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PRINCIPAL INVESTIGATOR: David Stern, Ph.D.

CONTRACTING ORGANIZATION: Yale University
New Haven, Connecticut 06520-8047

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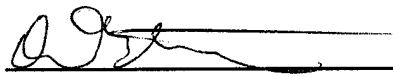
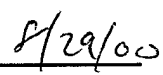
 
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5) INTRODUCTION

We originally hypothesized that components of DNA-related checkpoint pathways in addition to members of the *ATM* protein/lipid kinase family are conserved in all eukaryotes. This is based on functional similarities in the pathways and the conservation between the evolutionarily disparate budding and fission yeasts. Our goal was to identify additional regulators of mammalian DNA checkpoints by virtue of structural and functional homology with known checkpoint genes in budding yeast. We had proposed to use both structural and functional screens to identify human homologs of yeast damage checkpoint proteins Rad53 and Rad9. Once identified, such components would be ordered into pathways for mammalian checkpoint function, with emphasis on p53 regulation, cell cycle regulation, and complementation of *ATM* defects.

	<i>Saccharomyces cerevisiae</i>	<i>Schizosaccharomyces pombe</i>	<i>Homo sapiens</i>	
	Mec1 Tel1	rad3#	Atr Atm	PI kinase family; protein kinase
	Ddc2?	rad26 (*P)#		
DAMAGE PATHWAY				
	Rad9 (*P)	Crb2/Rhp9(*P)	Brcal? p53BP1? Nibrin? KIAA0170?	BRCT domains
	Rad24 Mec3/Pip3	Rad17#	Rad17 (RFC4?)	
		Hus1# *P	Hus1	
	Rad17	Rad1#	rad1	
	Ddc1 (*P)	Rad9 #	Rad9	
	DPB11	cut5		BRCT domains replication upstream Rad53
	Sld2/DRC1			replication upstream Rad53
	POL2		Pol	replication upstream Rad53
	RFC5		RFC	replication
	PRI4		Pol	replication Pol α -Primase downstream Rad53
	Rad53 *P	cds1 *P	cds1/Chk2*P	protein kinase FHA domain(s)
	Chk1	Chk1*P	Chk1*P	protein kinase

Table 1. Conservation of yeast and mammalian DNA checkpoint genes. *P indicates that damage induces protein phosphorylation.

(6) BODY

The major objective for this work has now shifted from identification of mammalian DNA checkpoint genes that are homologs of yeast genes, to their characterization. As we hypothesized, the major components of DNA checkpoint pathways have been shown to be conserved between budding yeast and humans. At the time of submission of the original proposal, the only human ortholog of a yeast checkpoint gene was Atm. Since then, this list has been extended to the genes listed below, which includes a homolog of yeast Rad53, which we originally sought (Table 1). With the identification of the yeast Rad53 homolog, Chk2/Cds1 [1-4], we have now begun to investigate how Chk2 is regulated, and how it regulates targets. This work is directly relevant to breast cancer, since it is now clear that Chk2 is an intermediary linking DNA checkpoint pathways from Atm to p53; since Chk2 phosphorylates and modulates Brca1 function [5], and since Chk2 mutations are found in variant p53+ forms of Li-Fraumeni syndrome, which predisposes to breast cancer and other cancers [6].

Connection with Approved Technical Objectives.

Technical Objective 1. Protein-interaction screens for mammalian Rad53 and Rad9 homologs.

Technical Objective 2. Cloning of mammalian DNA checkpoint genes by complementation of defects in yeast.

Technical Objective 3. Screening based upon protein sequence homology.

Technical Objective 4. Characterization of genes in mammalian cells.

As described in the previous annual report, we had already made substantial progress on Objectives 1 and 3. Objectives 1,2, and 3, which were various approaches to identifying mammalian homologs of Rad53 and Rad9 were made partially superfluous by the discovery of the Rad53 homolog Chk2. Continued work on Rad9 homologs and on Objective 4, is the subject of the present and future work. Since this new focus entailed a shift from work in yeast to work on mammalian homologs of the checkpoint genes, this has entailed development of entirely new reagents and techniques in the lab, so that most of the work in the current cycle has been "setup" work.

