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Mammalian TIMELESS and Tipin are Evolutionarily Conserved Replication Fork-associated Factors

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The function of the mammalian TIMELESS protein (TIM) has been enigmatic. TIM is essential for early embryonic development, but little is known regarding its biochemical and cellular function. Although identified based on similarity to a *Drosophila* circadian clock factor, it also shares similarity with a second family of proteins that is more widely conserved throughout eukaryotes. Members of this second protein family in yeast (*S.c.* Tof1p, *S.p.* Swi1p) have been implicated in DNA synthesis, S-phase-dependent checkpoint activation and chromosome cohesion, three processes coordinated at the level of the replication fork complex. The present work demonstrates that mammalian TIM and its constitutive binding partner, Tipin (ortholog of *S.c.* Csm3p, *S.p.* Swi3p), are replisome-associated proteins. Both proteins associate with components of the endogenous replication fork complex, and are present at BrdU-positive DNA replication sites. Knock-down of TIM also compromises DNA replication efficiency. Further, the direct binding of the TIM-Tipin complex to the 34 kDa subunit of replication protein A provides a biochemical explanation for the potential coupling role of these proteins. Like TIM, Tipin is also involved in the molecular mechanism of UV-dependent checkpoint activation and cell growth arrest. Tipin additionally associates with peroxiredoxin2 and appears to be involved in checkpoint responses to H_2O_2, a role recently described for yeast versions of TIM and Tipin. Together, this work establishes TIM and Tipin as functional orthologs of their replisome-associated yeast counterparts capable of coordinating replication with genotoxic stress responses, and distinguishes mammalian TIM from the circadian-specific paralogs from which it was originally identified.

Keywords: TIMELESS; Tipin; DNA replication; checkpoint signaling; chromosome cohesion

Introduction

Mammalian TIMELESS (TIM) proteins were originally identified and named based on weak similarity to *Drosophila* TIM,[1–3] a protein essential for circadian rhythms in the fly.[4] While it has been difficult to pinpoint the specific role of TIM in the circadian clock, database mining and phylogenetic sequence analysis has suggested that mammalian TIMs may not be the true orthologs of the circadian clock-specific proteins found in insects, since they share even greater similarity to a second family of proteins that are more widely conserved in eukaryotes. These include *Drosophila* TIMEOUT (or TIM-2), *Saccharomyces cerevisiae* Tof1p, *Sschizosaccharomyces pombe* Swi1p, and *Caenorhabditis elegans* TIM (reviewed by Gotter[5]). Where studied, these proteins appear to be involved in molecular pathways important for efficient cell growth and/or development. Similarly, knockout of mouse TIM results in embryonic lethality just after blastocyst implantation,[6] and down-regulation of TIM disrupts epithelial cell morphogenesis important for tubule formation in the developing mammalian lung and kidney.[7,8]

The molecular pathways through which mammalian TIM exerts its vital functions have not been

Abbreviations used: TIM, TIMELESS protein; Tipin, TIM interacting protein; RPA, replication protein A; ssDNA, single-strand DNA; ATR, ataxia-telangiectasia and Rad3-related; BrdU, 5′-bromo-2′-deoxyuridine; IP, immunoprecipitation.

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determined. An initial step in understanding the protein's biochemical roles was made with the identification of its binding partner, Tipin (TIM interacting protein). Isolated by yeast two hybrid screening, Tipin avidly associates directly with TIM, and is co-expressed with *Tim* in proliferating embryonic and adult tissues.[9] Orthologs of Tipin are found throughout eukaryotes and include *S. cerevisiae* Csm3p and *S. pombe* Swi3p. Remarkably, yeast versions of Tipin bind directly to orthologs of TIM,[10–13] demonstrating the evolutionary conservation of this interaction.

