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Schizosaccharomyces pombe rad3+ is homologous to the human ATM gene, which when mutated, causes the autosomal recessive human disease ataxia-telangiectasia. A-T patients suffer from a variety of symptoms, including a predisposition to cancers such as leukemias and lymphomas. A-T heterozygotes may also have an increased risk of developing cancers, in particular breast cancer. ATM and Rad3p are both structurally and functionally similar, as cells from A-T patients and *rad3-* yeast mutants both have sensitivities to ionizing radiation, and defects in cell cycle checkpoints. Since fission yeast Rad3p is believed to be involved in monitoring the state of the DNA, we have investigated genetic interactions between Rad3p and DNA replication proteins by using both genetic and biochemical approaches. We have also performed a structure/function analysis of *rad3*$^{+}$, which has identified domains important for normal *rad3*$^{+}$ function.

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Carolyn R Chapman 1/25/99

PI - Signature Date

Table of Contents

Introduction

I. Ataxia-Telangiectasia: Elevated Cancer Risk

Ataxia-telangiectasia (A-T) is a debilitating and progressive autosomal recessive human disease in which homozygotes suffer a predisposition to cancer, such as lymphomas and leukemias, and a marked sensitivity to ionizing radiation. In addition, the patients exhibit a variety of other symptoms; in particular, the immune system, nervous system, and skin are affected (7)(8).

Certain studies have found that individuals heterozygous for ataxia-telangiectasia have an increased risk of cancer (4); in particular, female heterozygotes may be five times more likely to develop breast cancer than control populations (22). However, this point is still not resolved, as some groups have found that ATM mutations do not confer a predisposition to early onset of breast cancer (6). It is clear that research on the biology of the A-T gene is likely to increase our understanding of one potential cause of breast cancer and may improve our ability to provide the most appropriate methods of diagnosis and treatment for these patients.

II. The *ATM* Gene: Homologous to *S. pombe rad3*$^+$

ATM (ataxia-telangiectasia mutated), the gene responsible for causing ataxia-telangiectasia, has been cloned and sequenced (18, 19). ATMp is homologous to a family of proteins which includes *S. pombe* Rad3p (the gene product of the *S. pombe rad3*$^+$ gene) (3, 18). Both ATMp (the predicted protein product of the *ATM* gene) and Rad3p contain leucine zipper motifs, which may mediate their dimerization or interaction with other proteins (19). They also each contain a kinase domain at their C-termini (19). Rad3p has been shown to be capable of autophosphorylation (3), while ATM has been shown to be capable of phosphorylating protein substrates (11). *ATM* and *rad3*$^+$are also homologous to the *S. cerevisiae MEC1/ESR1* gene (18). In addition to sharing substantial sequence homology, *ATM*-deficient cells and *rad3*$^-$ and *mec1*$^-$ mutants exhibit remarkable phenotypic similarities.

III. The *ATM* gene family: Functionally and structurally similar proteins in a range of organisms

Human *ATM*, *S. pombe rad3*$^+$ and *S. cerevisiae MEC1* are cell cycle checkpoint genes. These genes maintain the proper order of events in the cell cycle by preventing cell cycle progression at inappropriate times (9). For example, to ensure that a euploid set of chromosomes is transferred to the next generation, DNA synthesis must be completed before cells divide. To maintain the fidelity of genetic information, cells must not initiate mitosis in the presence of damaged DNA. Both *rad3*$^-$ and *mec1*$^-$ mutants fail to maintain these dependencies (5)(26)(12)(1). Although the checkpoint deficiency is most well-studied at G2/M in these organisms, there is evidence that the cells lack other cell cycle controls as well. Compared to wild-type cells, *mec1*$^-$ mutants exhibit a reduced ability to slow DNA replication in response to alkylating agents (17). *rad3* function is also believed to contribute to S-phase arrest in *S. pombe* (14).

A-T cells also lack the ability to execute a number of checkpoint controls which normally act to prevent cell cycle progression in the presence of damaged DNA (8)(23). These cells lack a G1/S checkpoint, the ability to delay entry into S phase in the presence of DNA damage induced by ionizing radiation . A-T cells also exhibit a defect in their G2/M checkpoint, which prevents cells with damaged DNA from entering mitosis (20)(2). Also like *mec1*$^-$ and *rad3*$^-$ mutants, A-T cells exhibit a deficiency in slowing the rate of DNA synthesis following exposure to DNA-damaging agents such as ionizing radiation (16)(10).