This ongoing work is divided into two components, upstream regulators and effectors. The work is being performed by two postdoctoral fellows who joined the lab during this grant cycle, Lyuben Tsvetkov and Xinghi Xu. Both are highly experienced molecular/cell biologists. Dr. Tsvetkov has a background in DNA damage responses and cell cycle control. Dr. Xu has worked on receptor-regulated signaling pathways. Both have substantial publication records.

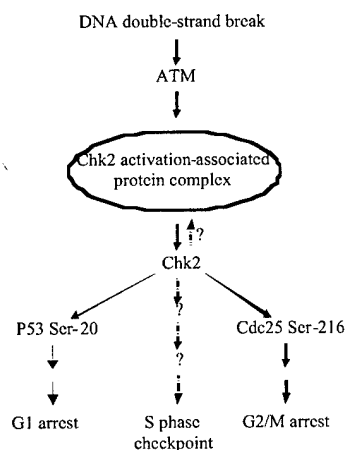


Fig. 1. Chk2 regulation by DNA damage, involving hypothetical activation-associated protein complex analogous to Rad53/P-Rad9 complex.

Mechanisms of Chk2 activation in response to DNA damage

In *S. cerevisiae*, *MEC1* and *TEL1* are functionally and structurally related to the human tumor suppressor *ATM*. *MEC1* and *RAD53*, two essential genes, play a central role in DNA damage checkpoints at all cell cycle stages. Our lab showed that Rad9 is a regulator coupling DNA damage to signaling through Mec1 to activate Rad53 and Chk1 in G1 and G2 checkpoints but not in the S-phase checkpoint [7, 8]. Phosphorylation of both Rad53 and Rad9 in response to DNA damage is *MEC1*-dependent. Rad53 interacts with Rad9 through the Rad53 FHA2 domain. This interaction is phosphorylation-dependent and is required for Rad53p activation [8]. The only recognizable sequence motif in Rad9 lies in its C-terminal two BRCT (Brcal-related C-terminus) domains, which are critical for Rad9 function. Rad9 has a homologue, *crb2*, in *S. pombe* [9]. Identification of its human homologue is critical for understanding how the Atm complex senses and transduces the DNA-damage signal to Chk2.

Chk2 has a N-terminal SQ/TQ cluster followed by a FHA (forkhead-associated) domain, and a C-terminal kinase domain. It differs from yeast Rad53 in that the latter (uniquely among FHA-containing protein kinases) has a second FHA domain. The Chk2 SQ/TQ cluster contains seven potential phosphorylation sites for Atm. Threonine 68 is one major Atm phosphorylation site in response to ionizing irradiation [1]. Earlier work in our lab showed that FHA domain-containing proteins mediate interactions with phosphoproteins through FHA domains, based on the phospho-Rad9/Rad53 interaction [8].

The most conserved region between Rad9p and *crb2* is the BRCT domains. Therefore, we would expect that the putative human homologue has similar C-terminal BRCT domains, functionally interacts with Chk2 through the Chk2 FHA domain in an *ATM*- and phosphorylation-dependent manner, and is required for Chk2 activation in response to DNA damage. The known human proteins that have similar C-terminal tandem BRCT repeats include p53 binding protein 1 (*53BP1*) [10], *BRCA1*, and EST clone *KIAA0170*, which also has an N-terminal FHA domain. We tested the possibility that, like Rad53 with phospho-Rad9, Chk2 would associated with these proteins after DNA damage. Our preliminary experiments did not detect any association between Chk2 and 53BP1 or KIAA0170. Brcal appears not to be required for Chk2 activation in response to gamma irradiation in HCC1937 cells, which have a homozygous truncation of the BRCT domains in Brcal [2]. As discussed below, we have continued work on a complex containing Nibrin, another protein with a Brct domain. Therefore, in the next cycle, we will systematically screen for Chk2 interacting proteins, particularly those that interact with the FHA domain in Chk2, using two hybrids and physical association screens.

Fig. 2. Chk2, but not kinase-deficient mutant, interacts with nibrin. A) 293 cells transiently transfected with various HA-tagged Chk2 constructs. Nibrin was quantified by immunoblotting. B) Protein extracts were immunoprecipitated with anti-HA and probed for with anti-nibrin (upper) or anti-Chk2 (lower).

