Phosphorylation of the *Oxytricha* telomere protein: possible cell cycle regulation

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ABSTRACT

In the macronucleus of the ciliate *Oxytricha nova*, telomeres end with single-stranded (T₄G₄)₂ DNA bound to a heterodimeric telomere protein ($\alpha\beta$). Both the α and β subunits (α -TP and β -TP) were phosphorylated in asynchronously growing *Oxytricha*; β -TP was phosphorylated to a much higher degree. *In vitro*, mouse cyclin-dependent kinases (Cdks) phosphorylated β -TP in a lysine-rich domain that is not required for specific DNA binding but is implicated in higher order structure formation of telomeres. Therefore, phosphorylation of β -TP could modulate a function of the telomere protein that is separate from specific DNA binding. Phosphoamino acid analysis revealed that the mouse Cdks modify predominantly threonine residues in β -TP, consistent with the observation that β -TP contains two consensus Cdk recognition sequences containing threonine residues. In *Xenopus* egg extracts that undergo cell cycling, β -TP was phosphorylated in M phase and dephosphorylated in interphase. This work provides the first direct evidence of phosphorylation at telomeres in any organism, as well as indirect evidence for cell cycle regulation of telomere phosphorylation. The Cdc2/cyclin A and Cdc2/cyclin B kinases are required for major mitotic events. An attractive model is that phosphorylation of β -TP by these kinases is required for the breakdown of telomere associations with each other and/or with nuclear structures prior to nuclear division.

INTRODUCTION

Telomeres, structures found at the natural ends of eukaryotic chromosomes, consist of a canonical G-rich repeated DNA sequence and associated protein (reviewed in 1-3). Telomere-bearing chromosomes are protected from exonucleolytic degradation and are stabilized against end-to-end fusion that can occur

at free ends generated by chromosome breakage (4,5). Telomeres may play a role in nuclear architecture by localizing chromosomes to the nuclear envelope (6-8), or the nuclear matrix (9). Activity of the telomere-replicating enzyme telomerase may be an obligatory step in tumor expansion, as telomerase activity is detected in extracts of human tumor cells but not in extracts of non-transformed cells (10, see 11 for review).

The involvement of telomeres in cell cycle regulation is an unfolding story. In one striking demonstration in the ciliate *Tetrahymena*, the alteration of even a single nucleotide in the T₄G₂ telomeric repeat sequence was found to disrupt cell division and result in senescence (12). In *Saccharomyces cerevisiae* telomeric DNA is replicated late in S phase (13) when long single-stranded tails of telomeric DNA are synthesized (14), presumably by telomerase. Loss of a single telomere causes cell cycle arrest in G₂ (5). Thus, telomere replication may serve as a checkpoint for completion of S phase (11). Studies of the cell cycle specific positioning of centromeres and telomeres in the fission yeast show that telomeres are associated with the nuclear periphery in G₂ (15) and are clustered at the leading end of pre-meiotic chromosome movement (16). It is clear, then, that telomere DNA synthesis and telomere structure are subject to constraints imposed by cell cycle regulation. As yet, it is unclear how regulatory signals generated during cell cycle progression alter telomere structure and function.

In many cases telomeres exist as nucleoprotein complexes (17), and it is likely that at least some telomere functions are imparted through telomere-specific proteins. The observation that the DNA at chromosomal termini in *Oxytricha nova* is protected from exhaustive nuclease digestion and chemical modification (18) led to the identification of a telomere protein (19,20). Genes encoding its α and β subunits (α -TP and β -TP) were subsequently cloned and sequenced (21,22). This telomere protein binds tenaciously to the single-stranded overhang present at the telomere, forming a nucleoprotein complex that is stable to >2 M NaCl. The complex is extremely long-lived, with a half-life >100 h *in vitro* (23). Because the cell cycle time is 8 h at maximum

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growth rate, this nucleoprotein complex is theoretically capable of enduring several rounds of the cell cycle intact.

