Nakamura et al., 1997). However, human orthologs of other yeast telomerase components, such as Est1p (Lundblad and Szostak, 1989) and Est3p (Lendvay et al., 1996), have not been found so far.

The lack of sequence similarity between the vertebrate TRF proteins and the yeast telomeric protein scRap1p (Shore and Nasmyth, 1987) is particularly striking given that both are duplex telomeric DNA binding factors and both act as negative regulators of telomere length (Kyrion et al., 1992; Marcand et al., 1997; van Steensel and de Lange, 1997; Smogorzewska et al., 2000). Rap1p is the major telomeric DNA binding activity in yeast (Buchman et al., 1988; Klein et al., 1992) and every chromosome end in yeast contains ~20 high-affinity Rap1p binding sites within its irregular TG_{1-3} telomeric repeat tract (Wang et al., 1990; Gilson et al., 1993). Additional Rap1p binding sites occur upstream of genes for many ribosomal proteins and glycolytic enzymes (for review, see Buchman et al., 1988; Capieaux et al., 1989), and Rap1p contributes to the regulation of these genes through a transcriptional activation domain located just C-terminal to its DNA binding domain (Hardy et al., 1992a). However, based on indirect immunofluorescence, Rap1p is concentrated at telomeres (Klein et al., 1992) and the telomeric function of Rap1p is better understood than its role in transcriptional regulation. Rap1p acts at telomeres by tethering two sets of proteins, the Sir proteins (Sir3p and Sir4p) (Moretti et al., 1994; Cockell et al., 1995; Liu and Lustig, 1996) and the Rif proteins (Rif1p and Rif2p) (Hardy et al., 1992b; Wotton and Shore, 1997). Recruitment of the Sirs to telomeres mediates the transcriptional repression of subtelomeric genes, whereas the Rifs control telomere length. Each of these four proteins binds to the C terminus of Rap1p, and tethering of this domain to telomeres in themselves can result in resetting of telomere length (Marcand et al., 1997). A second putative protein interaction domain, the BRCT (BRCA1 C-terminal) domain, was recognized in the N terminus of scRap1p based on sequence comparison (Bork et al., 1997; Callebaut and Mornon, 1997), but this part of scRap1p has yet to be ascribed a function.

It was previously noted that scRap1p, like TRF1 and TRF2, binds telomeric DNA with two Myb-type helix-turn-helix motifs (Chong et al., 1995; Konig et al., 1996; Bianchi et al., 1997, 1999; Smith and de Lange, 1997). However, this shared DNA binding mode is achieved using substantially different strategies. In scRap1p, two

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DNA binding modules are positioned in tandem in the middle of the polypeptide, while TRF1 and TRF2 each contain a single C-terminal Myb domain and use homodimerization to place two Myb domains on DNA (Bianchi et al., 1997, 1999; Broccoli et al., 1997). As a consequence of their different architecture, TRF1 and scRap1p recognize telomeric DNA in different ways. The two Myb domains of scRap1p recognize two tandem GGTGT sites at 8 bp distance (Konig et al., 1996), a sequence arrangement frequently represented in the yeast telomeric $TG_{1–3}$ repeat tracts. The two Myb domains in the TRF1 dimer, by contrast, interact with their YTAGGGTTR half sites regardless of their relative orientation and distance, as if the Myb domains are tethered by unusually flexible linkers (Bianchi et al., 1999). A Myb-type DNA binding domain is also present in the *S. pombe* protein Taz1, another telomeric protein that functions as a negative regulator of telomere length, but no other overt sequence similarity to the TRFs or Rap1p was recognized (Cooper et al., 1997; Vassetzky et al., 1999; Spink et al., 2000).

A search for TRF2-interacting proteins has revealed a novel human telomeric protein with a role in telomere length regulation that has significant sequence identity (24%–25%) with scRap1p in three different domains. Since this human protein has both structural and functional similarity to yeast Rap1p, we refer to it as hRap1. In the context of the identification of human Rap1, we reexamined the structural similarities of the telomeric protein complex in *S. cerevisiae*, *S. pombe*, and vertebrates, revealing previously unappreciated relationships and suggesting a model for the evolution of eukaryotic telomeres.

Results

A TRF2-Interacting Protein with Sequence Similarity to Yeast Rap1p

A standard yeast two-hybrid screen of HeLa cDNAs with part of the human TRF2 protein as bait yielded a partial 1.35 kb cDNA clone (referred to as M50–27) that was used to isolate a 2.67 kb cDNA (Figure 1A). This cDNA appears to represent the full-length mRNA which is detectable as an ~2.5 kb species with a ubiquitous expression pattern (Figure 1B). The open reading frame in this cDNA predicts a 399 aa protein of ~47 kDa and a Profile Scan motif search revealed that the ORF contained an N-terminal BRCT domain and a central Myb-type helix-turn-helix motif. The ORF also predicted an acidic C terminus (aa 214–382, pI around 3.8) featuring a predicted 33 aa coiled–coil region and a bipartite NLS (Figures 1A and 1C). Since yeast Rap1p has an N-terminal BRCT domain (Bork et al., 1997; Callebaut and Mornon, 1997), central Myb domains (Konig et al., 1996), and an acidic C terminus, we examined the sequence similarity of the TRF2-interacting protein and Rap1p in more detail. Alignments revealed an additional region of sequence similarity in the C termini of the human and yeast proteins that coincides with the main protein–protein interaction domain of scRap1p (Hardy et al., 1992b; Moretti et al., 1994; Cockell et al., 1995; Liu and Lustig, 1996; Wotton and Shore, 1997) (Figure 1F). Thus, the TRF2-interacting protein has three domains in common with Rap1p, including a region unique to Rap1p, suggesting that it is a human ortholog of Rap1p. We therefore refer to this protein as hRap1.

The BRCT domain is an ~95 aa presumed protein–protein interaction motif first found in BRCA1, p53BP1, and Rad9p (Koonin et al., 1996) and now recognized in a large number of other proteins linked to DNA repair (Bork et al., 1997; Callebaut and Mornon, 1997). X-ray crystallography of the XRCC1 second BRCT domains revealed an autonomously folded domain composed of a four-stranded parallel β sheet surrounded by three α helices (Zhang et al., 1998). Although the BRCT domains of the three Rap1p orthologs are highly diverged, most of the conserved patches of hydrophobic residues can be recognized by alignment to the BRCT domains of XRCC1 (Figure 1D).

Yeast Rap1p contains two HTH DNA binding domains as revealed by structural analysis (Konig et al., 1996). The first of these domains (called R1) can be recognized as a Myb motif in sequence alignments, although it is highly diverged from the Myb consensus sequence (Larson et al., 1994; Lipsick, 1996; Konig and Rhodes, 1997). ScRap1p R1 has some notable peculiarities, including a long linker between helix 1 and helix 2, and the presence of phenylalanine and tyrosine residues instead of the three highly conserved tryptophan residues typical of Myb domains (Konig et al., 1996; Konig and Rhodes, 1997). Similarly, the hRap1 Myb motif has phenylalanine and tyrosine at two of the three tryptophan positions and it carries an insert of ~10 aa between helix 1 and 2, consistent with a close relationship between the human and yeast Rap1 genes (Figure 1E). Furthermore, the Myb domains of human and yeast Rap1 both lack the conserved glycine just beyond helix 1 and they do not have a patch of aliphatic residues beyond helix 2. Finally, both human and yeast Rap1 have a histidine residue at the position of the highly conserved arginine preceding helix 3. In order to examine the relationship between several relevant Myb domains, we removed the variable spacers between the α helices that can bias sequence comparisons to favor similarly sized polypeptides. The resulting alignment underscores a stronger similarity of the human and yeast Rap1 Myb domains compared to the Myb domains of TRF1, the *S. pombe* Taz1, and *S. cerevisiae* Tbf1, each of which are more closely related to R1/R2 of c-Myb. It was previously pointed out that the Myb domains of the TRF/Taz/Tbf group show certain similarities in their recognition helix (helix 3) (Bilaud et al., 1996), and some of these (notably the motif LKD) are also present in hRap1. However, the Myb domains of human and yeast Rap1 are clearly more closely related to each other than to the Myb domains of the TRF/Taz1/Tbf1 group.

