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ABSTRACT

Genomic DNA is the fundamental hereditary material that passes from generation to generation. In cells, the integrity of DNA is vulnerable to environmental hazards such as radiation and certain chemicals, which can result in DNA damage and thus genetic alterations. Such DNA damage may also arise from attack by intracellular, chemically reactive molecules which are byproducts of normal metabolic processes. Unrepaired DNA often leads to conditions that are favorable for development of abnormalities in cells and organisms, or diseases in humans such as cancer. As a result, the cell has evolved an effective way to detect and repair DNA damage before it divides. ATM is a key player in DNA damage responses. Together with a number of other damage control proteins, it detects DNA damage and sends the damage signal to temporarily halt cell division and mobilize cellular enzymes to fix DNA molecules. We show some evidence that mTOR/FRAP, a member of the ATM kinase family, may have a role in DNA damage control by directly interacting with DNA. This observation suggests that rapamycin, a highly specific inhibitor of mTOR/FRAP, may be used to sensitize ionizing radiation therapy of breast cancer.

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US Army Idea Award (DAM17-02-1-0614) Year Three Report

(June 01, 2004-May 31, 2005)

Principal Investigator: Xiao-Feng Steven Zheng, Ph.D.

Introduction

Rapamycin is a bacterially produced macrolide with potent anti-cancer activity. Ester-derivatives of rapamycin, including CCI-779 and RAD001 are under multiple clinical trials for breast cancer treatment. These compounds are collectively called rapamycins that have the same mechanism to inhibit cell growth. Preliminary results show excellent efficacy among cancer patients (Huang and Houghton, 2002). Mammalian target of rapamycin (mTOR), also named FKBP12-rapamycin-associated protein (FRAP) is the physiological target of rapamycins. mTOR is a 289kDa protein serine/threonine kinase that is essential for cell growth in response to nutrients and growth factors (Schmelzle and Hall, 2000). Although the primary function of mTOR is in growth control, it belongs to a group of atypical protein kinases that are sensors of DNA damage, which includes ATM, ATR and DNA-dependent protein kinase (DNA-PK) (Hunter, 1995; Keith and Schreiber, 1995). mTOR/FRAP is well known as a regulator of protein synthesis (Gingras et al., 2001). On the other hand, ATM, ATR and DNA-PK are key checkpoint kinases that transduce DNA damage signals to regulate cellular responses, including stoppage of cell cycle progression and activation of DNA repair mechanisms. In the extreme event when DNA damage is severe, they mediate signals that trigger program cell death or apoptosis.

The fact that mTOR is related to DNA damage checkpoint kinases suggests that mTOR has a role in DNA damage signal transduction. Indeed, we found that Rad53, the yeast ortholog of DNA checkpoint kinase Chk2, is genetically linked to TOR signaling in budding yeast. Inhibition of the yeast TOR by rapamycin results in phosphorylation of Rad53. Additionally, rapamycin treatment induces the expression of several DNA repair genes, including RAD28, RAD51, CHK1 and MUS81. These results indicate a role of TOR in the DNA damage response. A compelling model for TOR function in DNA damage signaling is to coordinate cell growth and DNA repair. Like cell cycle progression, the cell may need to complete DNA repair before committing to resuming cell growth. Our major goal during the reporting period is to (1) study whether DNA damage inhibits mTOR activity; (2) investigate whether TOR interacts with DNA; (3) determine the phosphorylation sites on Chk2. My laboratory has recently moved from Washington University to UMDNJ-Robert Wood Johnson Medical School since July/August/September, 2004. In addition to the time-consuming physical move, two other factors has resulted considerable delays in work in this area. First, two people working on this project decided to stay in St Louis. We have not been able to find suitable replacements until recently. Second, there was a long delay in the transfer of this grant from Washington University to UMDNJ-Robert Wood Johnson Medical School, which also affected our ability to hire researchers in a timely manner. Nonetheless, we have made one breakthrough discovery: we found that the target of rapamycin (TOR) protein is indeed a DNA-binding protein and its DNA binding ability is sensitive to rapamycin. We have just reinitiated work in the two other areas and hope to make significant progress during the coming year.