Higher order structure of telomeres has been observed in the macronuclei of *Oxytricha* (24), but the role of the telomere protein in the formation/dissolution of these structures is not yet clear. Protease-sensitive higher order structures, in which the telomeres are self-associated, are observed in the macronuclear chromatin of *Holosticha*, a hypotrichous ciliate related to *Oxytricha* (25). These telomeres are associated in large bundles, with the internal, coding DNA extruded out in loops. Consistent with these observations, the *Oxytricha* telomere protein can induce higher order (multimeric) protein-DNA complexes *in vitro* (26). Multimeric complex formation is mediated by the lysine-rich C-terminal domain of β -TP (23); the nature of these complexes is not understood. In addition, telomeric DNA can adopt G-DNA structures—dimers and tetramers held together by guanine quartets (27–29)—and β -TP can promote the formation of these structures (30,31).

The existence of protein-mediated higher order structures and a tenacious, long-lived protein-DNA complex at chromosome ends suggested to us that telomeric DNA may be enzymatically inaccessible in *Oxytricha*, as it is in yeast (32). To replicate telomeric DNA and therefore proceed through S-phase, the telomere structure may need to be altered either on the level of telomere–telomere association or on the level of the individual telomeric complex, or both, to allow access by the telomere replication machinery. In addition, telomere–telomere or telomere–nuclear matrix associations may require alteration to enable karyokinesis. One way to alter this nucleoprotein complex would be by post-translational modification of the telomere protein. Because the phosphorylation of chromosomal and nuclear proteins controls major mitotic functions (33–37), we initiated studies to determine whether the *Oxytricha* telomere protein is modified by phosphorylation. In addition to observing phosphorylation of both subunits, we found that β -TP is a substrate for cyclin-dependent kinases *in vitro*. This suggests that phosphorylation of β -TP may be subject to regulation during progression through the *Oxytricha* cell cycle.

MATERIALS AND METHODS

Metabolic labeling with $^{32}\text{P}_i$

Oxytricha were cultured according to Swanton *et al.* (53). *Oxytricha* (41) kept at 12°C were washed, fed *Chlorogonium*, and placed into 1 l of phosphate-free medium, in which CaPO_4 was replaced by CaCl_2 . Five mCi $^{32}\text{P}_i$ were added and cells were incubated overnight at 25°C. Cells were then concentrated to 50 ml by filtration, pelleted by centrifugation, and washed in phosphate-containing medium. Macronuclei were isolated (53) and lysed by the addition of NaCl to 2 M and storage at 0°C for 60 min with occasional stirring. Macronuclear debris was removed by a 60 s spin at 16 000 g. Unincorporated radioactivity was then removed from the supernatant (300 μl) by centrifugation through 3 ml of Sephadex G-50 equilibrated in 10 mM Tris pH 7.5, 0.1 mM EDTA, 2 M NaCl, and pre-centrifuged (54). The eluted material was then fractionated by HPLC size exclusion in a column equilibrated with 0.1 M NaPO_4 pH 7.0, 2 M NaCl (DuPont Zorbax, 250 000 MW exclusion limit, run at 1 ml/min on a Waters System 600 HPLC). HPLC fractions (0.5 ml) were collected and 5 μg calf thymus DNA and 5 μg BSA were added.

Fractions were then concentrated to 50 μl and the buffer changed to 10 mM Tris pH 7.5, 0.1 mM EDTA, 125 mM NaCl (Millipore Ultrafree MC filters, 30 000 MW cut-off membranes, spun at 4000 g). Portions of each fraction were analyzed by SDS–PAGE (55), stained with silver (GELCODE silver staining system, Pierce Chemical Co.), and finally dried onto Whatman 3MM paper and autoradiographed.

