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Research Article

Phosphorylation and cellular function of the human Rpa2 N-terminus in the budding yeast Saccharomyces cerevisiae ☆

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ABSTRACT

Maintenance of genome integrity is critical for proper cell growth. This occurs through accurate DNA replication and repair of DNA lesions. A key factor involved in both DNA replication and the DNA damage response is the heterotrimeric single-stranded DNA (ssDNA) binding complex Replication Protein A (RPA). Although the RPA complex appears to be structurally conserved throughout eukaryotes, the primary amino acid sequence of each subunit can vary considerably. Examination of sequence differences along with the functional interchangeability of orthologous RPA subunits or regions could provide insight into important regions and their functions. This might also allow for study in simpler systems. We determined that substitution of yeast Replication Factor A (RFA) with human RPA does not support yeast cell viability. Exchange of a single yeast RFA subunit with the corresponding human RPA subunit does not function due to lack of inter-species subunit interactions. Substitution of yeast Rfa2 with domains/regions of human Rpa2 important for Rpa2 function (*i.e.*, the N-terminus and the loop 3–4 region) supports viability in yeast cells, and hybrid proteins containing human Rpa2 N-terminal phospho-mutations result in similar DNA damage phenotypes to analogous yeast Rfa2 N-terminal phospho-mutants. Finally, the human Rpa2 N-terminus (NT) fused to yeast Rfa2 is phosphorylated in a manner similar to human Rpa2 in human cells, indicating that conserved kinases recognize the human domain in yeast. The implication is that budding yeast represents a potential model system for studying not only human Rpa2 N-terminal phosphorylation, but also phosphorylation of Rpa2 N-termini from other eukaryotic organisms.

☆Replication Protein A (RPA) is called Replication Factor A (RFA) in budding yeast. Therefore, RFA refers to the yeast complex, and RPA refers to all other eukaryotic forms of the complex. Furthermore, individual human subunits are referred to as Rpa1, Rpa2, and Rpa3, and the homologous yeast subunits are referred to as Rfa1, Rfa2, and Rfa3. Finally, genes are denoted in *italics* and proteins are denoted in sentence case.

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Importance

The accumulation of mutations in the cellular genome can lead to cellular disease. To prevent mutation, prokaryotic and eukaryotic cells have systems to recognize and repair DNA lesions before they become mutations. Replication Protein A (RPA) is the eukaryotic form of single-stranded binding (SSB) protein essential for proper DNA duplication and maintenance. In human cells, the 32-kilodalton (kDa) subunit of RPA, called Rpa2, is hyper-phosphorylated on its N-terminus in response to DNA damage. It is important to examine Rpa2 phosphorylation in other eukaryotic organisms to fully understand how phosphorylation contributes to Rpa2 function. When expressed in a single-celled eukaryotic organism (budding yeast), the human Rpa2 N-terminus is recognized and phosphorylated similarly to that observed in human cells. We propose that budding yeast could be a powerful tool to study phosphorylation of Rpa2 N-termini from other eukaryotes where technical limitations to studying phosphorylation currently exist.

Introduction

Understanding the basic mechanisms by which the integrity of genomic DNA is maintained is central to understanding how mutations are prevented. There are numerous ways in which DNA integrity can be compromised, including errors in DNA replication, exposure to environmental stresses, and progression through natural physiological processes. Cells have numerous mechanisms to prevent DNA lesions and to deal with DNA lesions that do arise. Despite this, some DNA lesions can remain unrepaired or be repaired incorrectly, resulting in permanent changes (mutations) in the DNA.

Many DNA processes in the cell are carefully coordinated in an effort to maximize efficiency and minimize errors in the cellular genome. Processes such as DNA replication and DNA repair/recombination result in the formation of a DNA intermediate (single-stranded DNA; ssDNA) that ultimately must be processed to an intact double-stranded DNA (dsDNA) form. At the center of these DNA processing events is the heterotrimeric protein complex Replication Protein A (RPA). The major biochemical activity of RPA is to bind ssDNA with high affinity and low sequence specificity through multiple oligonucleotide/oligosaccharide -binding (OB)-fold domains (*i.e.*, DNA binding domains; DBDs) located within the three subunits [1–3]. The largest subunit, Rpa1 (Rpa70; 70 kDa), is the major contributor to high-affinity ssDNA binding [4,5], while the smallest subunit, Rpa3 (Rpa14; 14 kDa), appears to be important for heterotrimeric complex formation [6]. The medium subunit, Rpa2 (Rpa32; 32 kDa) is thought to not only contribute to ssDNA binding [7,8], but also to regulate function of the RPA complex, especially in response to DNA damage, through multiple post-translational modifications (PTMs) [9–14].

