UNDERSTANDING THE ROLE OF CLASPIN IN DNA DAMAGE CHECKPOINTS

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ABSTRACT

FUNDA SAR: Understanding the Role of Claspin in DNA Damage Checkpoints
(Under the direction of Aziz Sancar, Ph.D)

DNA damage and replication checkpoints are signal transduction pathways that provide constant surveillance to maintain genome integrity. Checkpoints control a variety of cellular responses including DNA repair, chromatin remodeling and gene transcription. Claspin is an essential protein for the ATR-dependent activation of the DNA replication checkpoint response in *Xenopus* and human cells. The presence of stalled replication forks leads to phosphorylation of Claspin in both of the organisms and phosphorylated Claspin interacts with Chk1 and this interaction is essential for phosphorylation of Chk1 and checkpoint activation. Here we describe the purification and characterization of human Claspin. The protein has a ring-like structure and binds with high affinity to branched DNA molecules. These findings suggest that Claspin may be a component of the replication ensemble and plays a role in the replication checkpoint by directly associating with replication forks and with the various branched DNA structures likely to form at stalled replication forks due to DNA damage. In addition, we analyzed the importance of Claspin DNA binding for Chk1 activation by testing whether Claspin recruits Chk1 to DNA. These studies led to identification of Chk1 DNA binding activity. Chk1 possesses low DNA binding affinity to certain branched DNA structures and Claspin does not recruit Chk1 to DNA.

To my family,
You are the gifts of my life

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LIST OF ABBREVIATIONS

ATM	ataxia telangiectasia mutated
ATR	ATM- and rad3-related
ATRIP	ATR-interacting protein
BRCA1	breast cancer susceptibility gene 1
BRCT	Brca1-carboxy terminus
DNA-PKcs	DNA-dependent protein kinase
dNTP	deoxyribonucleotide
DRF1	Dbf4-related factor 1
FHA	forkhead associated domain
HEAT	huntington, elongation factor 3, A subunit of phosphotase 2A and Tor1
HU	hydroxyurea
IR	ionizing radiation
kDa	kilodalton
mTOR	mammalian target of rapamycin
Mrc1	mediator of replication checkpoint
µl	microliter
µM	micromolar concentration
ml	milliliter
mM	millimolar concentration

min	minute
nM	nanomolar concentration
PI3K	phosphotidyl-inositol 3 kinase-like
Tim	timeless
TopBP1	topoisomerase IIβ binding protein 1
TRRAPs	transformation/transcription domain associated protein
UV	ultraviolet radiation
9-1-1	Rad9/Rad1/Hus1

CHAPTER I

INTRODUCTION

Neoplastic transformation of a normal cell requires accumulation and acquisition of a series of genetic mutations in tumor-suppressor and proto-oncogenes (Hahn and Weinberg 2002). Studies with colorectal carcinomas revealed that occurrence of seven independent genetic lesions are sufficient for cancer formation (Lengauer et al. 1998; Loeb 1991). Although the human body has trillions of cells with potential to transform, the probability of having cancer is very low. In support of this, the calculations of spontaneous mutation rates showed that it would be difficult to acquire sufficient number of genetic alterations to develop cancer during the lifetime of the organism (Cahill et al. 1999; Hahn and Weinberg 2002; Loeb 1991). Occurrence of cancer can be explained by the existence of mutator genes that give rise to genetic instability, leading to accumulation of sufficient number of genetic alterations (Cahill et al. 1999; Loeb 1991). Therefore, accuracy in DNA replication and error-free maintenance and inheritance of the genome are essential for cellular survival and to constrain cancer formation.

Byproducts of cellular metabolism and external factors such as ultraviolet radiation (UV), ionizing radiation (IR), or chemicals place cells under constant genotoxic stress. All eukaryotes have evolved surveillance mechanisms, named as DNA damage checkpoints to monitor the integrity of the genome constantly. DNA damage checkpoints are signal

transduction pathways that operate throughout the cell cycle. They ensure genome integrity by delaying cell cycle progression in the presence of DNA damage and/or in the presence of errors in DNA replication (Abraham 2001; Nyberg et al. 2002; Sancar et al. 2004). After the activation of checkpoints, the cells arrest at the phase of the cell cycle, in which the DNA damage has been encountered until the damage is repaired (Sancar et al. 2004). If the damage cannot be repaired, apoptosis is induced by the checkpoint mechanism. In addition to a delay in cell cycle progression, checkpoints regulate diverse cellular responses from DNA repair to chromatin remodeling and gene transcription (Lukas et al. 2004). The importance of checkpoints in proliferating cells has been emphasized by the findings that defects in checkpoints result in a mutator phenotype due to genomic instability. Mutations in checkpoint genes have detected in several human malignancies (Kastan and Bartek 2004). Two recent studies demonstrated that DNA damage checkpoints are activated early in tumorigenesis by oncogenic stress. Checkpoint activation is thought to occur before genome instability and entry into a malignant state, suggesting that by constraining the clonal expansion of damaged cells checkpoints limit the tumor progression (Bartkova et al. 2005; Gorgoulis et al. 2005). Interestingly, in addition to proliferating cells, some of the checkpoints function in quiescent and terminally differentiated cells (Lukas et al. 2001; Pandita et al. 2000).