The C-terminal quarter of scRap1p can be divided into a transactivation domain of about 65 aa and a more terminal domain (sometimes referred to as silencing domain, SD) that confers telomeric silencing and length regulation (Hardy et al., 1992a; Kyrion et al., 1992, 1993; Liu et al., 1994; Buck and Shore, 1995; Liu and Lustig, 1996). This part of Rap1p binds Rif1p and Rif2p and interacts with Sir3p and Sir4p (Hardy et al., 1992b; Moretti et al., 1994; Cockell et al., 1995; Liu and Lustig, 1996; Wotton and Shore, 1997). Alignment of hRap1 with the C terminus of scRap1p reveals significant sequence

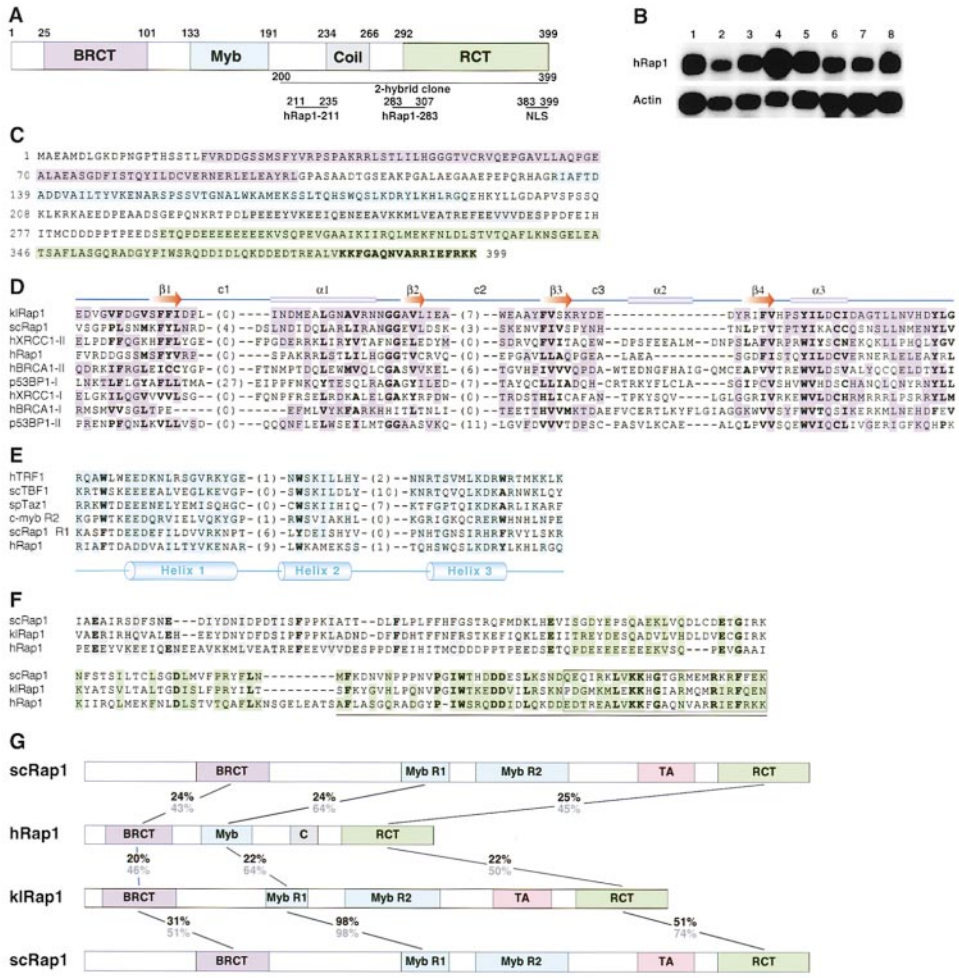

Figure 1. Sequence and Expression of the Human Ortholog of Yeast Rap1p

(A) Domain structure of hRap1. BRCT, BRCA1 C terminus; Myb, Myb-related HTH motif; Coil, predicted coiled domain; RCT, homology to the Rap1p C terminus; NLS, nuclear localization signal. The original two-hybrid clone and peptides used for raising antibodies are indicated.

(B) Ubiquitous expression of hRap1 mRNA. Northern blot (Clontech) probed with the original hRap1 two-hybrid clone or β-actin. Lane 1, spleen; lane 2, thymus; lane 3, prostate; lane 4, testis; lane 5, ovary; lane 6, small intestine; lane 7, colon (mucosal lining); lane 8, peripheral blood leukocytes.

(C) Amino acid sequence of hRap1. The BRCT, Myb, Coil, and RCT domains are colored as in (A). The putative NLS is shown in bold.

(D) Alignment of BRCT domains in hRap1 and several other proteins based on the XRCC1 BRCT structure (Bork et al., 1997; Zhang et al., 1998). Four β sheets (red arrows) and three α helices (blue bars) are marked. Bold residues are highly conserved (Bork et al., 1997), and other sequence similarities (Kyte-Doolittle) are shaded.

(E) Alignment of the Myb domains of Rap1 with human c-Myb R2, TRF1, spTaz1, and scTbf1p using ClustalW. The position of three α helices in c-Myb (Ogata et al., 1994) are indicated. Omitted amino acids between the α helices are indicated in parentheses. The three most conserved tryptophans are in bold. Shaded residues highlight sequence similarities (Kyte-Doolittle).

(F) Alignment of the C-terminal region of three Rap1 proteins using ClustalW. Green shading highlights sequence similarities in the RCT domain (represented by the C-terminal 108 aa of hRap1). Identities in all three Rap1s are in bold. The most conserved 52 aa are underlined. The boxed region indicates the C-terminal tail domain of scRap1p (Liu and Lustig, 1996).

(G) Overview of the sequence similarities in three Rap1 proteins listing percentage identities (black) and similarities (gray) in each pair-wise comparison.

similarity in the final 108 aa of these proteins (Figure 1F), which we refer to as the *R*ap1 *C T*erminus (RCT) domain. The highest level of conservation is found in the final 52 aa where there is 30% identity between human and scRap1p and 49% identity between the Rap1p proteins from *K. lactis* and *S. cerevisiae* (Figure 1F). Although its structure is not yet known, this part of the Rap1 proteins is likely to have an identical fold.

An overall summary of the similarities between the human and yeast Rap1 proteins is given in Figure 1G. We have not found significant similarity between human and yeast Rap1p outside the three domains mentioned above. Notably, the *trans*-activation domain of yeast Rap1p is not represented as such in hRap1p. This region, positioned between the Myb domain and the RCT domain, is not conserved in sequence and hRap1 has a predicted coiled domain that is not present in the yeast Rap1ps. Furthermore, we were unable to detect