Assay of kinase activity in isolated nuclei

Isolated macronuclei were pelleted by centrifugation and resuspended at 0°C at a density of $\sim 5 \times 10^7$ nuclei/ml in a buffer compatible with kinase activity: 0.25 M sucrose, 0.02 M glucose, 0.005 M MgCl_2 , 0.01 M Tris pH 8.0 (49). Macronuclei were used either immediately or frozen in 20 μl aliquots. After equilibration for 5 min at room temperature, reactions were initiated by the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (15 μCi per 20 μl reaction) in combination with unlabeled ATP such that final [ATP] was 10 μM . After 15 min at room temperature, labeling reactions were quenched by addition of NaCl to 2 M and unlabeled ATP to 1 mM. Macronuclei (2×10^7) previously isolated from *Oxytricha* were added as a carrier for macronuclear extract preparation and HPLC fractionation was carried out as described above.

Kinase purification

Cyclin-dependent kinases (Cdks) were purified from Mouse Mammary Tumor cells (line FM3A) grown in suspension (41), with additional purification over a Phenyl Superose column. Silver stained SDS–PAGE analysis of the eluted kinase fractions indicated significant purification beyond that reported by Hamaguchi *et al.* (41), with only a few bands observed beyond the Cdc2 and cyclin bands (Swank *et al.*, in preparation). Briefly, whole cell extracts were prepared by dounce homogenization of pelleted cells. The extracts were treated with polyethyleneimine and the resultant precipitate was salt extracted and ammonium sulfate precipitated. The pellet was solubilized, dialyzed and chromatographically separated on three columns. Four kinases were isolated and, by Western blot analysis, could be identified as: A = Cdc2/cyclin A; B = Cdk2/cyclin A; C = Cdc2/cyclin B; M = Cdc2/unidentified cyclin. The cyclin of kinase M has the same apparent molecular weight as cyclin A, but does not cross-react with antibodies to cyclin A. Cyclin antibodies were a gift from Tony Hunter (Salk Institute).

Telomere protein phosphorylation by Cdks

Reactions occurred in a volume of 25 μl at 32°C and contained 50 mM Tris pH 7.4, 10 mM β -mercaptoethanol, 10 mM MgCl_2 , 10 mM β -glycerophosphate, 0.1 mM EDTA, 0.1 mM ATP and 1.25 μCi $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Telomere protein subunits were purified as described (23) and were present at 3–4 μM . The single-stranded DNA, O2T (23), has 20 nt of non-telomeric DNA followed by 16 nt of $\text{T}_4\text{G}_4\text{T}_4\text{G}_4$ at its 3' end and was present at 6 μM . After a 10 min incubation the reactions were terminated by addition of 825 mM phosphoric acid to a final concentration of 75 mM. Phosphorylation was then quantitated using phosphocellulose (p81) filter binding followed by liquid scintillation counting. The samples were also denatured in Laemmli sample buffer and applied to SDS–12.5% polyacrylamide gels for separation and autoradiography, and the dried gel was also quantified using a BioRad Molecular Imager with PhosphorAnalyst software.

Truncated forms of telomere protein β N122 and β C232 were expressed and purified according to Fang *et al.* (23) and assayed *in vitro* as described above.

Phosphorylation in *Xenopus* egg extracts

Extracts of *Xenopus laevis* eggs, prepared from freshly laid eggs and capable of oscillating between M-phase and interphase, were prepared as described (42). The extracts, designated cytosolic factor or CSF extracts, are arrested in metaphase II prior to release with CaCl_2 . Telomere protein (20 pmol) either as the free β subunit or as the α - β -DNA ternary complex, and 120 μCi [γ ^{32}P]ATP were added to 200 μl freshly prepared egg extract. Aliquots were taken after 20 min. The extracts were then released from metaphase II arrest by addition of CaCl_2 to 0.4 mM. After 40 min of incubation, the extract had advanced into interphase, and another aliquot was taken. Extracts were monitored microscopically by addition of demembraneated sperm nuclei and staining with DAPI (56). Each aliquot was diluted 20-fold into lysis buffer (56) and frozen for later immunoprecipitation. Aliquots (1 μl) were also frozen at each time point to assay histone H1 kinase activity.