Rapidly accumulating evidence from yeast and *C. elegans* has demonstrated that orthologs of TIM and Tipin are DNA replication fork-associated factors that are not only involved in DNA synthesis, but also participate in S phase-dependent checkpoint signaling and chromosome cohesion. Within the replisome, both factors are involved in preventing the collapse of replication forks that have stalled adjacent to DNA damage, natural replication barriers and mating type loci. Both genes were identified in *S. pombe* for their role in mating type switching. Mutations in either Swi1p or Swi3p are associated with a failure of replisomes to stably stall adjacent to mating-type loci[14,15] and rDNA sites.[16,17] At the biochemical level, these proteins have been postulated to physically coordinate helicase progression with DNA polymerase activity such that mutations in either gene are associated with the appearance of abnormally large regions of ssDNA adjacent to replication origins under both normal and replication stress conditions.[17–20] TIM and Tipin orthologs may also coordinate leading and lagging strand synthesis as genetic interactions have been observed between genes for these proteins and Polα and Polδ.[12]

Yeast orthologs of TIM and Tipin also function in intra-S-phase checkpoint activation. As replication forks encounter DNA damage or metabolic stress in the form of reduced dNTP levels, checkpoint signaling cascades are activated to stabilize stalled replication forks and to arrest cell cycle progression, which ultimately allows damaged DNA to be repaired or dNTP levels to be restored.[21–24] TIM and Tipin orthologs in yeast are involved in molecular and cellular responses to UV irradiation, methyl methanesulfonate (MMS)-induced DNA damage, or hydroxyurea (HU)-induced depletion of dNTP stores. These roles include checkpoint kinase activation, cell cycle arrest, and maintaining the integrity of replication forks stalled by these treatments.[20,25–27] Like yeast Claspin (Mrc1p), TIM and Tipin orthologs are likely to mediate these functions by coordinating kinases at the replisome while providing a structural link between helicase unwinding and polymerase-mediated elongation activity.[18,19]

Protein factors involved in DNA replication and checkpoint signaling also regulate the initiation and maintenance of chromosome cohesion,[28,29] a process crucial for proper segregation of replicated sister chromatids and recombination-mediated repair of double-strand DNA breaks. Three independent genetic screens identified yeast orthologs of TIM and Tipin as being involved in meiotic and mitotic chromosome segregation.[11,30,31] In *C. elegans*, TIM-1 has been found to loosely associate with SMC1 of the Cohesin complex, and is involved in loading Cohesin subunits onto newly replicated chromosomes.[32]

Previous uncertainty regarding the evolutionary origin and circadian function of mammalian TIM has clouded the potential relevance of the replication fork-associated functions of yeast and *C. elegans* orthologs. One recent article by Ünsal-Kaçmaz *et al.*[36] showed human TIM to be involved in HU-induced CHK1 phosphorylation and UV-dependent checkpoint responses. Here we demonstrate that the TIM-Tipin complex associates with the replisome through a direct interaction with replication protein A (RPA) and is important for efficient DNA synthesis. Like TIM, Tipin also appears to be involved in checkpoint signaling and cell growth arrest occurring in response to UV irradiation and additionally, oxidative stress. These studies demonstrate that mammalian TIM is a functional ortholog of a widely conserved family of proteins that are distinct from the circadian-specific factors restricted to insects.

Results

Tipin and TIM associate with replication protein A

To link the mammalian TIM-Tipin complex with known biochemical pathways, a yeast two-hybrid approach was used to identify proteins capable of interacting with mouse Tipin. Screening a mouse embryonic cDNA library identified of seven unique clones that were classified as true positive interactors (Figure 1(a)). Two of these positive clones encoded portions of the peroxiredoxin 2 and axotrophin proteins. The remaining five positives, which exhibited strong reporter activity, encoded overlapping portions of the same protein: the 34 kDa subunit of replication protein A (RPA34). Examination of the insert sequences encoding these five RPA34 fragments enabled us to delineate the Tipin binding region contained within the RPA34 protein. The smallest Tipin-interacting clone encoded the C-terminal 84 amino acid residues of the full-length RPA34 protein (Figure 1(b)), a region containing the 34-residue XPA (xeroderma pigmentosum complementing factor A)-binding domain.[33] Conversely, the site on XPA known to bind RPA34 has also been delineated,[33] and resembles a 48-residue stretch of the Tipin protein sequence. These observations indicate that Tipin and RPA34 have interaction sites for one another, suggesting that RPA34 is a *bona fide* Tipin-interacting protein.

The interaction of RPA34 with Tipin was of particular interest given the known cellular roles