The above evidence suggests that the *ATM*, *rad3*$^+$, and *MEC1* gene products work by similar mechanisms to detect and respond to DNA damage by activating repair pathways and arresting cell cycle progression.

The ATMp family appears to define a novel class of proteins involved in communicating information about DNA to the cell. Their function is undoubtedly extremely important, as evidenced by the drastic phenotypes caused by loss-of-function mutations in these genes. I have initiated genetic and biochemical analysis to elucidate the molecular mechanism of action of the *rad3*+ gene. Any information we gain about *rad3*+ can be applied to further our understanding of the functions of *ATM*.

IV. The *Schizosaccharomyces pombe* checkpoint pathway

rad3+ is one of a number of checkpoint genes that have been identified in *Schizosaccharomyces pombe* (for a recent review, see (21)). The *S. pombe checkpoint rad* mutants, which include *rad1*, *rad3*, *rad9*, *rad17*, *rad26*, and *hus1*, all have similar phenotypes; they fail to arrest the cell cycle in the presence of either DNA damage or incomplete DNA replication. *chk1* and *cds1* encode kinases which also function in checkpoint control in fission yeast (25)(15). In contrast to the *checkpoint rad* mutants, *chk1* mutants appear to be specifically defective in responding to DNA damage, while *cds1* mutants exhibit the reciprocal problem, failure to recover from S phase arrest. Chk1p and Cds1p function downstream of the *checkpoint rad* genes (25)(15).

Rad3p is believed to play a central role in cell cycle checkpoint control in *Schizosaccharomyces pombe*. Evidence suggests that Rad3p is integrally involved in monitoring the DNA and relaying information about DNA damage and incomplete DNA replication to other cellular proteins such as DNA repair enzymes and the cell cycle machinery. My studies are aimed at achieving a better understanding of how *S. pombe* *rad3*$^+$ functions, as a model system for understanding the biology of human *ATM*. This report describes my work while on the grant, and is focused on details of experiments which were completed over the past year (January 1, 1998-December 31, 1998).

Body

I. Identification of gene products that interact with Rad3p: DNA replication proteins

As detailed in my last annual report, I have found interesting genetic and physical interactions between Rad3p and components of the DNA replication machinery. I discovered a gene and allele-specific genetic interaction between *rad3* and *cdc6* (*polδ*), the leading strand DNA polymerase. When I overexpress *rad3+* in either *cdc6-23* or *polδts1* alleles, the phenotypes of the *cdc6* mutants are worsened, and they now elongate at their permissive temperature. This data indicates that *rad3+* and *cdc6* exhibit a synthetic dosage phenotype. Synthetic dosage lethality provides evidence for an interaction between two genes (13). Overexpression of other checkpoint genes is unable to cause this phenotype, and overexpression of *rad3+* in other DNA replication mutants does not cause such a synthetic phenotype. Although I have been unable to observe any physical interactions between Rad3p and polδ, we do believe that the specificity of the genetic interaction indicates that these two proteins may interact in a special way. I have also discovered both physical and genetic interactions between Rad3p and PCNA, the processivity factor for polδ, which I also described in my last report. We believe that one of the normal functions of Rad3p may be to interact with components of the DNA replication machinery, possibly in order to slow DNA synthesis in the presence of DNA damaging agents, as discussed above.

In the past few months, I have performed an experiment to address why overexpression of *rad3* in specific mutants of *cdc6* (*polδ*) causes the polδ mutants to have a lower nonpermissive temperature (or a synthetic dosage phenotype). To distinguish whether *rad3+* overexpression causes DNA damage or whether it interferes with DNA replication, we investigated whether *rad3+* causes elongation in *cdc6-23* mutants in different checkpoint deficient backgrounds. *cdc2-3w* mutants are specifically defective in the DNA replication checkpoint, while *chk1-* mutants are mainly deficient in the DNA damage checkpoint. If the overxpression causes DNA damage, the DNA damage checkpoint deficient cells should undergo aberrant mitoses or "cuts." If the overexpression interferes with DNA replication, "cuts" should form in DNA replication checkpoint-deficient backgrounds. We overexpressed *rad3+* and vector controls in *cdc6-23, cdc6-23 cdc2-3w*, and *cdc6-23 chk1::ura4+* mutants and investigated the phenotype at the permissive temperature by looking at cell morphology and counting percent aberrant mitoses. In the *cdc6-23 chk1-* background, overexpression of *rad3+* does not result in aberrant mitoses, suggesting that large amounts of DNA damage are not present. The large