Components of DNA damage checkpoints

DNA damage checkpoints are multi-component signal transduction cascades, which contain five major classes of proteins; i) DNA damage sensors, ii) apical signal transducers,

iii) mediators, iv) distal signal transducers and v) effectors (**Figure 1.1**) (Lukas et al. 2004). Before explaining each class of checkpoints, it is important to mention that checkpoints do not operate as classical linear signal transduction pathways. Due to its complex network and presence of feedback loops, several of its components can be classified into more than one class, which makes it difficult to define upstream and downstream components of checkpoints.

Recognition of DNA damage or stalled replication forks is a key step in DNA damage responses such as DNA repair and DNA damage checkpoints. Rapidity and sensitivity of damage recognition has great importance in the activation of downstream signaling cascades resulting in the cell cycle arrest. Despite its importance, damage recognition is the least well characterized step of DNA damage checkpoints. Damage sensor proteins detect the presence of DNA lesions and relay this signal to downstream components; therefore, they associate with different or specific DNA lesions and provide the efficient detection of DNA damage. They may serve as recognition complexes to recruit, localize and activate downstream proteins.

Distinction between DNA damage sensors and apical signal transducers is not clear, as some of the signal transducers seem to have dual roles in damage sensing and amplification of the signal. So far, two groups of proteins functioning in checkpoints have been suggested to act as damage sensors, which are two PI3K (phosphotidyl-inositol 3 kinase-like) family members ATR (ATM- and Rad3- related), ATM (ataxia telangiectasia (AT)-mutated), Rad17, and 9-1-1 (Rad9/Rad1/Hus1) complexes (Sancar et al. 2004). Nonetheless, the general criterion to classify a protein in the damage sensor class includes the physical association of the protein with the DNA lesion or the stalled replication forks

(Petrini and Stracker 2003). This physical association may occur only after DNA damage or alternatively, the sensor protein may be localized to undamaged DNA and DNA damage may increase its binding to DNA. Based on these criteria, several checkpoint proteins were initially classified as damage sensors. Recent studies showed that several downstream checkpoint proteins possess DNA binding activity; therefore the importance of DNA binding should be determined before classifying a protein as a damage sensor. A physiological role of the DNA binding can be ascertained by identification of mutations or conditions changing the DNA binding properties that cause defects on activation of checkpoints. Nonetheless, none of the sensor proteins have been characterized well in terms of the type of lesions they bind and the relative DNA binding affinities to different DNA lesions. Characterization of the DNA binding properties of sensor proteins will also provide the insights into how rapidity and sensitivity of damage recognition is achieved.

Checkpoint mediators are a newly emerging class of proteins that modulate checkpoint activation through protein/protein interactions and/or recruitment of substrates, providing a bridge between major signal transducers and effector proteins. Mrc1 (Mediator of replication checkpoint) in budding and fission yeast has been shown to be required for the activation of distal signal transducer kinases, scRad53 and spCds1, respectively (Alcasabas et al. 2001; Tanaka and Russell 2001). In a damage dependent manner, scRad9 regulates the activation of scRad53 in yeast (Sancar et al. 2004). Claspin has been identified as a Chk1 binding protein and classified as a mediator protein (Kumagai and Dunphy 2000).

Signal transducers are the kinases that are activated in the presence of DNA damage. They amplify and propagate the signal for the DNA damage and activate the effector proteins. The effector proteins generally function in cell cycle progression. Phosphorylation

of these proteins causes changes in their activity so that the cell cycle is delayed and damage can be repaired.

Depending on the type of DNA lesion and phase of the cell cycle different checkpoint pathways are activated. Two serine/threonine kinases, ATR and ATM are central to these pathways and they belong to the PI3K (phosphotidyl-inositol 3 kinase-like) family, which also contains DNA-PKcs (DNA-dependent protein kinase), mTOR (mammalian target of rapamycin), ATX/hSMG-1 and TRRAPs (transformation/transcription domain associated protein). Except TRRAPs, other family members function in signal transduction pathways. DNA-PK plays a role in nonhomologous end-joining and in checkpoints (Ma et al. 2002). mTOR regulates protein translation and cell growth, depending on the nutrient availability (Yamashita et al. 2001). ATX/hSMG-1 has recently been shown to be required for the mRNA surveillance (Schmelzle and Hall 2000).