Immunoprecipitation

Diluted *Xenopus* egg extract was pre-treated using 25 μl 50% v/v protein A-Sepharose CL 4B beads (Pharmacia) at 4°C for 1 h, and the beads were then removed by centrifugation (54). One μl undiluted polyclonal rabbit antiserum #13232 raised against bacterially expressed β -TP (Macromolecular Resources, Ft Collins, CO) was then incubated with the diluted extract with shaking at 4°C for 1 h, followed by addition of 25 μl 50% protein A-Sepharose CL 4B beads. After 1 h at 4°C, beads were recovered by centrifugation and washed according to Izumi and Maller (56) except that protein A-Sepharose beads replaced Omniosorb. After the final wash, remaining buffer was aspirated using suction through a 23 gauge hypodermic needle and 25 μl Laemmli sample buffer was added to the beads. The solubilized material was then separated on an SDS-10% polyacrylamide gel, stained with Coomassie Blue, dried and autoradiographed.

RESULTS

The telomere protein is phosphorylated *in vivo* and in isolated nuclei

To determine whether the *Oxytricha* telomere protein is phosphorylated, we grew a culture of *O. nova* overnight in phosphate-free medium along with $^{32}\text{P}_i$ and its algal food source, *Chlorogonium*. Nuclei were then isolated and lysed in 2 M NaCl, a concentration at which telomere protein remains DNA-bound (19,20). The resulting soluble material was fractionated on an HPLC size exclusion column in 2 M NaCl, in an adaptation of methods used to purify telomere protein (19,20). It is expected that DNA-bound telomere protein will elute in the void volume of this column due to the large size of the associated DNA.

Figure 1A (lane 1) indicates that in the void volume, two bands migrating at ~56 and ~45 kDa are stained with silver. These are identified as α -TP and β -TP because they are the only abundant polypeptides that co-elute with the DNA in high salt (19,20) and because they have the correct apparent molecular weights (Fig.

Figure 1. Phosphorylation of telomere protein *in vivo* and in isolated nuclei. (A) Soluble material from salt-lysed nuclei of *Oxytricha* metabolically labeled by $^{32}\text{P}_i$ was separated by HPLC size exclusion chromatography, and analyzed by SDS-PAGE. Silver staining (lane 1) and autoradiography (lane 2) of the void volume fraction reveal the telomere protein subunits α -TP and β -TP. β -TP stains faintly relative to α -TP due to its susceptibility to proteolytic degradation. BSA was added to each fraction as a carrier, subsequent to HPLC fractionation. ^{14}C protein markers are at right. Positions of α - and β -TP migration are indicated with arrowheads. (B) Isolated nuclei were labeled with [γ ^{32}P]ATP and analyzed as in (A) except that the gel was stained with Coomassie Blue.

1A). β -TP, due to its protease sensitivity (20), is only faintly visible.

The autoradiogram of the same gel (Fig. 1A, lane 2) reveals a ^{32}P -labeled species that co-migrates with the faintly staining band migrating at 45 kDa. This band is identified as β -TP, still bound to DNA in this phosphorylated form. In addition, a ^{32}P -labeled doublet is visible at 56–58 kDa, corresponding to the α -TP doublet seen by silver stain. [α -TP purified from *Oxytricha* is subject to proteolysis at its N-terminus (J. Gray and B. Hicke, unpublished results) and can migrate as a doublet in polyacrylamide gels (J. Gray, unpublished observation).] The ^{32}P -specific activity appears much greater in the upper band of this doublet, as its autoradiographic intensity is about equal to that of the lower band while its silver staining is much fainter.

Because *Oxytricha* must be fed with a live food source, the specific activity of ^{32}P is greatly diluted, making it currently impractical to obtain enough protein labeled *in vivo* for further analysis. Therefore, *Oxytricha* nuclei were isolated by centrifugation, resuspended in a buffer compatible with kinase activity, and incubated with [γ ^{32}P]ATP for 15 min at 20°C. DNA-bound telomere protein was then chromatographically separated as described above. Coomassie staining (Fig. 1B, lane 1) revealed that α -TP and β -TP are present in the void volume. The autoradiogram (lane 2) indicates that both subunits are labeled with ^{32}P . β -TP's specific activity appears much greater than that of α -TP, given the weak Coomassie staining of β -TP in lane 1. A control experiment, in which [γ ^{32}P]ATP was added to nuclei after beginning salt extraction, resulted in no labeling, indicating that ^{32}P is not transferred to telomere protein subsequent to salt extraction of the nuclei (data not shown). In summary, the telomere protein is labeled *in vivo* by the addition of $^{32}\text{P}_i$ and in nuclei by the addition of [γ ^{32}P]ATP.