percentage of aberrant mitoses in the *cdc6-23 cdc2-3w* cells suggests that overexpression of *rad3+* interferes with DNA replication in *cdc6-23* cells. The ability of *rad3+* to interfere with DNA replication is a novel finding.

In the next few months, we hope to prepare a manuscript which describes the interactions we have observed between Rad3p and the DNA replication proteins. We would also like to perform a few more experiments to further our understanding of these interactions. Specifically, I would like to confirm that *rad3+* is interfering with DNA replication in these *cdc6* alleles using another method. I will perform CHEF gels to investigate the status of the DNA in *cdc6-23* alleles with and without *rad3+* overexpression. We expect that at the permissive temperature, *cdc6-23* alleles will be able to replicate their DNA, and thus the *S. pombe* chromosomes will enter the CHEF gel. We will look to see whether overexpression of *rad3+* interferes with DNA replication by checking to see whether the chromosomes fail to enter the gel in this condition. We will also confirm the Rad3p/PCNA physical interaction using tagged versions of the proteins.

II. Regulation of Rad3p protein and activity-- Structure/Function Analysis of Rad3p

As discussed in my previous report, we have taken a structure/function approach to understand the regulation of Rad3p protein and activity. In collaboration with Sarah Evans, another graduate student in my lab, I recently submitted a manuscript "Requirement of Sequences Outside the Conserved Kinase Domain of Fission Yeast Rad3p for Checkpoint Control," to *Molecular Biology of the Cell* which described these studies. (I submitted a copy of this manuscript to Patricia Modrow at U.S. Army Medical Research and Materiel Command, as well, in accordance with the procedures outlined in the grant.) In this manuscript, we showed that the kinase domain is necessary but not sufficient for Rad3p function. C-terminal Rad3p constructs which contain the complete Rad3p kinase domain cannot complement a *rad3* deletion strain, nor do they have kinase activity *in vitro*. As discussed in the last report, I found that a construct which contained the first 775 amino acids of Rad3p caused a dominant negative checkpoint phenotype in wild-type cells, when overexpressed. This shortened Rad3p construct probably functions as a dominant negative by titrating important Rad3p interactors into nonfunctional complexes. By performing deletional analysis into the N-terminal construct, we identified two sites N-terminal to the conserved kinase domain that are essential for Rad3p function. One of these sites is the putative leucine zipper, which is conserved throughout the PI3KR family. The other is a novel motif, which we call the Psite, which may also potentially mediate Rad3p protein-protein interactions.

Interestingly, full-length *rad3* alleles which harbor deletions in these sites, *rad3-LZdel* and *rad3-Pdel*, fail to complement *rad3Δ* cells, although they retain kinase activity. This suggests that kinase activity is not sufficient for complementation of the *rad3Δ* checkpoint deficient phenotype. In addition, I found that *rad3-LZdel* is still capable of Rad3p homomultimerization, indicating that the leucine zipper is not required for the ability of Rad3p to self-associate. The ability of Rad3p to self-associate was first reported by others (3). I have also found that *rad3-LZdel* is still capable of binding to Chk1p, an interaction that was recently described by others in the literature. This indicates that the leucine zipper may be mediating binding to another factor that has not yet been identified.

• Composition of the Rad3p Complex: Self-Association Studies

Recently, I have engaged in studies aimed at addressing the composition of the Rad3p complex. As mentioned above, others have demonstrated that Rad3p can homomultimerize (3), and I have confirmed their findings. I have started to investigate what domains of Rad3p are required for these interactions. Localizing the self-association domain in Rad3p would be very interesting because we could access whether self-association was required for the normal function of Rad3p by performing complementation studies. In addition, we would also be able to determine if dimerization is necessary for Rad3p kinase activity. We could also examine the affect mutation of the domain has on dominant negative activity. Although we were suprised that the leucine zipper mutant did not abrogate the ability of Rad3p to self-associate (see above), we have preliminary data that suggests that a Rad3p self-association domain might be located between amino acids 184-372 of the protein.

