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Award Number: DAMD17-98-1-8023

TITLE: Alternative DNA Damage Checkpoint Pathways in Eukaryotes

PRINCIPAL INVESTIGATOR: Yi-Chen Li, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

REPORT DATE: April 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2000	Annual Summary (23 Mar 99 - 22 Mar 00)

4. TITLE AND SUBTITLE
Alternative DNA Damage Checkpoint Pathways in Eukaryotes

5. FUNDING NUMBERS
DAMD17-98-1-8023

6. AUTHOR(S)
Yi-Chen Li, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030

E-MAIL:
yili@bcm.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Our laboratory has previously shown that a human cDNA *CHES1* (checkpoint suppressor 1) suppresses multiple mutants along the primary DNA damage checkpoint pathway in *Saccharomyces cerevisiae*. Our hypothesis is that *CHES1* does so by activating an alternative DNA damage-induced checkpoint pathway. The objectives of this project are to identify, characterize, and clone the genes in this pathway, and to isolate human homologs and analyze their structure and expression in human breast cancers.

We have constructed a temperature- and UV-sensitive strain SCP2. Both phenotypes can be partially rescued by *CHES1*. Approximately 220,000 colonies of SCP2 have been mutagenized and screened for the mutant phenotypes in the presence of *CHES1*. Three *chb* (for checkpoint bypass) mutants were isolated. Among them, *chb13* has a strong mutant phenotype and both *chb16* and *chb57* are weaker alleles. We have confirmed that all three mutants are recessive and belong to the same complementation group. In the process of cloning this gene we encountered some difficulties, therefore we also tried the candidate gene and the yeast 2-hybrid approaches but with no success. In this report, we proposed alternative methods in cloning the new pathway genes. We will also focus on the characterization of *CHES1* in mammalian cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Checkpoints, Mutagenesis, Radiation Resistnace, Tumorigenesis, Cell Cycle	10
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

DNA damage checkpoint pathways are important regulatory mechanisms in cancer development. Breast cancer, in particular, has been linked to several DNA damage checkpoint genes. Most of these genes are conserved across evolution. For example, the mammalian *ATM* gene has a counterpart in *Saccharomyces cerevisiae* called *MEC1*, and one in *Schizosaccharomyces pombe* called *Rad3*. Many of the pathways merge, split, or work in parallel to form a complex DNA damage regulatory network. This project studies a *MEC1*-independent DNA damage checkpoint pathway in budding yeast *Saccharomyces cerevisiae*. Since yeast is a terrific genetic tool, the goal here is to use yeast as a model to identify new pathway genes, and then search for homologs in higher eukaryotes.

Body

A human cDNA *CHES1* (checkpoint suppressor 1) was isolated previously in our laboratory by high copy suppression of the DNA damage checkpoint mutant *mec1-1* in the budding yeast *Saccharomyces cerevisiae*. It was also shown that *CHES1* suppresses multiple other mutants along the same pathway. Our hypothesis is that *CHES1* does so by activation of an alternative DNA damage-induced checkpoint pathway in *S. cerevisiae*. The objectives of this project are to perform a mutagenesis screen to identify the genes in the alternative DNA damage checkpoint pathway, to characterize and clone these genes, and to isolate the human homologs of these genes and analyze their structure and expression in human breast cancers.

We have constructed a *cdc9-8*, *rad9Δ* double mutant strain SCP2, for which the permissive temperature is 30°C in the presence of *CHES1* and 23°C in the absence of *CHES1*. The UV sensitivity due to a *rad9Δ* mutation in this strain is also partially rescued by the presence of *CHES1*. Approximately 220,000 colonies of SCP2 were mutagenized by EMS at a 50%-killing condition, and screened for temperature-sensitivity at 30°C in the presence of *CHES1*. Three *chb* (for checkpoint bypass) mutants were isolated. Among those, *chb13* is highly temperature sensitive whereas *chb57* and *chb16* are weaker alleles. These mutants were also subjected to a second UV-sensitivity test. The results showed that *chb13* and *chb57* are sensitive to UV radiation despite the presence of *CHES1* but the effect of *chb16* is minimal when compared to the controls. Overall, *chb13* appears to have a strong mutant phenotype that has lost all response to *CHES1* by our assays. Strain *chb57* is a moderate mutant that has one strong phenotype. Strain *chb16* is in general an unhealthy strain. We have confirmed that all three mutants are recessive by mating to wild type strains. We have also checked for complementation groups among the three mutants and found they all belong to the same group, i.e., they all contain mutations in the same gene. Therefore, we decided to clone the gene mutated in *chb13*, since it has the strongest phenotype.