Body

TOR is a member of the ATM kinase family that includes ATM, ATR and DNA-dependent protein kinase (DNA-PK). ATM, ATR and DNA-PK are known DNA-binding proteins. The fact that these proteins interact with DNA suggests that TOR can be a DNA-binding protein as well. Despite such obvious connection, however, there has been little effort to investigate this possibility. This is primarily because of the apparent role of TOR in mediating extracellular growth signals (nutrients and growth factors). It has been always presumed in the field that TOR functions only in the cytoplasm. If mTOR functions as a DNA-binding protein, at least a fraction of mTOR proteins must reside in the nucleus. To confirm this, we have recently conducted immunofluorescence staining of mTOR in different cell lines (Drenan et al., 2003). Indeed, mTOR is a nuclear protein in certain human cells, including breast cancer (MCF7) and ovarian cancer cells (A2774)(Figure 1). The amount of mTOR found in the nucleus can be variable, depending on the cell types. We have also observed that Tor1 is localized in the yeast nucleus (Figure 2). These data demonstrate that Tor proteins have a conserved nuclear function, possibly in monitoring DNA integrity.

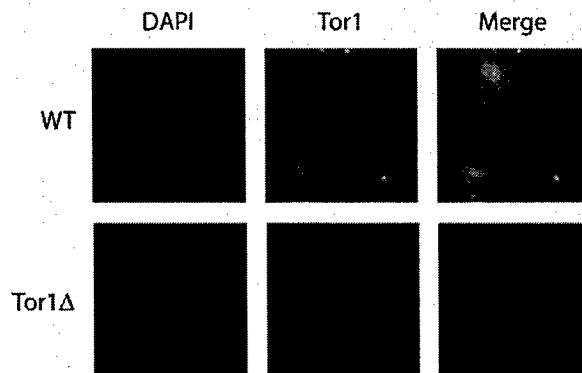
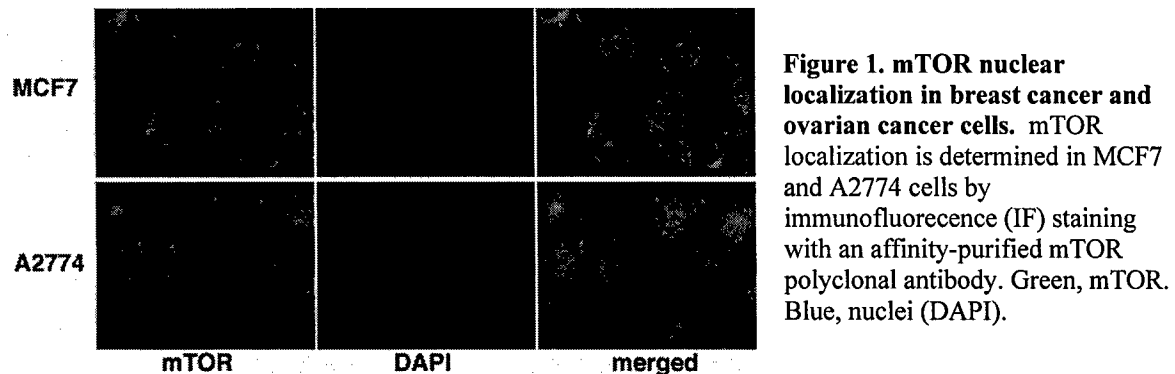


Figure 2. Tor1 nuclear localization in yeast cells. Tor1 localization in the wild type or *tor1* null (*Tor1Δ*) yeast cells is determined by immunofluorescence (IF) staining with an affinity-purified Tor1 polyclonal antibody. Green, Tor1. Blue, nuclei (DAPI). There is no Tor1 staining in the *Tor1Δ* cells, demonstrating the specificity of the antibody.

The above observations prompted us to further investigate whether TOR interacts with DNA. Chromatin immunoprecipitation (ChIP) assay is a powerful tool to study protein-DNA interaction in vivo. In this assay, cells are treated with formaldehyde, a chemical that cross-links proteins with DNA. If a protein binds to a specific DNA sequence, it will be cross-linked with this DNA sequence, which can be co-immunoprecipitated with the protein and detected by polymerase chain reaction (PCR). This assay is highly sensitive and reliable. In contrast, the in vitro protein-DNA assay we have

proposed in our original proposal is much less sensitive and is prone to produce artifact results. We decided to validate this hypothesis in yeast. This is primarily because we have generated a high affinity antibody against the yeast Tor1 that works very well with immunoprecipitation/Western blot/IF. We chose to assay for Tor1 binding to the ribosomal DNA (rDNA). The advantage of using rDNA is that there are ~150 tandem copies of rDNA genes on chromosome XII, which significantly increases the sensitivity of this assay. We found that rDNA is precipitated in the presence of the Tor1-specific antibody and the Tor1 protein. However, no or little rDNA is found in the absence of Tor1 or in the presence of a control antibody. These results demonstrate that Tor1 can specifically interact with rDNA. Moreover, we found that rapamycin inhibits the ability of Tor1 to bind to DNA, suggesting that rapamycin may mimic DNA damage condition. A working model for increase of radiosensitivity by rapamycin is that rapamycin causes TOR dissociation from DNA, which enhances cellular sensitivity to DNA damage.