In vitro labeling by cyclin-dependent kinases (Cdks)

A search of the amino acid sequence of β -TP for potential phosphorylation sites revealed two closely spaced copies of the consensus sequence for phosphorylation by Cdks (38,39). These sequences, KYTPSK and KDTPQK (where the potential phosphorylation sites are underlined; see Fig. 2 for their location within the β -TP sequence), are found within a region of β -TP that exhibits slight but significant sequence similarity to a sea urchin

Figure 2. β -TP's domain structure and putative phosphorylation sites. The two functional domains of β -TP are separable by a truncation at residue 232 as indicated (23). The C-terminal two thirds of β -TP is 23% lysine and shares slight sequence similarity with a sea urchin histone H1 (21). The 15 amino acid stretch indicated here contains two closely spaced consensus sites for phosphorylation by cyclin-dependent kinases; the two candidate Thr residues are underlined.

Figure 3. Phosphorylation of recombinant telomere protein subunits by cyclin-dependent kinases purified from mouse cells. (A) Coomassie stained SDS-PAGE of substrate telomere protein after the phosphorylation reactions. (B) Autoradiogram of the same gel seen in (A). M = Cdc2/unidentified cyclin; this kinase is a *bona fide* Cdk (see text); A = Cdc2/cyclin A; B = Cdc2/cyclin B; C = Cdc2/cyclin C.

histone H1 (21). The version of α -TP that has been overexpressed in *E. coli* contains no such site (22), although a different version contains the sequence TASPEG near its N-terminus (40).

We tested (both as free subunits and as protein-DNA complexes) purified, recombinant α - and β -TP as substrates for four Cdk from mouse cells (Fig. 3). Whereas α -TP is not phosphorylated, β -TP is indeed a Cdk substrate, and there are differences among the kinases in the efficiency of β -TP phosphorylation. In particular, kinase M had almost no activity on β -TP, although it

Figure 4. Differences in phosphorylation rate of telomere protein β subunit by different kinases. Quantitation of data from Figure 3B, and additional data using histone H1 as a control substrate. For the histone H1 data, label incorporation into histone H1 by Cdk2/cyclin A (kinase B) was set at 100, and other kinases normalized to that value. For comparison of the first two data sets with the control data set (histone H1), phosphorylation of unbound β -TP by Cdc2/cyclin A (kinase A) was set equal to phosphorylation of histone H1 by the same enzyme. This normalization permits comparison of labeling of DNA-bound versus unbound β , and permits comparison of relative substrate utilization by the four enzymes, but does not allow comparison of histone H1 and telomere protein as substrates for these kinases.

phosphorylated histone H1 efficiently. We have not yet identified the cyclin subunit of kinase M, but kinase M appears to be a *bona fide* Cdk by the following criteria: it phosphorylates histone H1 at the same sites where H1 is phosphorylated by Cdc2 *in vivo*; the non-Cdc2 subunit has the same apparent molecular weight as cyclin A, and it co-purifies with Cdc2 on p13 Sepharose (41, R. Swank, unpublished observation). Figure 4 depicts quantitation of phosphorylation, using histone H1 as a standard to measure the specific activity of each enzyme preparation. Whereas the differences in labeling of histone H1 by the four kinases are relatively small, the kinase composed of Cdc2 and the yet-unidentified cyclin is extremely inefficient at recognizing telomere protein. The DNA-bound form of β -TP is phosphorylated 2.8-fold less efficiently than the free form by Cdc2/cyclin A, a ratio that is similar for the other two kinases that efficiently utilize β -TP as a substrate.