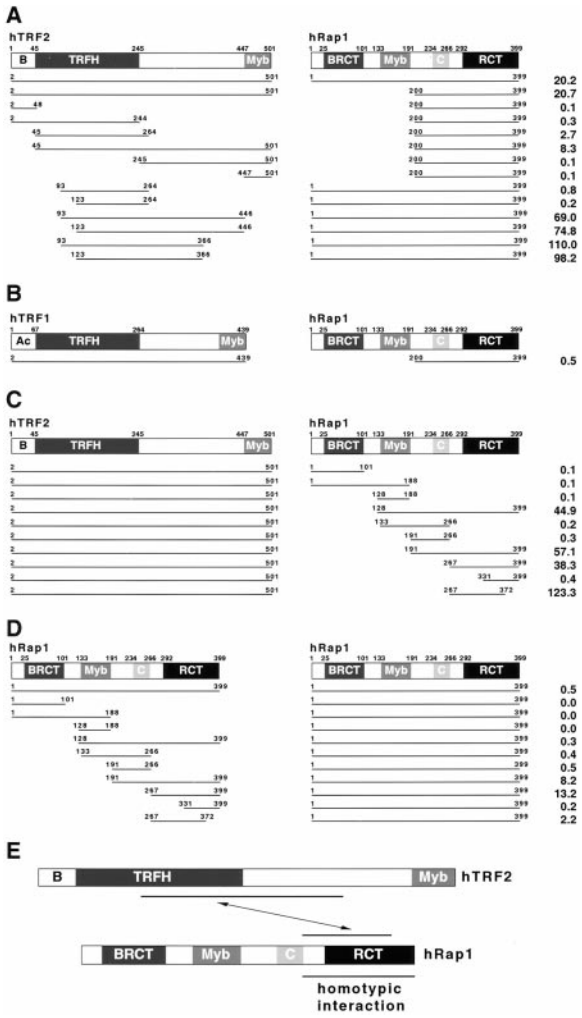

Figure 2. Two-Hybrid Mapping of hRap1–TRF2 and hRap1–hRap1 Interacting Domains

(A) Mapping the hRap1-interacting domain in TRF2. (B) hRap1 does not interact with TRF1. (C) Mapping the TRF2-interacting domain in hRap1. (D) hRap1 can interact with itself through the RCT domain. (E) Summary of the TRF2-hRap1 interactions. Constructs on the left in (A, B, and D) and constructions on the right in (C) show LexA fusions. No transactivation activity was detected for all LexA-hRap1 fusion proteins. Constructs on the right in (A, B, and D) and constructions on the left in (C) represent GAD fusions. β-galactosidase levels (right column) were measured as described in the Experimental Procedures. The values represent an average of three independent transformants. Values <0.05 are indicated as 0.

transactivation with this or other parts of hRap1 in a LexA fusion context (see below, Figure 2D).

The C Terminus of hRap1 Is a Protein Interaction Domain

The yeast two-hybrid system was used to delineate the regions in TRF2 and hRap1 responsible for their interaction. Deletion mapping indicated that hRap1 associated with a central domain of TRF2 located between positions 123 and 366 (Figure 2A). Although TRF2 and TRF1 show significant sequence similarity in the N-terminal half of this region (~29% in the region from aa 45 to 245 in TRF2 [Broccoli et al., 1997]), hRap1 does not interact with full-length TRF1 in a two-hybrid setting (Figure 2B).

The TRF2-interacting domain in hRap1 was mapped to the C terminus (Figure 2C). The RCT domain in scRap1p is required for interaction with Rif1p, Rif2p, Sir3p, and Sir4p (Hardy et al., 1992b; Moretti et al., 1994; Cockell et al., 1995; Liu and Lustig, 1996; Wotton and Shore, 1997). Similarly, deletion mapping indicated that a region between aa 267 and 372 in the C terminus of hRap1 is responsible for the binding to TRF2 (Figure 2C). Yeast two-hybrid experiments also suggested homotypic interactions in the RCT domain. The area required for homotypic interactions in hRap1 is more extensive than that needed for binding to TRF2 and requires sequences from aa 267 to the very C terminus of the protein, including the last 27 aa (Figure 2D). The final 8 aa of the scRap1p RCT domain have been shown to be required for the interaction with Sir3p (Liu and Lustig, 1996). Consistent with the detection of homotypic interactions in the yeast two-hybrid assay, recombinant hRap1 behaves as a dimer in gel-filtration chromatography (D. Rhodes, personal communication). A summary of all the interaction data is given in Figure 2E.

TRF2 Recruits hRap1 to Telomeres

The subcellular localization of hRap1 was examined by indirect immunofluorescence (IF) of cultured human cells (Figure 3). A polyclonal rabbit antibody (Ab 765) was raised against $[His]_6$-tagged hRap1 expressed in a baculovirus system and subjected to affinity purification. This reagent revealed a prominent punctate nuclear pattern in several different human cell lines (HeLa cervical carcinoma, HT1080 fibrosarcoma cells, and IMR90 primary fibroblasts) (Figures 3A and 3D and data not shown). The same telomeric pattern was seen in interphase nuclei stained with an hRap1-specific, affinity-purified, anti-peptide antibody (Ab 666 directed against aa 211–235, see Figure 1A) (Figure 3G), and exogenously expressed FLAG-tagged hRap1 also localized to telomeres (data not shown). Dual IF with antibodies for TRF1 showed that the majority of hRap1 colocalized with TRF1 in interphase nuclei (Figures 3A–3C). Since it was previously shown that TRF1 is found exclusively at sites of telomeric TTAGGG repeats (Chong et al., 1995), the colocalization of hRap1 with TRF1 indicated that hRap1 is predominantly located at telomeres. Similarly, hRap1 colocalized with TRF2 in all cells examined (Figures 3D–3F). The telomeric localization of hRap1 is further corroborated by IF analysis of metaphase chromosomes which showed an exclusively terminal signal with hRap1 Ab 666 (Figure 3J). We surmise from these data that, as in yeast, human Rap1 is concentrated at telomeric loci throughout the cell cycle.

We next asked whether the accumulation of hRap1 at telomeres depended on its interaction with TRF2, as suggested by the two-hybrid interaction of these proteins and their coimmunoprecipitation (X.-D. Zhu et al., submitted). To this end, we examined HeLa cells which were transiently transfected with the dominant-negative allele of TRF2 ($TRF2^{\Delta B \Delta M}$), a truncated version of TRF2 that forms inactive heterodimers with the endogenous protein and effectively removes TRF2 from telomeres (van Steensel et al., 1998). HeLa cells transfected with this construct were examined by IF for the presence of TRF2 on telomeres after gentle extraction with Triton X-100 to remove the nucleoplasmic proteins. A subpopulation of the cells showed a severe loss of the punctate

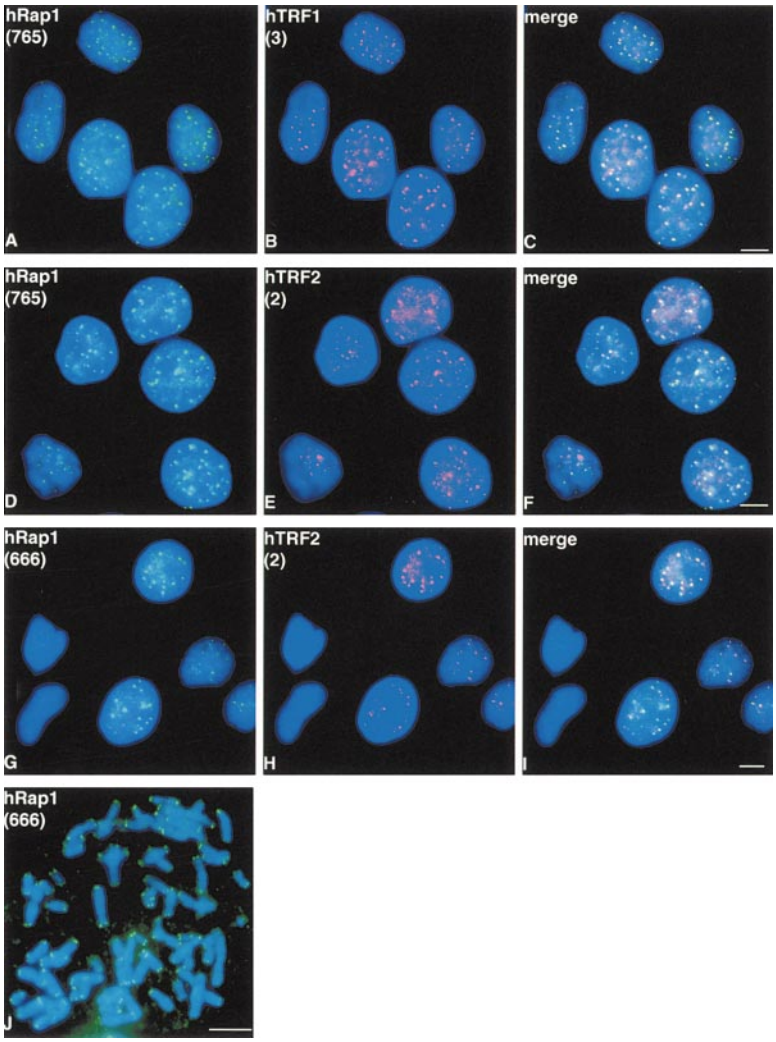

Figure 3. hRap1 Locates to Telomeres through Interaction with TRF2

(A and D) Localization of endogenous hRap1 using indirect immunofluorescence with antibody 765 (green) in interphase nuclei of HeLaI.2.11 cells. (B) Localization of TRF1 in the same cells as in (A) using mouse antibody against TRF1 (red). (C) is a superimposition of (A) and (B). (E) Localization of TRF2 in the same cells as in (D) using mouse antibody against TRF2 (red). This antibody shows some staining at nontelomeric sites. (F) is a superimposition of (D) and (E). (G) Localization of endogenous hRap1 using antibody 666 (red) in interphase nuclei of HeLaI.2.11 cells transiently transfected with TRF2$^{\Delta B \Delta M}$. The cells were extracted with Triton X-100 before fixation. (H) Localization of TRF2 in the same cells as in (G) using mouse antibody against TRF2 (green). (I) is a superimposition of (G) and (H). (J) Localization of endogenous hRap1 using antibody 666 (green) on metaphase chromosomes in HeLaI.2.11 cells. DNA was stained with DAPI (blue). Bars in (C), (F), (I), and (J) represent 5 μm.