DNA double-strand break repair complex and Rad53 regulation.

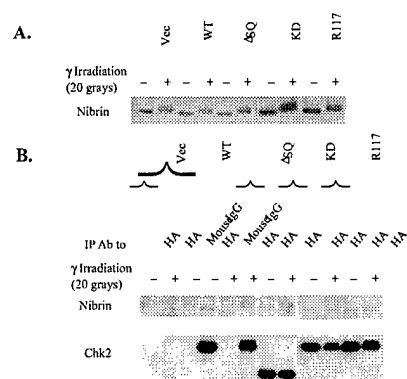
Loss of ionizing irradiation-induced S-phase checkpoint, which leads to radio-resistant DNA synthesis (RDS), is a common phenotype of both AT cells and cells derived from Nijmegen breakage syndrome (NBS) [11]. NBS is a rare cancer-predisposition disease. Mutation of the NBS1 gene is responsible for this syndrome [12]. This gene encodes a 95 KD protein, nibrin. Nibrin has an N-terminal FHA domain and an adjacent BRCT domain. Nibrin interacts with the Rad50-Mre11 complex. Mre11 alone has endonuclease activity, and its 3' to 5' exonuclease activity is increased when present in a complex with Rad50 or nibrin [13, 14]. The nibrin-Rad50-Mre11 complex plays pivotal roles in DNA double-strand break repair. Cellular factors repair broken ends very efficiently either by recombination with homologous template, or by connecting the ends directly (non-homologous end-joining, NHEJ). The NHEJ pathway is considered to be the most prominent in mammalian cells [14].

In *S. cerevisiae*, a structurally and functionally related complex, xrs2-Rad50-Mre11, is critical for the NHEJ pathway [15]. The xrs2-Rad50-Mre11 complex is a downstream target of Tel1p [16]. Each component of this complex is essential for Rad53 phosphorylation in gamma irradiation-induced G1 and G2 checkpoints, and bleomycin-induced S-phase checkpoint (N. Lowndes, personal communication). This indicates that the xrs2-Rad50-Mre11 complex is upstream of Rad53. The xrs2-Rad50-Mre11 and Rad9 may be located within an adaptor complex required for Rad53 activation. Alternatively, the xrs2-Rad50-Mre11 complex may process damaged DNA and then recruit Rad9 to relay damage signal to its downstream targets.

In NBS cells, a deficiency of p95 is correlated with an inability to form Mre11-Rad50 nuclear foci in response to ionizing radiation [12]. Mre11 is hyperphosphorylated in response to gamma irradiation, and this phosphorylation is nibrin-dependent [17]. Recently, nibrin was shown to be phosphorylated by the Atm kinase in response to gamma irradiation [18, 19]. Though this phosphorylation is not required for the association of nibrin with the Rad50-Mre11 complex, it is required for formation of the nibrin-Rad50-Mre11 nuclear foci in response to gamma irradiation. Mutation of certain phosphorylation sites of nibrin in NBS cells diminishes RDS, indicating that this phosphorylation regulates the S-phase checkpoint. The nibrin-Rad50-Mre11 complex does not have kinase activity. This complex may recruit ATM effector kinases such as Chk2 and/or Chk1 to relay DNA damage signal to S-phase checkpoint and/or to phosphorylate Mre11, which may be important for DNA repair.

In work accomplished during the current cycle, we have observed that Chk2 functionally interacts with the nibrin-Rad50-Mre11 complex:

- 1) Chk2 coimmunoprecipitates with nibrin in 293 cells and HT1080 cells (Fig. 2). We have generated a series of deletion mutants of Chk2 and nibrin. These mutants will be used to identify the domains responsible for this interaction. We have also generated GST fusion proteins with overlapping fragments of nibrin. These GST proteins will be used in kinase assays to determine if Chk2 is upstream of nibrin.



- 2) Chk2 coimmunoprecipitates with Rad50, but not Mre11.
- 3) Restoration of expression of nibrin in the nibrin-deficient cell line (GM07166) results in Chk2 hyperphosphorylation in response to ionizing irradiation. Basal levels of phosphorylation of Chk2 are seen in GM07166, but not in GM07166 transfected with NBS1 cDNA and irradiated. We have generated point mutants knocking out all the potential phosphorylation sites within the SQ/TQ cluster of Chk2, and add-back mutants retaining one potential phosphorylation site within the SQ/TQ cluster of Chk2. These mutants will help identify the phosphorylation sites in Chk2, which are nibrin-dependent, in response to DNA damage.