Phosphoamino acid analysis of free β -TP labeled by Cdc2/cyclin A indicated the presence of phosphothreonine, a lesser amount of phosphoserine, and no phosphotyrosine (data not shown). This result is consistent with the presence of threonine residues in the two β -TP consensus Cdk sites, and also suggests additional serine phosphorylation site(s). Ser319 is followed by a proline, forming an incomplete Cdk consensus sequence, and is therefore a candidate for the phosphoserine detected in the assay.

To maximize phosphate incorporation into free β -TP, time courses were performed using Cdc2/cyclin A (data not shown). A maximum of 0.8 mole phosphate could be incorporated per mole telomere protein. Thus, although phosphate incorporation could not be driven to completion, a large fraction of the available substrate polypeptide is phosphorylated (i.e., 80% of β -TP if singly phosphorylated or 40% if doubly phosphorylated).

Truncated versions of β -TP were used as substrates for Cdc2/cyclin A to define the region of β -TP that serves as a kinase substrate (Fig. 5). Removal of the first 122 residues of β -TP (N122) had no effect on phosphorylation. Removal of C-terminal residues 232–385 abolished phosphorylation. This effect cannot be ascribed to a misfolding of the polypeptide, since truncation at residue 232 has no effect on β -TP's ability to participate with

Figure 5. Phosphorylation of truncated recombinant β -TP identifies the domain phosphorylated *in vitro*. Protein purified from *E. coli* expressing β C232, β N122 and full-length β -TP was incubated with Cdc2/cyclin A in the presence of [γ - 32 P]ATP. Products were analyzed by SDS-PAGE and autoradiography. β C232, a C-terminal truncation of β -TP at residue 232; β N122, an N-terminal truncation of β -TP at residue 122. Some degradation products of β N122 are visible as lower molecular weight species in lane 3.

α -TP in DNA binding (23). Therefore, Cdc2 recognizes and phosphorylates one or more residues in the 232–385 region. This region contains the two consensus Cdk sites and represents a structural domain of β -TP that is dispensable for binding to telomeric DNA (23).

Cell cycle-dependent phosphorylation of telomere protein

Since the telomere protein is a substrate for purified Cdc2, we wished to know whether phosphorylation of β -TP could be subject to cell cycle regulation. Because *Oxytricha* have not been induced to grow synchronously, we adopted an *in vitro* model for cell cycling, the *Xenopus* egg extract (42). These extracts, called cytosolic factor (CSF) extracts, are initially arrested in M phase, having high Cdc2 activity; upon addition of calcium the extracts cycle into interphase, and have low Cdc2 activity.

β -TP and [γ - 32 P]ATP were added to a *Xenopus* egg extract, and after 20 min the β -TP was recovered by immunoprecipitation. Figure 6 indicates that β -TP was phosphorylated in M-phase and was dephosphorylated when the same extract was released from metaphase arrest into interphase by Ca^{2+} addition. There was virtually no phosphorylation of β -TP when it was incubated in an interphase extract with [γ - 32 P]ATP (data not shown). In a separate experiment, β -TP was shown to be re-phosphorylated upon progression to the second M-phase, indicating that phosphate is being added, removed, and added back to this protein during progression through the cell cycle in the *Xenopus* egg extract (data not shown). This eliminated the possibility that disappearance of labeled β -TP was due to proteolysis. In this experiment, it was found that the level of β -TP phosphorylation paralleled the level of histone H1 kinase activity in the extract. Additionally we found that β -TP was phosphorylated ~3-fold less efficiently when it was added to the extract in the form of the α - β -DNA ternary complex (data not shown), paralleling our observations using purified Cdk. Finally, maturation promoting factor purified from *Xenopus* eggs also phosphorylates β -TP (data not shown). In summary, we find that β -TP is subject to phosphorylation during

Figure 6. Cell cycle-dependence of phosphorylation. *Xenopus* egg extracts arrested in M-phase were prepared, and either β -TP (+) or buffer (–) was added along with [γ - 32 P]ATP. Extracts were radiolabeled for 20 min in M phase and aliquots removed. The extracts were then treated with Ca^{2+} , incubated for a further 40 min to allow progression into interphase, and samples removed. β -TP was immunoprecipitated from the extract, separated by SDS-PAGE, and exposed for autoradiography.