I have performed immunoprecipitation experiments which demonstrate that full-length Rad3p has the ability to bind to tagged versions of Rad3-N775, Rad3-N690, Rad3-N541, and Rad3-N372 N-terminal constructs (both proteins are overexpressed). N775-LZdel and N775-Pdel are also both capable of binding to full-length Rad3p. Rad3p may also be able to bind to the C-terminal kinase domain as well, but the interaction appears to be weaker than with the N-terminus. However, an N184 construct, which contains the leucine zipper motif, is unable to bind full-length Rad3p, suggesting that there may be a Rad3p self-association domain located between amino acids 184-372. Interestingly, there appears to be a domain which resembles a single TPR motif in this region. TPR motifs are thought to mediate protein-protein interaction. However, TPR motifs are extremely degenerate, and are usually present in multiples. I specifically looked for TPR motifs in Rad3p because a new PI3KR protein, TRAAP, contains a putative TPR motif.

rad3 Allele	Ability to Bind Full-Length Rad3p
rad3+	++
rad3-LZ	++ (Chapter I)
rad3-N775	++
rad3-C725	+
rad3-N775-P	++
rad3-N775-LZ	++
rad3-N690	++
rad3-N541	++
rad3-N372	++
rad3-N184	--
rad3-Δ1-183	? (hypothesis +)
rad3-Δ1-372	? (hypothesis --)
rad3-Δ220-441	? (hypothesis --)
rad3-184-775	? (hypothesis +)
rad3-373-775	? (hypothesis --)
rad3-N775Δ220-441	? (hypothesis --)

I am hoping to identify the Rad3p self-association domain located in the N-terminus. To do so, I am creating new mutants: Δ1-183 and Δ1-372. If the Rad3p self-association domain is indeed located between amino acids 184 and 372, we would expect Δ1-183 to be capable of binding to full-length Rad3p, and that Δ1-372 would be unable to do so. I have also created Δ220-441, chosen because of convenient restriction enzyme sites. If the Rad3p self-association domain is in the TPR motif, this mutant should eliminate Rad3p self-association. I will first observe the binding of the deletions in the context of the N-terminal domain (N775) alone, since there may be more than one self-association domain in Rad3p (for example, I have evidence that another one may exist in the C-terminus). I will then try the binding assays in the context of the full-length alleles.

If we can locate the Rad3p N-terminal self-association domain, we can determine the genetic phenotypes of *rad3* alleles which lack the domain, in both complementation studies and dominant negative assays. We will also investigate the kinase activity of the mutant(s). Rad3p self-association might be required for kinase activity, for example.

Progress Towards Statement of Work

Technical Objective 1: Identify gene products that interact with Rad3p

Task 1. Months 1-6. (Completed) We have determined if overexpression of known checkpoint genes can suppress *rad3-* mutations, and have been unable to observe cross suppression, except with *chk1* and *cds1*, which are already known to function downstream of Rad3p.

Task 2. Months 6-12. (Completed) As detailed in the previous report, we performed a screen for high copy plasmid-born suppressors of *rad3-* mutations. However, we only isolated very weak suppressors of *rad3-* mutants, and at this time, we are unsure of the relevance of their relation to the checkpoint pathway. This project has been put on hold for the time being.

Task 3. Months 12-16. (Completed) As detailed in the previous report, we sequenced the high copy suppressors we isolated in our screen. We compared the sequences to the data base. As mentioned in Task 2, we are not following up on these clones at the present time because they are not strong suppressors of *rad3-* mutants, and other areas of our research are proving more fruitful.

Task 4. Months 16-20 (No longer appropriate) As described above, because we are unsure of the relevance of the suppressors we isolated to the *rad3* checkpoint pathway, we are not following up on the clones. Therefore, we do not feel it is productive to create null alleles for these genes.

Task 5. Months 1-6. (Completed) Others in my lab have created *rad3* mutants with other checkpoint genes, and there are no phenotypes which indicate interesting epistatic relationships.