TRF2 pattern, as expected from previous results with the TRF2$^{\Delta B \Delta M}$ allele (van Steensel et al., 1998) (Figure 3H). In dual IF experiments for both TRF2 and hRap1, we found a close correlation between the accumulation of the two proteins in a punctate pattern (Figures 3G–3I). Specifically, all cells that had retained TRF2 at telomeres showed hRap1 at the same sites whereas cells that had lost the punctate TRF2 pattern (presumably due to expression of the TRF2$^{\Delta B \Delta M}$ allele) also lacked hRap1 dots. Based on these data, interaction with TRF2 appears to be the predominant manner by which hRap1 accumulates on telomeres. In agreement, telomeric accumulation was observed with a C-terminal fragment of hRap1 (aa 184 to the C terminus) which retains the TRF2 interacting domain, but lacks the BRCT and Myb domains (data not shown).

TRF2 and hRap1 Form a Complex on Telomeric DNA

Since hRap1 carries a Myb motif, it was pertinent to examine its interaction with DNA. Using a double-stranded [TTAGGG]$_{12}$ probe and a bandshift assay that allows binding of TRF2 to this probe, we failed to detect a protein–DNA complex with baculovirus-expressed hRap1 (Figure 4A). Although a large number of parameters were varied in additional experiments (for instance, gel conditions, reaction buffer, and protein preparations), we did not find evidence for the binding of telomeric repeat DNA by hRap1. We also failed to detect a complex of hRap1 with single-stranded telomeric DNA or with duplex probes containing the yeast telomeric TG$_{1–3}$ repeats (data not shown).

When hRap1 was incubated with TRF2 and a ds[TTAGGG]$_{12}$ probe, a ternary complex was formed (Figure 4B). Titration of TRF2 on DNA resulted in two complexes with different mobility (Figures 4A and 4B). The slower migrating complex appeared only at high protein concentrations and presumably represents a complex containing a larger number of DNA-bound TRF2 molecules. When hRap1 was added to the reaction, we observed an additional complex that migrated above the two TRF2 complexes (Figure 4B). The appearance of this new band was strictly dependent on the addition of hRap1 and was not observed in bandshift reactions with hRap1 and TRF1 (data not shown). A supershift assay with an affinity-purified, anti-peptide antibody directed to aa 283 to 307 of hRap1 (Ab 664, see Figure 1A) demonstrated that the hRap1-dependent

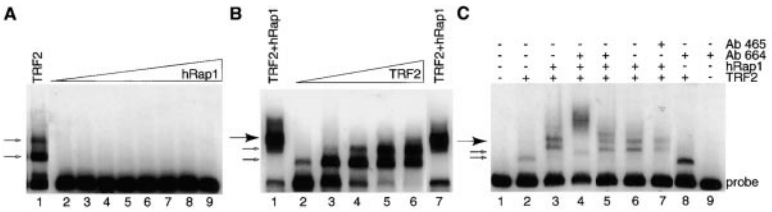

Figure 4. hRap1 Binds to TRF2-Telomeric DNA Complexes

(A) Lack of telomeric DNA binding activity for hRap1. Bandshift assays with hRap1 and a ds(TTAGGG)$_{12}$ probe. The highest concentration of hRap1 is 20 pM and the dilution series represents 3-fold steps. Lane 1 represents reaction with 1 pM TRF2. (B) hRap1 and hTRF2 form a ternary complex. Bandshift assays with increasing amounts of TRF2 (lanes 2 to 6, 3-fold steps up to 20 pM) or TRF2 and hRap1 protein (lanes 1 and 7, 2 pM of each protein). (C) The TRF2/hRap1 specific complex can be super-shifted by antibodies against hRap1. Bandshift assays with ~2 pM of TRF2 and hRap1 and antibodies to hRap1 (Ab 664) and tankyrase (Ab 465) as indicated above the lanes. Lanes 4, 7, 8, and 9 contain 250 ng of antibody. Lane 5 contains 50 ng of antibody. Small arrows, TRF2 complex. Large arrows, hRap1-TRF2 complex.

bandshift complex indeed contained hRap1 protein (Figure 4C). An antibody to the TRF1-interacting protein, tankyrase (Ab 465), served as a negative control in this experiment (Figure 4C). These data are consistent with the observation that hRap1 binds to telomeres in a TRF2-dependent manner.

Although hRap1 does not appear to bind DNA by itself, we considered the possibility that hRap1 might interact with DNA in the context of the TRF2 complex. Such additional protein–DNA contacts often result in cooperative binding. An example is the interaction of two DNA binding proteins, Mcm1 and α2, which bind to each other on certain yeast operators, and display strong cooperative effects (>10-fold enhancement) in bandshift assays with the appropriate target DNAs (Keleher et al., 1989; Smith and Johnson, 1992). However, we failed to detect significant cooperativity for TRF2 and hRap1 under the conditions that allow detection of the ternary complex. No enhanced binding was detectable when hRap1 was added to TRF2 band-shift reactions (for instance, compare lanes 4 and 7 in Figure 4B and lanes 2 and 3 in Figure 4C). In addition, we failed to detect an effect of hRap1 on the off-rate of the complexes (data not shown). We note that in some cases, additional protein DNA contacts are known to have a relatively minor cooperative effect. For instance, the binding of the second Myb domain in TRF1 homodimers only increases the affinity by a factor of 10 (Bianchi et al., 1999). Footprinting analysis of the TRF2-hRap1 complex might provide further insight into this issue.

hRap1 Affects Telomere Length

ScRap1p has two well-defined functions at yeast telomeres. First, scRap1p recruits Sir3p and Sir4p to telomeres, resulting in the establishment of a heterochromatic state that represses the expression of nearby genes (Moretti et al., 1994; Cockell et al., 1995; Liu and Lustig, 1996). Because we and others have failed to demonstrate telomeric silencing in human cells (Broccoli and Cooke, 1994; H. Tommerup and T. d. L., unpublished data), exploration of this potential role for hRap1 is currently not possible. The second known function of scRap1p at chromosome ends is the regulation of telomere length (Conrad et al., 1990; Lustig et al., 1990; Kyrion et al., 1992; Liu et al., 1994; Marcand et al., 1997). Tethering experiments have demonstrated that cells evaluate telomere length based on the number of scRap1p C-terminal domains present at chromosome ends (Marcand et al., 1997). The current "protein-counting" model for telomere length homeostasis dictates that long telomeres recruit a greater number of Rap1p molecules, resulting in inhibition of telomere extension in *cis*. How the C terminus of Rap1p regulates telomere elongation has not been clarified, but, the relevant part of Rap1p interacts with two proteins, Rif1 and Rif2, each of which contributes to telomere length homeostasis (Hardy et al., 1992b; Wotton and Shore, 1997).

In order to explore a function for hRap1 in telomere length regulation, we used a previously developed Tetracyclin (Doxycyclin)-inducible system in the human fibrosarcoma cell line HTC75 (an HT1080 derivative) (van Steensel and de Lange, 1997). HTC75 cells express high levels of telomerase, yet maintain their telomeres at a stable length setting over long periods of growth (300 population doublings [PD]). The stable length setting of HTC75 telomeres depends on TRF1 and TRF2 (van Steensel and de Lange, 1997; Smogorzewska et al., 2000). Overexpression of TRF1 or TRF2 results in gradual telomeric decline although telomerase levels are unaffected.