M phase and dephosphorylation during interphase in the *Xenopus* egg extract.

DISCUSSION

We have found that both subunits of the *Oxytricha* telomere protein incorporate ^{32}P -phosphate *in vivo* and in isolated nuclei. Many cellular enzymes, receptors, signaling molecules, transcription proteins, histones etc. are regulated by phosphorylation, and it now seems possible that telomere functions in replication or chromosome positioning might be similarly regulated. *Oxytricha* presents an unusually good opportunity for studying the structure and function of telomeres due to their abundance at 80 million per cell. However, these cells have not been synchronized nor can they be grown on phosphate-free medium in an axenic culture. Because of these limitations on the analysis of telomere protein phosphorylation *in vivo*, we employed *in vitro* systems derived from other organisms to examine the potential of the telomere protein subunits to serve as substrates for known protein kinases. We found that the lysine-rich domain of β -TP is a substrate for purified mouse cyclin-dependent kinases. In addition, the phosphorylation state oscillates with the cell cycle in *Xenopus* egg extracts, as β -TP is phosphorylated in M-phase and dephosphorylated in interphase.

While we have focused on β -TP because it is more heavily phosphorylated, the level of α -TP phosphorylation may increase in certain metabolic conditions or during certain phases of the *Oxytricha* cell cycle that were not studied here. Weakened DNA binding upon phosphorylation has been demonstrated for the transcription factor Oct-1 (43) and it is conceivable that phosphorylation of α -TP would decrease its affinity for chromosome ends. While it has been hypothesized that dislodging the telomere protein might be required for DNA to serve as a substrate for telomerase (44,45), recent experiments indicate that, at least in some fraction of the telomeres, telomerase can efficiently extend telomeric DNA bound to the telomere protein (46). Further investigation will be required to determine if phosphorylation regulates telomerase's ability to elongate telomeric DNA bound by the telomere protein.

Since β -TP contains two consensus sites for phosphorylation by cyclin-dependent kinases, we tested β -TP as a substrate for four different Cdk purified from mouse cells. Three of the Cdk phosphorylate β -TP. A fourth is extremely inefficient in utilization of telomere protein as a substrate; we have not yet identified the cyclin subunit of this Cdk. This is in contrast to histone H1, which is efficiently recognized by all four Cdk *in vitro*. This finding indicates that recognition of telomere protein as a kinase substrate depends strongly on the cyclin subunit present; the presence of consensus Cdk phosphorylation sites on β -TP is not sufficient to enable efficient recognition. Along similar lines, Peeper *et al.* (47) reported that while Cdk-cyclin A complexes and Cdk-cyclin B complexes are indistinguishable in terms of histone H1 phosphorylation, only Cdk-cyclin A complexes phosphorylate the Rb-related p107 *in vitro*.

Given that Cdk can recognize β -TP as a substrate, it was of interest to determine when this polypeptide is phosphorylated during the cell cycle. Because *Oxytricha* cannot currently be grown synchronously, we adopted a heterologous system, the *Xenopus* egg extract, to address this question. CSF metaphase-arrested extracts from *Xenopus* eggs oscillate from M-phase into interphase and back into M-phase after the addition of Ca^{2+} . We found that β -TP is phosphorylated in M-phase, dephosphorylated in interphase, and rephosphorylated upon entry into the next M-phase. This cell cycle-dependent modification occurred regardless of whether β -TP was introduced into the extract as a free protein or as a telomeric complex. It remains to be demonstrated whether the phosphorylation observed *in vivo* in *Oxytricha* and *in vitro* by the mouse and *Xenopus* Cdk occur at the same amino acid residues.