The *chb13* mutant strain was backcrossed twice, and selected for mutant phenotype in the presence of *CHES1*, to segregate out any unrelated mutations in the genome. The resulting strain was used to perform complementation by a CEN/TRP yeast genomic library. There was high background growth at 32°C even when the empty vector was used for transformation so identification of candidate clones was somewhat complicated. A total of 57,500 transformants were screened and 34 candidate clones were obtained. Most of these clones contain the *CDC9* genomic fragment. These clones, which can grow at 37°C since they now have a wild type copy of *CDC9*, were later eliminated by screening at 37°C. The remaining clones, however, when transformed back to the *chb13* mutant strain, could not complement the mutant phenotype. We have also screened 20,000 transformants of *chb57* and 26,300 transformants of *chb16*, and the same results as in *chb13* were obtained. None of the genomic fragments isolated from the candidate clones complemented the mutant phenotype. The fact that we have isolated *CDC9* gene multiple times indicates that the complementation strategy is working. One possible cause of the problem is that the gene mutated in these mutants is fairly big, as many of the checkpoint genes are, therefore, cloning may not be an easy task. The fact that we did not come across RAD9, which is a big gene and should also complement the mutant phenotype, in our screen also

validates the possibility. Moreover, the effect of this alternative pathway on DNA damage-induced cell cycle delay may be limited when compared with the primary pathway, so the phenotype is not as tight. This would further complicates the cloning process.

We also took the "candidate gene" approach to examine several genes that possibly play a role in the alternative pathway. *CHK1* was our first candidate for its role in DNA damage response in fission yeast *S. pombe* and in mammalian cells. We have knocked out the *CHK1* gene in the same strain background as the *chb* mutants and compared the mutant phenotype. Deletion of *CHK1* did not confer yeast the *chb* phenotype (temperature-sensitive in the presence of *CHES1*). We have also over-expressed wild type *CHK1* together with *CHES1* in *chb* mutants and it did not complement the mutant phenotype. Therefore, we conclude that *CHK1* is unlikely to be a part of the alternative DNA checkpoint pathway. The second candidate gene was *TEL1*, a gene involved in telomere length controlling, for its homology *to S. cerevisiae* checkpoint gene *MEC1* and mammalian checkpoint gene *ATM*. We first over-expressed *CHES1* in yeast and, in collaboration with Dr. Lunblad's laboratory, performed a telomere Southern blot analysis, which tests for the length of telomeres of chromosomes. *CHES1* did not seem to alter telomere length in either wild type or *tel1Δ* strains. We then over-expressed wild type *TEL1* with *CHES1* in *chb* mutant strains. Over-expression of *TEL1* did not complement the *chb* phenotype. Therefore, *TEL1* is not likely to play a role in the alternative pathway either.

In the effort to find the protein(s) that directly interact with *CHES1* in yeast, *RAS*-recruitment system (RRS) yeast 2-hybrid screen was done using *CHES1* as the bait and yeast cDNA library as the prey. Six and a half million yeast transformants were screened and more than 90 positive clones were obtained. However, after eliminating the bait plasmid from the cells, all of the candidate clones turned out to be bait-independent false positive clones. A possible explanation is that since we were using yeast library, we may have got wild type yeast *RAS* back from the screening, and these clones will grow independently of the *CHES1* bait. Over-expression of the genes downstream of *RAS* may also show the same effect.

An alternative way to search for the alternative pathway genes is to perform a transposon mutagenesis screen. The advantage of this method is that the genes being disrupted are fairly easy to isolate since there is now a "transposon tag" next to the gene of interest. The disadvantage is that this method can only generate null mutations and if the gene is essential, we may not be able to find it. Another possible method is the sectering screen, which utilizes genomic instability of the rad9Δ-/- strain and the ade2 pink phenotype to screen for pink/white sectered colonies, which have lost part of their genome. This also has the same disadvantage as the transposon mutagenesis.

In summary, we have confirmed the existence of an alternative *MEC1*-independent DNA damage checkpoint pathway in budding yeast and isolated 3 mutant strains in this new pathway. However, in the effort of cloning the genes mutated in these strains, we have encountered some unexpected difficulties. We have tried different ways to overcome the problem but with little success. We have also tried the candidate gene approach and the yeast 2-hybrid approach but neither gave us a positive result. We are proposing alternative methods in doing mutagenesis that should lead to easy isolation of the genes of interest. We will also focus on the characterization of *CHES1* in mammalian cells.