The major emphasis of studies of RPA post-translational modification have focused on phosphorylation of the human Rpa2 N-terminus (NT) on multiple serine/threonine (S/T) residues located within the first 40 amino acids (aa) [15,16]. Human Rpa2 is phosphorylated both *in vitro* and *in vivo* on specific residues by multiple kinases during DNA replication and in response to specific DNA damaging agents. While some of these targets are consensus sequences (S/TQ) for phosphatidylinositol–3 (PI3)-related kinases (ATM and ATR) involved in checkpoint regulation, others are phosphorylation targets of cyclin-dependent kinase (CDK) and DNA-dependent protein kinase (DNA-PK) [17]. Many Rpa2 orthologs contain an N-terminal region that is S/T-rich; however, it is not known whether these residues in most orthologs are (1) actual targets of phosphorylation or (2) important for RPA cellular function.

Studies of the cellular function(s) of human Rpa2 phosphorylation initially focused on the utilization of "extensive" phospho-mutants, where *all* S/T residues in the Rpa2 NT were mutated to mimic phosphorylation (all aspartic acids; Rpa2-D_x), to prevent phosphorylation (all alanines; Rpa2-A_x), or were removed completely (deletion of first 33 aa; Rpa2-ΔN_x) [9,18]. These mutants, along with mutation of individual or pairs of sites have been instrumental in implicating this region as important for human RPA function in DNA repair, cell cycle progression, and protein interactions [9–14]. For example, it is clear that lack of hyper-phosphorylation of the human Rpa2 NT, either by mutation of serines 4 and 8 (S4/S8) to alanines or by inhibition of DNA-PK activity, leads to defects in the cellular response to replicative stress, including premature replication restart, hyper-recombination, and defective checkpoint arrest [11,14]. Also, ATR-dependent phosphorylation of threonine 21 (T21) and serine 33 (S33) is important for disrupting RPA association with replication centers and preventing replication during replication stress [9,12,13]. Although none of these effects have been examined beyond a few cell generations due to experimental complexity in human cells, the defective phenotypes would suggest long-term detrimental effects on cells. This is supported by an increase in apoptosis following replicative stress in human Rpa2-T21A/S33A mutant cells [19].

In the budding yeast *Saccharomyces cerevisiae*, it is not as clear what, if any, role phosphorylation of Rfa2 (specifically the Rfa2 N-terminus) has on cells. Phosphorylation of this region by the damage-specific kinase Rad53 during mitosis has been reported, but only when the yeast cells contain a *set*1Δ mutation [20]. The Rfa2 N-terminus (NT) is also phosphorylated by the meiosis-specific kinase Ime2 during meiosis [21]. However, an unphosphorylatable yeast Rfa2 NT mutant (Rfa2-A_x) has no discernible phenotype in mitotic cell growth or in standard DNA damage assays, indicating that this domain does not have to be phosphorylated for proper function of RFA in response to DNA damage in yeast [22]. Furthermore, if mitotic phosphorylation is occurring in this region (in a *SET*1 background), it is below the level of detection by Western blotting and has not been previously detected by mass spectrometry. Mutation of the Rfa2 NT, either to a constitutively phospho-mimetic form (Rfa2-D_x; analogous to human Rpa2-D_x) or to a form where the N-terminus has been removed (Rfa2-ΔN_x; analogous to human Rpa2-ΔN_x), leads to DNA damage-sensitivity [22]. However, removal of the Rfa2 N-terminus has also been reported to partially-suppress the damage-sensitive phenotype observed in *mec*3Δ or *set*1Δ cells, possibly through de-repression of expression of repair genes [20]. Taken together, this suggests that this domain is (1) necessary for the damage response (at least in *SET*1 cells) and (2) if phosphorylated, may need to be dephosphorylated for a proper response to DNA damage (based on the *rfa2-A*$_x$ damage-resistant phenotype). There is precedence for dephosphorylation being important in human cells (and in the yeast *Candida albicans*; [23,24]), as human