We derived five independent clonal HTC75 cell lines (F9, F11, F13, F14, and F17) overexpressing full-length, FLAG-tagged hRap1 in an inducible manner. In each cell line, Western analysis demonstrated inducible expression of an hRap1 protein that appeared slightly larger than endogenous hRap1, as expected from the presence of the FLAG tag (Figure 5A). Induction of hRap1 in these cell lines did not affect the expression of endogenous hRap1 (Figure 5A) or other telomeric proteins (including TRF1, TRF2, and tankyrase) or telomerase activity (data not shown).

The effect of hRap1 on telomere length homeostasis was determined by measuring telomere length changes during prolonged culturing of the cells. A control cell line (B31) transfected with the empty vector displayed no changes in telomere length in the presence or absence of doxycyclin (Figure 5B). Of five hRap1 expressing lines, one (F11) showed erratic telomere length changes under uninduced conditions and was not studied further. The other cell lines showed consistent changes in telomere dynamics, in each case demonstrable as a gradual elongation in the mean length of the telomeric restriction fragments (Figures 5C–5E and data not shown). The growth of the telomeres was largely dependent on the induction of hRap1 expression, although leaky expression of hRap1 under repressed conditions may have led to gradual telomere extension in the lines F9 and F13. The maximum telomere elongation rate in cells overexpressing hRap1 was 40–50 bp/PD (based on the early stages of the F9 and F17 growth

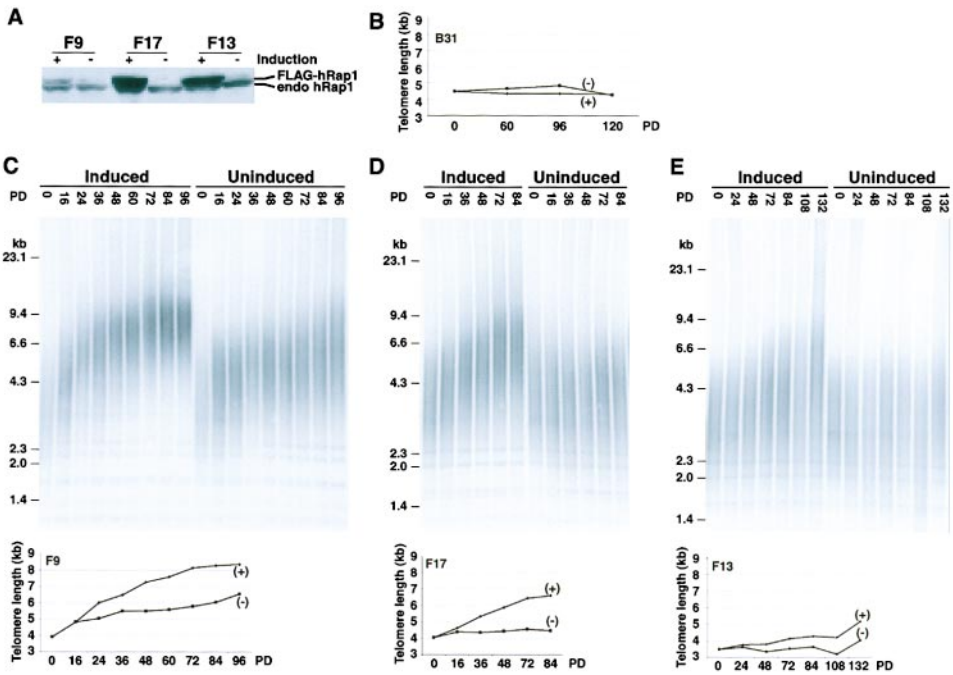

Figure 5. Overexpression of Full-Length hRap1 Results in Telomere Elongation

(A) Western analysis of the inducible expression of the FLAG-tagged hRap1 in three F cell lines. Extracts were prepared from cells grown in parallel in the presence (uninduced) or absence (induced) of doxycyclin for four population doublings and probed with the hRap1 antibody 765 that detects both the endogenous hRap1 and the induced, FLAG-tagged hRap1.

(B) Telomere length is maintained stably in clonal HTC75 line B31 containing empty expressing vector pUHD10–3. Chart showing the median telomere lengths versus PD in cells grown under induced (+) or uninduced (−) conditions.

(C–E) Time course of changes in telomere length in clonal HTC75 lines expressing FLAG-tagged hRap1 (F9 in C, F17 in D, and F13 in E) grown in the absence and presence of doxycyclin (+ and − induction, respectively). Top, genomic blotting analysis of telomeric restriction fragments in HinfI/RsaI digested DNA, probed with a TTAGGG repeat probe. Bottom, chart showing the changes of median telomere lengths in each corresponding cell line.

curves). Telomere growth occurred at a rate of 30 bp/PD in F14 (data not shown). However, much more modest rates of telomere elongation were found in F13, which showed extension at a rate of 17 bp/PD (Figure 5E). Similar clonal variations in response were previously observed with HTC75 clones expressing TRF1 (van Steensel and de Lange, 1997).

Taz1 Is a Member of the TRF Protein Family

Prompted by the identification of the ortholog of yeast Rap1p at human telomeres, we examined the possibility of other such relationships. Taz1 was identified in *S. pombe* by a one-hybrid screen using telomeric DNA and also by a screen for loss of telomeric silencing (Cooper et al., 1997; Nimmo et al., 1998). The ORF of Taz1 showed a Myb domain near the C terminus but no other sequence similarity to TRFs or Rap1p had been recognized. The identification of the key sequences conserved in scRap1 and hRap1 showed that Taz1, which lacks the BRCT and the RCT domains, is not a member of this family.

TRF1 and TRF2 have been identified in mice and humans and sequence alignments have shown that in addition to their C-terminal Myb motif, the TRFs have a defining sequence motif near their N termini. This domain, called TRF homology (TRFH) domain here, is a region of ~200 aa with unknown function, although it overlaps with protein–protein interaction domains in both TRF1 and TRF2 (Bianchi et al., 1997; Broccoli et al., 1997; Kim et al., 1999). The mammalian TRFs have approximately 29% sequence identity in this region (Broccoli et al., 1997; Smith and de Lange, 1997). Alignment of Taz1 with this part of TRF1 and TRF2 revealed significant similarity to this central domain (Figure 6A). Using standard ClustalW settings, Taz1 can be aligned to TRF1 yielding 24% sequence identity and 47% similarity over 180 aa (Figures 6A and 6B).

Thus, Taz1 has two sequence motifs in common with TRF1 and TRF2: a Myb domain that is substantially more similar to that of the TRFs than to scRap1p R1 (Figure 1E) and the TRFH domain (Figure 6A). Furthermore, the overall architecture of Taz1 seems to resemble that of the TRFs since each of these proteins bind to DNA as a homodimer (Bianchi et al., 1997; Broccoli et al., 1997; van Steensel et al., 1998; Spink et al., 2000). Finally, Taz1, like the TRFs, acts as a negative regulator of telomere length (Cooper et al., 1997). Based on these structural and functional similarities, we suggest that Taz1 is a member of the TRF family of telomeric proteins.