Key research accomplishments

- Performed a mutagenesis screen and isolated three mutants that are defective in the alternative DNA damage checkpoint pathway
- Confirmed the existence of an alternative DNA damage checkpoint pathway in yeast *S. cerevisiae*
- Characterized the three mutants and showed that they are recessive mutants and that they belong to the same complementation group

Reportable outcomes

Abstracts/Presentations:

Sharon E. Plon, Dabananda Pati, and Yi-Chen Li. Analysis of *CHES1*, a human checkpoint suppressor. Poster presentation. International Meeting on Forkhead/Winged Helix Proteins. November 14-15, 1998

Yi-Chen Li and Sharon E. Plon. Isolation of mutants that are defective in an alternative DNA damage checkpoint pathway. Poster presentation. American Society for Microbiology Conference: Yeast Genetics and Human Disease II. Vancouver, British Columbia, Canada. June 24 – 27, 1999.

Development of yeast strains:

SCP1: MATa, cdc9-8, can1-100, ade2, his3, leu2, trp1, ura3

SCP2: MATa, cdc9-8, rad9Δ::HIS3, can1-100, ade2, his3, leu2, trp1, ura3

SCP3: MATα, cdc9-8, can1-100, ade2, his3, leu2, trp1, ura3

SCP4: MATα, cdc9-8, rad9Δ::HIS3, can1-100, ade2, his3, leu2, trp1, ura3

SCP1A: MATα, cdc9-8, rad9Δ::HIS3, chk1Δ::Kan, can1-100, ade2, his3, leu2, trp1, ura3

SCP5A: MATa, cdc9-8, rad9Δ::HIS3, chk1Δ::Kan, can1-100, ade2, his3, leu2, trp1, ura3

chb13: MATa, cdc9-8, rad9Δ::HIS3, chb13, can1-100, ade2, his3, leu2, trp1, ura3

chb13-8a2-4a2-2a2: MATa, cdc9-8, rad9Δ::HIS3, chb13, can1-100, ade2, his3, leu2, trp1, ura3

chb13-8a2-4a2-2α1: MATα, cdc9-8, rad9Δ::HIS3, chb13, can1-100, ade2, his3, leu2, trp1, ura3

chb16: MATa, cdc9-8, rad9Δ::HIS3, chb16, can1-100, ade2, his3, leu2, trp1, ura3

chb57: MATa, cdc9-8, rad9Δ::HIS3, chb57, can1-100, ade2, his3, leu2, trp1, ura3

inversely proportional to the amount of TEP1 that is present in the cell. The tep-strains have also shown a slight resistance to 7ug/ml and 10ug/ml concentrations of wortmannin, and there is some indication that overexpression of other genes in the phosphatidylinositol pathway can be synergistic with Tep1p ablation at uncovering the activity of TEP1 in other pathways.

103) In Vitro Amyloid-like Fibril Formation by Yeast Ure2p

M. SCHLUMPBERGER (1), H. WILLE (1), I. HERSKOWITZ (2) AND S.B. PRUSINER (1,2)
(1) Institute for Neurodegenerative Diseases, (2) Dept. of Biochemistry and Biophysics, Univ. of California, San Francisco, CA 94143-0518

It has been suggested that the Ure2 protein from *Saccharomyces cerevisiae* can undergo a prion-like autocatalytic conformational change which leads to inactivation of the protein, thereby generating the [*URE3*] phenotype (Wickner, Science 264:566-569, 1994). The first 65 amino acids, which are dispensable for the cellular function of Ure2p in nitrogen metabolism, have been shown to be both necessary and sufficient for this phenomenon (Masison and Wickner, Science 270:93-95, 1995). This N-terminal domain has therefore been designated the Ure2 prion domain (UPD). We have expressed GST-Ure2p and GST-UPD fusion proteins in *E. coli*. Both fusion proteins were reasonably soluble, but upon cleavage by thrombin to release the GST moiety, a heavy precipitate formed. The released UPD formed ordered arrays, which displayed amyloid-like fibrillar morphology by electron microscopic observation and tinctorial characteristics such as green-gold birefringence after Congo red staining. FTIR spectroscopy demonstrated a high β-sheet content in these aggregates. Kinetic studies of fiber formation, using the specific fluorescence emitted by thioflavine T in the presence of ordered, β-sheet rich aggregates, showed a concentration-dependent lag phase between release of the UPD fragment and polymerization into fibrils. This lag phase could be abolished by seeding with preformed fibrils, demonstrating the autocatalytic nature of the polymerization process. Under the same conditions, the released, full-length Ure2 protein formed aggregates more slowly, and only a minority of Ure2p appeared to aggregate in fibrils of uniform size and morphology. Seeding with preformed UPD fibrils accelerated the aggregation process substantially and increased the amount of high density, proteinase resistant aggregates formed. We suggest that inhibition of prion domain aggregation by the GST moiety is mainly due to steric hindrance. In the case of full-length Ure2p, it appears that the C-terminal, functional domain of the protein prevents polymerization of the prion domain, but partial refolding can allow formation of amyloid-like fibrils. The same principle of autocatalytic formation of amyloid fibrils could underlie the conversion from the wild-type phenotype to the [*URE3*] state in yeast. Unraveling the mechanism of prion conversion in yeast might serve to advance our understanding of prions in mammals.