We delimited the region of β -TP that is labeled *in vitro* by Cdc2-cyclin A to the C-terminal 153 residues of β -TP, a lysine-rich region that contains two consensus Cdk sites. If the phosphorylation of β -TP on its lysine-rich C-terminal domain observed *in vitro* and in isolated nuclei occurs also *in vivo*, then the fact that β -TP so modified remains DNA-bound is not surprising. This lysine-rich domain is dispensable for binding to telomeric DNA (23) and can be proteolytically removed from intact telomeric complexes during their isolation from *Oxytricha* without disrupting the complexes (B. Hicke, unpublished observation).

What then is the function of β -TP's lysine-rich region and why should it be phosphorylated? Higher order structures form at ciliate telomeres *in vitro* (26,23) and *in vivo*, as judged by electron microscopy of lysed macronuclei (25). The *in vivo* structures are protease sensitive, and therefore thought to be higher order protein-DNA complexes. *In vitro*, higher order, multimeric protein-DNA complexes require β -TP and specifically its lysine-rich region (23). This structural domain of β -TP exhibits sequence similarity to a sea urchin histone H1 and may perform functions analogous to those of histone H1's basic tails in promoting higher order chromatin structure (48). Phosphorylation of β -TP could regulate higher order telomere structures by analogy to the regulation of higher chromatin structure by histone phosphorylation (36). Additionally, the lysine-rich region of β -TP promotes dimerization and tetramerization of single-stranded telomeric DNA through guanine quartets (G-DNA), another potential form of higher order structure *in vivo* (30,31). In the yeast S-phase, long single-stranded telomeric DNA strands are synthesized (14), which have the ability to fold into G-DNA structures. Phosphorylation of the G-DNA structure-promoting

domain of β -TP could regulate its activity, and thus influence the folding/unfolding of G-DNA structures if they exist *in vivo*.

In the *Xenopus* egg extract, we find that β -TP is phosphorylated in M-phase. The dominant M-phase kinase, Cdc2, triggers large structural alterations upon entry into M-phase (38), including nuclear envelope breakdown (phosphorylation of nuclear lamins), cytoskeletal rearrangements (phosphorylation of pp60^{c-src}) and chromatin condensation (phosphorylation of histone H1). As the phosphorylation of H1's basic tails by Cdc2 correlates with changes in higher order chromatin structure, so might the phosphorylation of β -TP's basic tail alter higher order telomere structure in *Oxytricha*. Although the *Oxytricha* macronucleus undergoes amitotic division, cell cycle regulation of telomeres in *Oxytricha* may model mitotic nuclei. A Cdc2-related kinase is found in the *Tetrahymena* macronucleus (49), suggesting that Cdk are required for cell cycle progression in amitotic nuclei as in mitotic nuclei.

The yeast protein, RAP-1, is multifunctional, serving as a transcriptional repressor or activator, being involved in mating type silencing and binding at telomeres. Interestingly, RAP-1, like the *Oxytricha* telomere protein, has been shown to promote formation of G-DNA (50). RAP-1 appears to be phosphorylated (51,52). The binding of RAP-1 to gene regulatory sequences on DNA has been inferred to be influenced by phosphorylation, based on phosphatase treatment experiments (51) and experiments in which the level of protein kinase A was increased or decreased *in vivo* (52). While these authors discussed their findings in terms of RAP-1's role as a regulator of gene expression, it will be important to ascertain whether telomere-bound RAP-1 is phosphorylated and whether such modification affects the binding or activity of RAP-1 at yeast telomeres.

While the phosphorylation of *Oxytricha* β -TP by Cdk *in vitro* makes the protein a viable candidate for similar phosphorylation *in vivo*, this hypothesis remains to be tested. Furthermore, whether phosphorylation of β -TP affects telomere function needs to be addressed. Although the absence of genetics is a hindrance, it should be possible to determine whether phosphorylation affects β -TP's G-DNA-promoting activity or higher order structure formation *in vitro*, and to study the relationship of phosphorylation of β -TP to cell division and DNA synthesis in *Oxytricha*.

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