It is not clear whether Taz1 is more closely related to TRF1 or to TRF2. Although the TRFH and Myb domains of Taz1 are more similar to those of TRF1, the region between the TRFH and the Myb domain bears more similarity to TRF2. The TRF1 and TRF2 proteins are

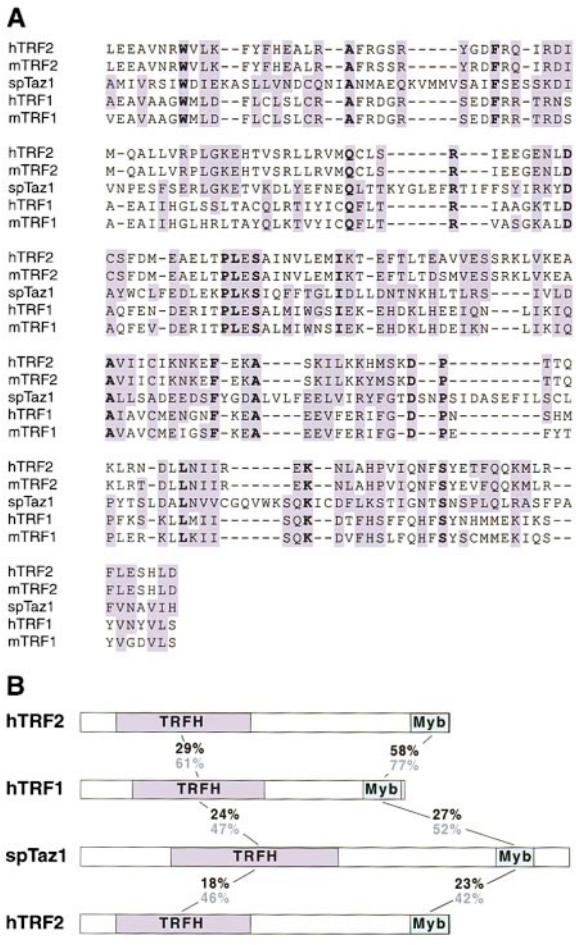

Figure 6. *S. pombe* Taz1 Is a Member of the TRF Family

(A) Alignment of the TRFH domain of mouse and human TRF1, mouse and human TRF2, and *S. pombe* Taz1 using ClustalW. Identical residues are in bold; sequence similarities are shaded (Kyte-Doolittle).

(B) Overview of the sequence similarities in TRF and Taz1 proteins, listing percentage identity (black) and similarity (gray).

paralogs with strikingly different N termini (Chong et al., 1995; Broccoli et al., 1997). The first 45 aa of TRF2 are very basic, while the N terminus of TRF1 is acidic. In contrast, the N terminus of Taz1 has no overriding acidic or basic nature.

Discussion

Proteins that bind to the duplex part of the telomeric repeat array have been studied in vertebrates and in the yeasts *S. cerevisiae* and *S. pombe*. The identification of the human ortholog of the budding yeast telomeric protein Rap1p now suggests an evolutionary relationship between the telomeric complexes in these three systems. We propose that telomeres in vertebrates and in *S. pombe* represent an ancestral situation in which a TRF-like protein and a Rap1-like factor both functioned at telomeres, whereas the arrangement found in *S. cerevisiae* is apparently different, representing telomeres without the TRF module. We propose that the telomeric complex in *S. cerevisiae* evolved through a combination of gene loss and nonorthologous gene displacement and that these changes were accompanied by an alteration in the telomeric DNA sequence. Our findings may allow a more meaningful comparison of telomere biology in yeast, vertebrates, and other eukaryotes.

Structural and Functional Similarities of Human and Yeast Rap1

Human Rap1 has three domains in common with yeast Rap1p. It contains a central Myb motif with specific sequence features also present in the yeast Rap1p R1 Myb domain. In addition, human Rap1, like yeast Rap1p, carries a BRCT domain at the N terminus and the Rap1 proteins share a conserved motif (the RCT domain) in their C termini. Functionally, hRap1 also displays strong similarities to scRap1p. Like the yeast protein, hRap1 is an integral component of the telomeric complex and the majority of the protein is detectable at chromosome ends by indirect immunofluorescence. Furthermore, in both yeast and human Rap1, the C terminus of the protein functions to bind other telomeric proteins.

Yeast and human Rap1 may also be similar with regards to their effect on telomere maintenance. Yeast Rap1p acts as a negative regulator of telomere length. A number of rap1 mutants in yeast display telomere elongation phenotypes (Kyrion et al., 1992; Liu et al., 1994) and mutation of the Rap1p binding sites in yeast telomeres can result in run-away telomere elongation (McEachern and Blackburn, 1995; Krauskopf and Blackburn, 1996). Furthermore, the scRap1p-interacting factors, Rif1p and Rif2p, display characteristics consistent with a negative regulatory pathway that limits telomere elongation (Hardy et al., 1992b; Wotton and Shore, 1997). Since targeting the Rap1p C-terminal domain to subtelomeric sites results in shortened telomeres (Marcand et al., 1997), it was suggested that Rap1p is a *cis*-acting negative regulator of telomere maintenance and that cells measure telomere length through counting the number of Rap1p C-terminal domains at individual chromosome ends.

Moderate overexpression of scRap1p results in slight telomere elongation (Conrad et al., 1990), most likely because the increased abundance of scRap1p titrates a limiting component required for inhibition of telomere extension (e.g., one of the Rif proteins). The phenotype of overexpression of human Rap1 is very similar. Increased hRap1 level in the telomerase positive cell line HTC75 resulted in a moderate telomere elongation. It is possible that the mild telomere extension phenotype is due to titration of an hRap1-interacting protein that is required for stable telomere length maintenance. It will be necessary to establish the effect of diminished hRap1 activity to settle this issue.

Although the hRap1-interacting partner TRF2 is required for the protection of chromosome ends (van Steensel et al., 1998; Karlseder et al., 1999), it has yet to be established whether hRap1 contributes to this function. A possible role for yeast Rap1p in maintaining telomere integrity is suggested by the instability of telomeres in rap1–17 cells (Kyrion et al., 1992). In addition, overexpression of yeast Rap1p induces a mild chromosome instability phenotype (Conrad et al., 1990) and

several rap1^{ts} mutants have effects consistent with scRap1p acting to protect chromosome ends (Lustig et al., 1990). Like inhibition of TRF2, complete loss of Rap1p in yeast is lethal but this phenotype may be primarily due to nontelomeric functions.

A BLAST search with full-length hRap1 against the budding yeast genome did not yield scRap1p or klRap1p. Similarly, a search with Taz1 yielded several Myb motif proteins but not the TRFH domains of any of the four mammalian TRFs represented in the nonredundant database. These findings illustrate current difficulties in finding highly diverged but biologically informative orthologs. Precise definition of the most conserved motifs in the TRF and Rap1 families may facilitate the identification of telomeric proteins in other organisms.

S. pombe Taz1 Is a Member of the TRF Family

We propose that Taz1 is a member of the TRF family of duplex telomeric DNA binding proteins. This family is defined by the presence of two regions of sequence similarity: a Myb motif that is closely related to c-Myb R1/R2 and a TRF homology domain. TRF family proteins undergo homotypic interactions and bind telomeric DNA as dimers or higher order oligomers (Bianchi et al., 1997; Broccoli et al., 1997; Spink et al., 2000). In addition, each of these proteins have a negative effect on telomere length maintenance in telomerase expressing cells (Cooper et al., 1997; van Steensel and de Lange, 1997; Smogorzewska et al., 2000).

Taz1 has two additional functions. First, it suppresses a telomerase-independent pathway of telomere maintenance, possibly by interfering with recombination at telomeres (Nakamura et al., 1998). This function is reminiscent of the protective activity of TRF2, which appears to suppress inappropriate DNA repair at mammalian telomeres (van Steensel et al., 1998). Secondly, Taz1 plays a role in meiosis. Taz1 mutants display a defect in the horse-tail stage of meiosis I in *S. pombe* (Cooper et al., 1998; Nimmo et al., 1998), in which telomeres associate with the spindle pole body and facilitate the rapid migration of the meiotic nucleus through the cell (Chikashige et al., 1994, 1997; Kohli, 1994). This telomere-led movement critically depends on Taz1, with taz1^{-} cells showing loss of clustering of telomeres at the spindle pole body and a marked drop in meiotic recombination and spore viability. It will be of interest to determine whether TRF1 or TRF2 has a role in mammalian meiosis, which features an analogous chromosome reorganization in the bouquet stage of meiosis I (see for review de Lange, 1998).

Evolution of Eukaryotic Telomeres

The recognition of *S. pombe* Taz1 as a member of the TRF family suggests that a TRF-like protein originally was engaged on telomeres in the last common ancestor of fungi and vertebrates. According to this view, *S. cerevisiae* most likely lost the TRF-like telomere binding component at some point during the divergence of the Hemi- and Eu-ascomycetes (represented in Figure 7B by budding yeasts and *Neurospora*, respectively).