We have also observed that Chk2 forms a large protein complex:

- 1) Chk2 forms an oligomer, since HA tagged Chk2 coimmunoprecipitates FLAG-tagged Chk2 in 293 cells. This is consistent with our earlier work on Rad53, since a two-hybrid screen with Rad53 as bait pulled out Rad53 [8].
- 2) Our preliminary gel filtration experiments indicate that the Chk2-containing complex is in the range of 500 – 700 kDa. Chk2 co-fractionates with nibrin, Rad50, and Brca1. We are in the process of purifying this Chk2-containing complex.

Our observations indicate three possible models of the functional relationships between Chk2 and the nibrin-Rad50-Mre11 complex:

- 1) the nibrin-Rad50-Mre11 complex is upstream of Chk2 and regulates Chk2 activation in checkpoint controls
- 2) Chk2 is upstream of the nibrin-Rad50-Mre11 complex and regulates DNA repair
- 3) Chk2 may be essential for both checkpoint controls and DNA repair.

Work in the coming cycle will test these possibilities.

Proteins targeted for phosphorylation by Chk2.

Known effector substrates for Chk2 include Cdc25C and p53. Both of these proteins are also targeted by the structurally distant kinase Chk1, which operates in parallel to Chk2 in many organisms. A major present goal is to identify new substrates for Chk2, some of which may turn out to be Chk2-specific. In order to begin this work, we have produced a number of reagents:

- -GST-Chk2 and GST-Chk2 D368A (kinase-inactive) proteins. They have been examined by *in vitro* kinase assays for their activities. GST-Chk2 can autophosphorylate by incorporation of ^{32}P γ -ATP, while GST-Chk2D368A cannot.
- HA-Chk2 and HA-Chk2 D368A (kinase-inactive) plasmids which have been used for transient transfections and for stable transfection of HT-1080 cells (with mutated p53). Chk2 proteins immunoprecipitated from those cells were used for *in vitro* kinase assays for autophosphorylation to examine the kinase-inactive mutant (successfully) and for transphosphorylation of p53 and RPA - unsuccessfully so far.
- Preparation of α -GST-Chk2 polyclonal rabbit antibodies. Those antibodies both immunoprecipitate and immunoblot Chk2.
- Phosphospecific antibody for P-T68 in Chk2. 16 AA oligopeptide that resembles a region around phosphorylated T68 in Chk2 has been cross-linked to KLH protein and used for immunization of two rabbits. We have verified that the antiserum produced recognizes Chk2 protein only when it is phosphorylated on T68 (active form). This phosphorylation is probably mediated *in vivo* by Atm, and Atr.

CHK2 and RPA

Unpublished work from our laboratory has suggested the possibility that the DNA replication factor RPA2 in yeast is a substrate for Rad53. Human RPA2 is also heavily phosphorylated in response to DNA damage [20]. Hence, we have determined if RPA and Chk2 can interact. We have found that a small population of RPA2 co-immunoprecipitates with HA-Chk2 by IP with α -Chk2 and α -HA antibodies.

Treatment of HT-1080 cells with 1 μ M camptothecin (CPT), a topoisomerase I inhibitor, for 1 hour causes RPA2 hyperphosphorylation which can be prevented by 15 min pre-treatment of cells with 5 μ M a protein kinase inhibitor UCN-01 (7-hydroxystaurosporine). This concentration of UCN-01 is quite different from the one that inhibits Chk1 (IC50- 15nM). We have found that treatment of HT-1080 cells with 1 μ M CPT causes gel mobility shift of Chk2. Also, we have found that pre-treatment with 5M UCN-01 diminishes Chk2 mobility shift caused by 10 Gy γ -radiation and 1 μ M CPT.