Task 6. Months 6-12 (Completed) We use a classical genetics approach to identify a synthetic dosage phenotype between *rad3* and *cdc6* (pol∂), the leading strand DNA polymerase. We have also used overexpression studies to identify genetic interactions between *rad3* and PCNA.

Task 7. Months 12-18 (Completed) *cdc6* and PCNA are known genes, which have already been cloned and genetically characterized by others.

Task 8. Months 18-24 (Completed) Because *cdc6* and PCNA have already been cloned and sequenced by others, we do not have to clone these genes.

Task 9. Months 24-30. (Completed). See Task 8.

Task 10. Months 30-36 (Completed) *cdc6* and PCNA are both essential genes. The null alleles have been published in the literature. We have also obtained many different alleles of *cdc6*.

Task 11. Months 1-6 (Completed) We have constructed yeast strains expressing epitope-tagged versions of Rad3p.

Task 14. Months 1-8. (Completed). We have prepared polyclonal antibodies to bacterially expressed Rad3p. We were unable to have synthetic peptides of Rad3p created, for technical reasons. Our antibodies recognize Rad3p when overexpressed from moderate strength promoters. Unfortunately, we have been unable to visualize Rad3p at endogenous levels.

Task 15. Months 8-24 (In progress). We are using epitope-tagged versions of Rad3p to analyze Rad3p complexes by immunoprecipitation. For example, we have looked at the ability of Rad3p to self-associate and to interact with Chk1p and PCNA (and pol∂). We are currently investigating the domains required for the Rad3p self-association.

Task 16. Months 25-36 (In progress). See Task 15.

Technical Objective 2: Regulation of Rad3p

Task 15. Months 8-12 Unfortunately, we have been unable to examine expression levels of Rad3p in wild-type and checkpoint defective cells because we are unable to visualize endogenous levels of the protein.

Task 16. Months 8-14. (In progress) Because we are unable to visualize endogenous levels of protein, it is difficult to assess the subcellular localization of Rad3p. However,

preliminary experiments to try to develop immunofluorescence assays for Rad3p localization have been performed.

Task 17. Months 14-24. (In progress) We have checked for a shift in Rad3p migration on SDS-PAGE gels in response to HU and in response to bleomycin, which might indicate a change in phosphorylation state. We have been unable to observe any changes in mobility of the protein. We are currently studying Rad3p complexes, as mentioned above.

Task 18. Months 24-36 (Not yet addressed)

Task 19. Months 24-36 (Not yet addressed)

Task 20. Months 36-42 (Not yet addressed)

Task 21. Months 42-48. (Partially completed) As stated above, we have prepared a manuscript describing work relating to the regulation and structure/function analysis of Rad3p, and submitted it to *Molecular Biology of the Cell*. We have provided a copy of this manuscript to the Department of Defense.

Conclusions

I. Identification of gene products that interact with Rad3p

We have discovered genetic interactions between Rad3p and both polϑ and PCNA, as well as a physical interaction between Rad3p and PCNA. In future experiments, we hope to further understand exactly how these proteins might function together. Our current model is that Rad3p can inhibit DNA replication in certain circumstances, possibly by direct physical interaction with members of the DNA replication complex.

Over the next year, we hope to perform experiments which will lead to a manuscript detailing the interactions we have observed between Rad3p and components of the DNA replication machinery.

II. Regulation of Rad3p protein and activity

In order to further our understanding of regulation of Rad3p protein and activity, we have performed a structure/function analysis on *rad3*. Our studies indicate that the leucine zipper motif, the kinase domain, and a site we call the "Psite," are all required for proper Rad3p function. We have analyzed the mutants we have generated by measuring their phenotypes quantitatively by performing hydroxyurea time courses, radiation curves, and assessments of their effects on Rad3p kinase activity (using an assay developed by Sarah Evans in the lab). We have compiled our work into a manuscript entitled "Requirement of Sequences Outside the Conserved Kinase Domain of Fission Yeast Rad3p for Checkpoint Control," which we have submitted to *Molecular Biology of the Cell.* (A copy of this manuscript was also sent to Patricia Modrow at the Department of the Defense, postmarked December 5, 1998).

Our future studies will aim to identify the domains required for the self-association of Rad3p, as described above. We believe our studies are furthering our understanding of how Rad3p functions, and hope that our work will be used as a model for ATM.

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