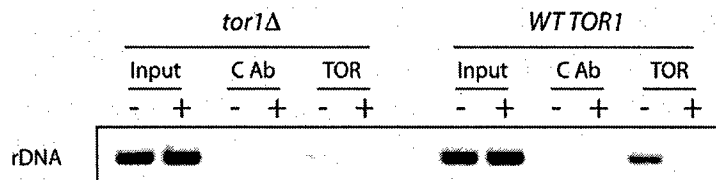


Figure 3. Tor1 binding to ribosomal DNA (rDNA) in a rapamycin-sensitive manner. Tor1 binding to rDNA is assayed by ChIP. Actively growing yeast cells or yeast cells treated with rapamycin for 1 hr were fixed with formaldehyde to cross-link DNA with proteins. Tor1 is immunoprecipitated and its bound rDNA is detected by PCR with a pair of rDNA specific primers. C Ab, control antibody; TOR, Tor1-specific antibody. *tor1Δ*, *tor1* null cell; *WT TOR1*, wild type cell.

By sequence analysis, we have identified three potential DNA-binding motifs in Tor1, two leucine zipper sequences (LZ I and LZ II) and a helix-turn-helix (HTH) motif. The HTH motif, but the two leucine zippers, is well conserved among Tor1, Tor2 and mTOR (Figure 4A). Interestingly, deletion of the HTH motif, but not the two leucine zippers significantly inhibits the ability of Tor1 to bind to rDNA (Figure 4B). Taken together, these data indicate that Tor1 binds to DNA via a conserved HTH motif, and suggest that this DNA-binding property is conserved in humans. These studies laid a foundation for future mechanistic understanding of mTOR in DNA damage response.

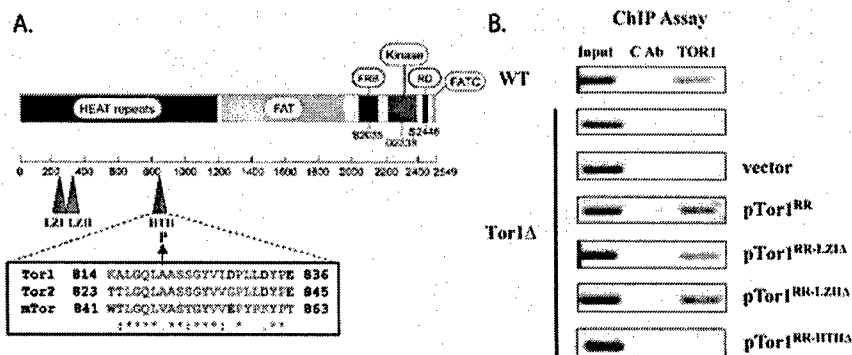


Figure 4. Identification of a conserved helix-turn-helix (HTH) that mediates Tor1 binding to DNA. (A) Potential DNA binding motifs in Tor1. The alignment shows that the HTH motif is conserved. (B) Tor1 binding to rDNA is assayed by ChIP. *tor1Δ* null cells carrying no plasmid, a control plasmid vector, or plasmid expressing Tor1 or Tor1 mutants that have deleted different putative DNA-binding motifs are assayed for their ability to bind to rDNA. C Ab, control antibody.

Key Research Accomplishments

- Demonstrated that TOR proteins are localized in the nucleus in both yeast and human cells, including breast cancer cells
- Demonstrated that Tor1 binds to DNA in vivo
- Identified a conserved HTH motif that mediates Tor1 binding to DNA

Reportable Outcomes

We have observed that Tor1 interacts with DNA in vivo. This is a significant advance in the TOR field. It is potentially a breakthrough discovery toward understanding the mechanism by which TOR is involved in DNA damage response.

Conclusions

We have shown that TOR binds to DNA using a conserved HTH motif, a classical DNA binding sequence. It provides an important starting point for investigating the ability of mTOR to bind to DNA in human cells in response to DNA damages.

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