Given that human TRF2 facilitates the binding of hRap1 to telomeres, this may have been one of the original functions of the ancestral TRF (Figure 7A). Thus, it is possible that Taz1 also recruits a Rap1 to telomeres. Complete genome information on *S. pombe* may reveal the existence of a Rap1 ortholog. It will be of interest to determine whether such an *S. pombe* Rap1 has one or two Myb domains. ScRap1p has two Myb domains and binds to telomeres through a direct interaction with the telomeric DNA, while human Rap1 has a single Myb motif and has no known telomeric DNA binding activity. Thus, either scRap1p has gained a Myb domain or hRap1 has lost one. Determination of the telomeric DNA binding activity and the number of Myb domains in Rap1 in *S. pombe* or another fungus could provide insight into this issue. It is of obvious interest to establish the function of the single Myb motif in hRap1.

The genome of *S. cerevisiae* encodes one protein with a TRF-like Myb domain, the TTAGGG repeat binding

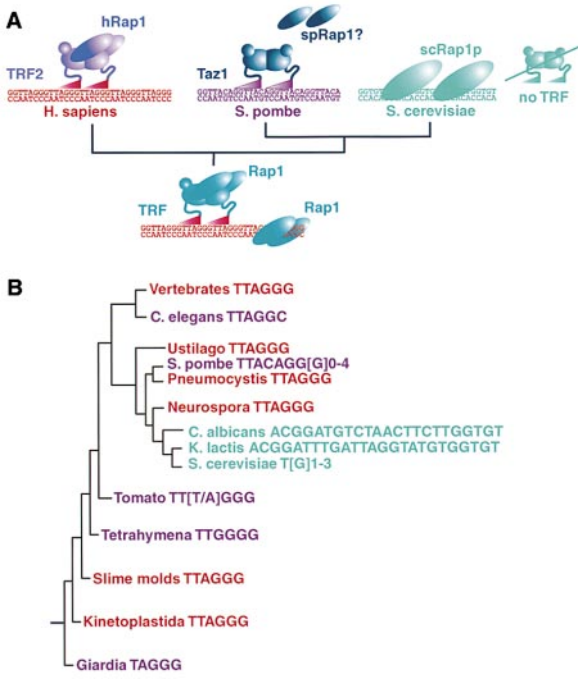

Figure 7. Model for the Evolution of Eukaryotic Telomeres

(A) A model for the evolution of duplex telomeric DNA binding proteins. See Discussion for details. TRF/Taz1 and Rap1 proteins are shown in the same corresponding shapes but different colors. Vertebrate telomeres have both Rap1 and a TRF module. The vertebrate Rap1 is drawn as not touching DNA for the sake of simplicity. Actual DNA contacts of hRap1 in vivo have not been analyzed. The *S. pombe* ortholog of Rap1 has not been identified and its presence at *S.pombe* telomeres is speculative. Loss of the TRF module is proposed to have occurred in budding yeasts. The proposed structure of the telomeric complex in the last common ancestor of fungi and vertebrates is shown to contain both a TRF-like protein as well as a Rap1-like factor. No prediction is made on whether the original Rap1 bound to telomeric DNA directly or via interaction with the TRF tether.

(B) Preponderance of TTAGGG-like repeats at telomeres of most eukaryotes with the exception of budding yeasts. For references on telomeric sequences, see Henderson (1995). Abbreviated approximate lineage of eukaryotes is based on the phylogeny listed in http://phylogeny.arizona.edu/tree/life.html. Line lengths do not represent evolutionary distances.

protein Tbf1p (Brigati et al., 1993; Bilaud et al., 1996). Tbf1p has not been ascribed a function at telomeres so far. It is unlikely that Tbf1p is a remnant of the budding yeast TRF gene since Tbf1p lacks a TRFH domain and an *S. pombe* Tbf1p ortholog is reported in the database.

Why does *S. cerevisiae* lack the TRF class of telomeric proteins? One possibility is that during the evolution of the budding yeasts, including *S. cerevisiae, K. lactis*, and several *Candida* species, the telomeric DNA sequence may have been altered such that the TRF component could no longer bind to the telomeric repeat array. A mutation in the telomerase RNA template could have caused this alteration, and binding of Rap1 to the new version of the telomeric DNA might have rescued the potential lethality of this change (McEachern and Blackburn, 1995; Krauskopf and Blackburn, 1996). An alternative scenario is that loss of the TRF gene resulted in selection of yeasts with specific compensatory mutations in their telomerase RNA template gene that allowed Rap1 to bind to telomeric DNA independently.

Perusal of the large collection of available telomeric DNA sequences indicates that the TTAGGG repeat of present day vertebrate telomeres is the most pervasive telomeric sequence in eukaryotes, also occurring in kinetoplastida, slime molds, and most branches of fungi (e.g., Ustilago, Pneumocystis, Neurospora, and Aspergillus) (reviewed in Henderson, 1995) (Figure 7B). The majority of other eukaryotes have telomeric sequences that are closely related to the TTAGGG repeats, for example TAGGG in Giardia, TT[T/A]GGG in tomato, TTAGGC in *C. elegans*, and TTGGGG in the ciliate Tetrahymena. Even the heterogenous telomeric repeat of *S. pombe* was recently recognized as being composed of a TTACAGG repeat motif (Hiraoka et al., 1998). Thus, the last common ancestor of all eukaryotes most likely carried TTAGGG repeats or a closely related sequence at its telomeres. Budding yeast telomeric DNA represents a striking departure from the TTAGGG motif, containing telomeric repeats that are very different in length and sequence (e.g., ACGGATTTGATTAGGTATGTGG TGT in *K. lactis* [McEachern and Blackburn, 1994]). Possibly the precursor of the Hemiascomycetes suffered a major change in the telomerase RNA template region (see also discussion in Brun et al., 1997).

We propose a model for the evolution of the telomeric complex in which the ancestral eukaryotic telomeres were composed of TTAGGG repeats and bound a TRF-like protein as well as a Rap1-like factor. During the evolution of *S. cerevisiae* and other budding yeasts, a telomerase mutation may have caused a shift in the telomeric sequence, resulting in loss of the tethering function of the TRF module (or vice versa). In agreement, TRF1 does not bind telomeric DNA from *S. cerevisiae* (Zhong et al., 1992). The optimal binding site for current day human TRF1 contains two (overlapping) copies of the sequence YTAGGGTTR (Bianchi et al., 1999), a precise representation of the TTAGGG repeat array, and minor changes in this sequence strongly inhibit TRF1 binding (Zhong et al., 1992; Hanish et al., 1994). The DNA binding activity of scRap1 may have rescued cells bearing telomeres containing Rap1 recognition sites. Once telomere function became independent of TRFs, divergence of the telomeric sequence could occur, as long as the Rap1 binding sites were preserved. Indeed, Rap1 sites appear to be the only common motif in the highly diverged telomeric sequences of budding yeasts (McEachern and Hicks, 1993; Krauskopf and Blackburn, 1996, 1998). The DNA binding activity of scRap1p is also important for its role as a transcriptional regulator. Although there is no indication that the ortholog of Rap1 described here has a similar function, it is not excluded that mammals have a second ortholog of Rap1 with a role in gene expression.

Experimental Procedures

Isolation of hRap1

A two-hybrid screen was carried out with the yeast reporter strain L40 (Hollenberg et al., 1995; Bianchi et al., 1997), using the human HeLa S3 matchmaker cDNA library (Clontech), and a LexA-fused hTRF2 cDNA as a bait. The TRF2 fusion contained an unintended frame shift mutation leading to a TRF2 protein with 444 aa that is mutated from aa 389. The hRap1 two-hybrid cDNA clone (aa positions 200 to 399, see Figure 1A) resulting from this screen was used as probe on a cDNA library made from a human breast cancer cell line (MDA) (Kratzschmar et al., 1996), yielding 26 positive clones. Coupled in vitro transcription/translation of the largest cDNA (M50–27MDA6–1) yielded a protein with an apparent MW of 63 kDa and its sequence (GenBank accession number: AF262988) encoded a predicted ORF of 399 aa.