104) Isolation of Mutants That Are Defective in An Alternative DNA Damage Checkpoint Pathway

Y.-C. J. LI and S. E. PLON
Baylor Coll. of Medicine, Houston, Texas

A human cDNA *CHES1* (checkpoint suppressor 1) was isolated previously in our laboratory by high copy suppression of the DNA damage checkpoint mutant *mec1-1* in budding yeast. It was also shown that *CHES1* suppresses multiple other mutants along the same pathway. Our hypothesis is that *CHES1* does so by activation of an alternative DNA damage-induced checkpoint pathway in *S. cerevisiae*. The objectives of this project are to identify, characterize, and clone the genes in this alternative pathway, and to isolate the human homologs of these genes and analyze their structure and expression in human cancers. We plan to identify these genes by a comprehensive mutagenesis screen in a *cdc9-8, rad9Δ* strain, where the permissive temperature is 30°C in the presence of *CHES1* and 23°C in the absence of *CHES1*. The UV sensitivity caused by *rad9Δ* of this strain is also partially rescued by the presence of *CHES1*. EMS generated mutants were screened for loss of both the temperature and the UV response to *CHES1*. The tentative name assigned to these mutants is *chb* for checkpoint bypass. We have mutagenized and screened approximately 220,000 clones. Three clones that are temperature sensitive in the presence of *CHES1* were isolated. Among those, *chb13* and *chb16* are highly temperature sensitive (no growth at 30°C) whereas *chb57* is a weaker allele. With regard to UV sensitivity, *chb13* and *chb57* are sensitive to UV radiation but the effect of *chb16* is intermediate when compared to the controls. Overall, *chb13* appears to have a strong mutant phenotype that has lost all response to *CHES1* by our assays. Both *chb16* and *chb57* are moderate mutants that have one strong phenotype. We will clone the genes that were mutated in the recessive *chb* strains by complementation. The analysis of these mutants has also allowed us to confirm that the *CHES1* -dependent pathway is at least partially parallel to the *RAD9* -dependent pathway. If *CHES1* acts on the *RAD9* -dependent pathway, it must act downstream of *RAD9* since *CHES1* restores the UV resistance in the *rad9Δ* strain. However, introduction of a wild type *RAD9* gene into all three *chb* mutants restores UV resistance. Thus, the *CHB* genes are apparently not simply downstream of *RAD9*. Therefore, the *CHB* genes, through which *CHES1* acts, are more likely to be in an alternative pathway. In summary, a DNA damage checkpoint pathway alternative to the primary *RAD9* -dependent pathway was confirmed and three *chb* mutant strains, which are defective in the alternative DNA damage checkpoint pathway, have been isolated.

105) Efficient translocation of Apn1 into yeast mitochondria depends on interaction with Pir1.

Vongsamphanh, R., David, J., and Ramotar, D.
Hôpital Maisonneuve-Rosemont, Centre de Recherche. Université de Montréal, Montréal, Québec H1T 2M4, Canada.

The yeast Apn1 DNA repair enzyme acts on apurinic/apyrimidinic (AP) sites in damaged DNA. Cells lacking Apn1 are unable to repair damaged DNA and possess high spontaneous mutation rates. The Apn1 C-terminus contains two clusters (I & II) of basic amino acids which function as a bipartite nuclear localization signal (Ramotar & Demple, 1996). Deletion of either clusters I or II of Apn1 prevents its entry into the nucleus (Ramotar et al., 1993). We show here with the yeast two-hybrid system that Pir1 (a protein of previously unknown function) interacts with the Apn1 C-terminal end (Apn1-CT). However, indirect immunofluorescence and western blots surprisingly revealed that the absence of Pir1 protein does not prevent Apn1 from entering the nucleus, but rather causes its accumulation in the nucleus and depletion from the cytoplasm. It should be noted that Pir1 function appears specific for Apn1, as the rate of uptake of another nuclear protein Imp2 is not altered by the absence of Pir1. It is not clear why Pir1 protein retains Apn1 in the cytoplasm, but we suspect that it may retard nuclear import to allow some of the protein to be transported into the mitochondria to repair mitochondrial damaged DNA. However, it was never shown whether mitochondria contain Apn1, but our preliminary results by western blots and enzymatic assays strongly indicate that the mitochondria derived from wild-type cell does contain Apn1. In the absence of Pir1, the Apn1 level is drastically reduced in the mitochondria. We conclude that Pir1 is required to ensure proper cellular distribution of Apn1 to the mitochondria. In the absence of Pir1, Apn1 distribution favors the nucleus. Relationship to human mitochondrial diseases will be discussed.