We have transfected transiently AT cells with an HA-Chk2 plasmid and treated those cells with CPT. Chk2 and RPA2 proteins were shifted, so those responses are ATM-independent. Dose/ response curves of RPA2 and Chk2 phosphorylation, after treatment with UCN-01 and CPT, are similar. We have pre-treated AT cells, transiently transfected with HA-Chk2 plasmid, with 10 μ wortmanin or 3mM caffeine and then treated those cells with CPT. Chk2 and RPA2 phosphorylation were caffeine-sensitive and wortmanin-insensitive, so more probably this pathway go through ATR than DNA-PK.

We have treated HT-1080 cells, stably transfected with HA-Chk2 and HA-Chk2D368A (kinase dead-dominant negative mutant), with CPT and then we have detected RPA2 hyperphosphorylation. But we could not detect a decrease of RPA2 hyperphosphorylation in cells stably transfected with dominant-negative Chk2 mutant. Since Chk2 is a protein kinase, it can be phosphorylated in trans, and also autophosphorylate. DNA damage induces both trans, and autophosphorylation. After treatment of HT-1080 cells, stably transfected with HA-Chk2 and HA-Chk2D368A, with 10Gy γ -radiation or 1 μ M CPT wild-type Chk2 was shifted while the kinase dead mutant has almost undetectable mobility shift.

In work to be conducted over the next cycle, we will continue to determine whether Chk2 is a proximal kinase for RPA2, and, if, so, whether this is associated with changes in RPA activity. Although mouse Chk2^{-/-} cells have been produced, we are planning to use somatic cell recombination to knock out CHK2 in human cells, so that we can analyze functionality of this kinase in a human background. Finally, a variety of approaches are being used to identify new substrates for this protein kinase.

(7) KEY RESEARCH ACCOMPLISHMENTS.

Major accomplishments include production of key reagents for work on Chk2 and other human checkpoint proteins, and pilot surveys of possible Chk2 interactors and substrates:

- Development of antibodies and epitope-tagged clones for analysis of Chk2, and Brc1 domain containing proteins Brc1, Nibrin, 53BP1, and KIAA0170. These antibodies include a phospho-specific antibody that exclusively recognizes active Chk2.
- Investigation of requirements for Atm, Nibrin, and Brc1 in regulation of Chk2.
- Evaluation of Chk2 association with Brc1, Nibrin, 53BP1, KIAA0170, Rad50.

- Production of deletion and other mutants of Nibrin for investigating Nibrin interactions with Chk2.
- Production of cell lines expressing wildtype Chk2 and a number of phosphorylation site and other mutants.
- Investigation of RPA as a new Chk2 substrate.
- Preparation of molecular clones to be used for recombinational inactivation of human *CHK2* in somatic cells.

(8) REPORTABLE OUTCOMES.

Work from the previous cycle was presented at the USAMRMC ERA of HOPE Meeting held in Atlanta, May, 2000.

(9) CONCLUSIONS

This proposal hypothesized that there would be a mammalian homolog of Rad53, and that this homolog would be important in regulation of DNA checkpoint pathways. The human homolog was discovered two years ago, and in the previous cycle we have set up to analyze regulation and targets of Chk2 in mammalian cells. We have now developed systems and reagents for expression and analysis of all of the pertinent proteins, and are making headway on association of Chk2 with DNA damage response complexes including FHA domain-containing proteins. We anticipate significant results over the forthcoming cycle of the grant.

Work on these genes and pathways is not simply an academic exercise. These DNA checkpoint proteins have important roles in breast cancer. Although no clear Rad9 ortholog has been identified, it does share a protein homology domain and, possibly, functionality, with the breast cancer tumor suppressor gene *BRCA1*. Mutations in the *MEC1* ortholog *ATM* are apparently responsible for a significant fraction of hereditary breast cancers. The *RAD53* ortholog *CHK2/CDS1* regulates breast cancer tumor suppressor proteins p53 and Brca1. Also, mutations in *CHK2* are responsible for a variant form of the breast cancer predisposing Li-Fraumeni syndrome, in which *TP53* is not mutated. Not only will progress on these problems enhance our understanding of carcinogenic processes, they may be important for optimization of breast cancer therapies. The response of tumor cells to genotoxic chemotherapeutic agents is greatly affected by the affects they have on the DNA checkpoint pathways including Chk2. It can be anticipated that fruits of this work will include better therapeutic modulators, and the ability to maximize utility of existing therapies.

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