Two-Hybrid Assays

The LexA-TRF2 full-length fusion was generated by inserting a PCR fragment encoding TRF2 aa 2–389 into pBTM116 (Bartel et al., 1993) at the EcoRI site, followed by replacement of the C-terminal half of this clone by a NarI/BamHI fragment of TRF2 encoding aa 31–501. Additional LexA-TRF2 fusions were generated by PCR amplification of sequences encoding the indicated amino acids from the LexA-TRF2 full-length clone followed by insertion into the polylinker region of pBTM116. GAD-TRF2 hybrid was generated by cloning the LexA-TRF2 StuI/BamHI fragment into pACT2 (Clontech). The LexA-hRap1 fusions were generated by PCR amplification using the hRap1 cDNA (M50–27MDA6–1) followed by insertion into the BamHI site of pBTM116. GAD-hRap1 fusions were generated similarly in pACT2. The LexA-fused full-length hTRF1 (Smith et al., 1998) and the original two-hybrid clone of hRap1 (M50–27) were used for testing interaction between TRF1 and hRap1. Expression of fusion proteins was verified by Western blotting using anti-LexA or anti-GAD monoclonal antibodies (Clontech).

Two-hybrid experiments were performed in the yeast strain L40. β-galactosidase activities were measured according to Clontech MATCHMAKER library protocol. The average value of three individual transformants for each set of plasmid constructs is reported. Values from individual transformants differed by $<$30% from the average.

Recombinant hRap1 and Generation of hRap1 Antibodies

N-terminally $[His]_6$-tagged full-length hRap1 protein was expressed in insect cells using the Bac-To-Bac Expression System (Monsato Corporate Research). The recombinant hRap1 protein was purified and used to immunize NZW rabbits (Covance). The resulting immune serum, Ab 765, was affinity purified using hRap1 coupled to sulfolink gel (Pierce). Peptides hRap1–211 (NH_2-RKAEEDPEAADSGEPQNKR TPDLPEC-COOH) starting at aa 211 and hRap1–283 (NH_2-DPP TPEEDSETQPDEEEEEEEEKVSC-COOH) starting at aa 283 of hRap1 were synthesized (BioSynthesis, Lewisville, TX), conjugated to KLH (Pierce, Rockford, IL), and used to immunize NZW rabbits (Covance). The resulting immune sera, Ab 666 (against hRap1-211) and Ab 664 (against hRap1-283), were affinity purified against sulfolink gel (Pierce) bound peptides (hRap1-211and hRap1-283 respectively) using standard procedures (Harlow and Lane, 1988).

Immunofluorescence and Metaphase Chromosome Spread

Preparation of metaphase chromosome spreads and indirect fluorescence techniques are described in Chong et al., 1995. In brief,

HeLaI.2.11 cells (van Steensel et al., 1998) were washed in phosphate-buffered saline (PBS), fixed in 2% formaldehyde in PBS for 10 min, and permeabilized with 0.5% Nonidet P-40 in PBS for 10 min. To prepare metaphase chromosome spread, HeLa I.2.11 cells were treated with colcemid (0.1 μg/ml, 90 min), harvested by trypsinization, washed with serum and PBS, hypotonically swollen in 10 mM Tris (pH 7.4), 10 mM NaCl, and 5 mM $MgCl_2$ (37°C, 15 min), and sedimented onto cover slips for 15 s at 3000 rpm in a Sorvall RT6000B tabletop centrifuge. Chromosome spreads were immediately fixed and permeabilized as described above. To examine the hRap1 localization in interphase HeLa1.2.11 cells transfected with $hTRF2^{\Delta B\Delta M}$, cells were washed with PBS, extracted with Triton X-100 buffer (0.5% Triton X-100, 20 mM HEPES [pH 7.9], 50 mM NaCl, 3 mM $MgCl_2$, and 300 mM sucrose) for 10 min, washed with PBS twice, fixed with 3% formaldehyde, 2% sucrose in PBS for 10 min, permeabilized with Triton X-100 buffer for 10 min, washed with PBS twice followed by antibody staining. Most nucleoplasmic proteins are extracted in this method. Residual signals represent predominantly DNA bound proteins.

Endogenous hRap1 was detected with polyclonal antibodies 765 and 666 (see above). TRF1 was detected with polyclonal mouse anti-TRF1 antibody (Ab 3, S. Smith and T. d. L., unpublished data), and TRF2 was detected with polyclonal mouse anti-TRF2 antibody (Ab 2, X.-D. Zhu and T. d. L., unpublished data). In untransfected HeLa1.2.11 cells, rabbit antibodies were detected with fluorescein isothiocyanate (FITC)-conjugated donkey-anti-rabbit antibodies (Jackson ImmunoResearch) and mouse antibodies were detected with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated donkey anti-mouse antibodies (Jackson ImmunoResearch). In HeLa 1.2.11 cells transfected with $hTRF2^{\Delta B\Delta M}$, rabbit antibodies were detected with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated donkey-anti-rabbit antibodies (Jackson ImmunoResearch) and mouse antibodies were detected with fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse antibodies (Jackson ImmunoResearch). DNA was stained with 4′, 6-diamidino-2-phenylindole (DAPI). Micrographs were recorded on a Zeiss Axioplan II microscope with a Photometrics Sensys camera using IP Lab program (for metaphase chromosome spread experiment) or with a Hammamatsu C4742–95 camera using Open Lab program (for other IF experiments). IP lab images were noise filtered, corrected for background, and merged using Adobe PhotoShop.

Cell Extracts and Western Blotting

Cells grown to semiconfluence on 15 cm dishes were trypsinized, washed with media, harvested by centrifugation, washed with ice-cold PBS twice, and incubated with buffer C (van Steensel et al., 1998) on ice for 30 min. The extract was cleared by centrifugation for 10 min in an Eppendorf microfuge at 14,000 g at 4°C and the supernatant was frozen in liquid nitrogen and stored at ~80°C. Protein content of extracts was measured using the Bradford assay (BioRad, Hercules, CA) using bovine serum albumin as a standard.

Proteins (65 μg) were separated on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membrane by electrophoresis. Ponceau S-staining confirmed equal loading of the samples. Blots were preincubated in 10% nonfat milk in PBS containing 0.5% Tween 20 for 30 min. All subsequent steps were carried out in 0.1% nonfat dry milk powder and 0.1% Tween 20 in PBS. Blots were incubated for 12–16 hr at 4°C with anti-hRap1 antibody 765, followed by three 10 min washing steps. Subsequently, blots were incubated for 30 min with horseradish peroxidase-conjugated donkey-anti-rabbit antibody (Amersham) and washed four times for 5 min each. Blots were then rinsed with PBS followed by H_2O. Bound antibody was detected using the enhanced chemiluminescence kit (Amersham).

Bandshift Assays

Purified baculovirus-expressed, $[His]_6$-tagged hRap1 and TRF2 proteins were used for bandshift assays. Binding reactions (in 4% Glycerol, 0.5 μg *E. coli* DNA, 20 mM Glycine-KOH [pH 9.0], 10 mM DTT, and 50 ng β-casein per 20 μl) were incubated at 4°C for 30 min and run on a 0.6% agarose gel in 0.1× TBE at 4°C. Gels were dried onto DE81 paper and analyzed by autoradiography or by exposure on a Phosphorimager. DNA probes were prepared as described previously (Zhong et al., 1992). In brief, the HindIII/Asp718 fragment containing $(TTAGGG)_{12}$ repeats was purified from pTH12 (Zhong et al., 1992) and filled in with [α-^{32}P]dCTP and Klenow enzyme.

Tetracyclin Inducible Expression of hRap1 in HTC75 Cells

A Tetracyclin controlled expression vector of full-length hRap1 carrying an N-terminal FLAG epitope tag was created by PCR cloning in pUHD10–3 (Gossen and Bujard, 1992). First, pUHD10–3-FLAG-5′ was generated by inserting paired oligos encoding a FLAG tag into BamHI site of pUHD10–3. A fragment representing the complete coding region of hRap1 was generated by PCR using Pfu-polymerase and the primer pair 5′-GCGCAGATCTGCGGAGGCGTTGGAT TTG-3′ and 5′-GCGCGGATCCTTATTTCTTTCGAAATTCAATCCT-3′ and cloned into the BamHI site. The resulting plasmid was introduced into HTC75 as described previously (van Steensel et al., 1998) and independent clonal cell lines (F lines) were isolated using cloning cylinders. Growth conditions of the cells and methods to measure telomere lengths were described previously (van Steensel and de Lange, 1997).

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