All PI3K family members are quite large, 270-450 kDa proteins (Perry and Kleckner 2003) with a C-terminal PI3K catalytic domain. Unlike PI3K lipid kinases, PI3Ks phosphorylate proteins on serine or threonine residues, followed by glutamine residues (SQ/TQ). In the amino terminus, PIKKs contain 40-50 HEAT (huntington, elongation factor 3, A subunit of phosphatase 2A and Tor1) repeats. A single HEAT repeat is composed of two interacting anti-parallel helices linked by a flexible loop (Perry and Kleckner 2003). HEAT repeats occur in series and form a domain with superhelical structure and they are thought to mediate protein-protein interactions, raising the possibility HEAT domains function as a landing pad for various proteins (Perry and Kleckner 2003).

ATR-Dependent Signaling Pathway

ATR is an essential gene, suggesting that it has an important role in normal cellular growth and/or early development. ATR-/- null mice die by embryonic day 7.5 (Brown and Baltimore 2000) and in cells obtained from ATR-/- mice apoptosis is induced (de Klein et al. 2000). Homologs of ATR in *C. elegans* and *Drosophila* have been shown to be required for normal development and cell cycle progression (Brauchle et al. 2003; Sibon et al. 1997). In humans, mutations in ATR are associated with Seckel syndrome (O'Driscoll et al. 2003). Seckel syndrome is an autosomal recessive disorder. Individuals with Seckel syndrome have intrauterine growth retardation, microcephaly, dwarfism, skeletal and muscle abnormalities. A hypomorphic, synonymous mutation (A2101G) in ATR affects splicing and therefore, reduces ATR protein level and impairs ATR-dependent signaling in response to UV or hydroxyurea (HU) (O'Driscoll et al. 2003).

ATR forms a stable complex with ATR-interacting protein (ATRIP) in humans (Cortez et al. 2001; Zou and Elledge 2003). Similarly, yeast homologues of ATR *Sp*Rad3 and *Sc*Mec1 exist in complex with *Sp*Rad26 and *Sc*Lcd1/Ddc2, respectively (Sancar et al. 2004). ATR and ATRIP are both mutually dependent each other for expression. ATRIP is phosphorylated by ATR and essential for ATR-dependent checkpoint signaling. The exact role of ATRIP in ATR signaling is not well-understood; however, recently, a motif has been identified in the C terminus of ATRIP. This motif is required for ATRIP-ATR interaction (Falck et al. 2005). Deletion of this region did not affect ATRIP binding to ssDNA but abolished ATR's binding to DNA and phosphorylation of Chk1 by ATR in response to UV damage (Falck et al. 2005), suggesting that ATR-ATRIP interaction is essential for ATR

activation and its recruitment to ssDNA. The function of this C terminal motif is evolutionary conserved as C terminal deletions in yeast, and *Drosophila* ATRIP results in ATRIP- and ATR- null phenotypes. In addition to ATRIP, RPA has been shown to be required for recruitment of ATR to ssDNA (Zou and Elledge 2003). RPA is a heterotrimeric protein important for DNA replication, repair and recombination. Several DNA repair pathways process DNA lesions in a way that generates RPA-coated ssDNA and during DNA replication ssDNA does covered by RPA. The higher affinity of ATRIP to RPA-coated ssDNA suggested a model in which ATR is recruited to stalled replication forks or DNA lesions processed to have RPA coated ssDNA. In support of this model, ATRIP and RPA are both required for DNA damage induced ATR foci formation in human cells (Barr et al. 2003; Cortez et al. 2001). In RPA-depleted *Xenopus* egg extracts, ATR can no longer associate with chromatin and can't be activated in response to topoisomerase inhibitors (Costanzo and Gautier 2003). Cimprich and colleagues showed that ssDNA is indeed generated in the presence of DNA lesions or replication inhibitors. Depending on the extent of ssDNA generated, Chk1 is phosphorylated and activated (Byun et al. 2005). This finding supported a model where functional uncoupling of DNA polymerase and helicase activity create RPA-coated ssDNA and this RPA coated ssDNA act as a signal for checkpoint activation (Byun et al. 2005). This model can explain how ATR can respond to such a variety of DNA damaging agents.

Although several studies have demonstrated the importance of RPA for ATR activation and downstream signaling, other findings have challenged its absolute requirement for checkpoint activation. Deletion of the RPA-interaction domain of ATRIP prevents its focus formation and binding to RPA-ssDNA, but does not affect Chk1 phosphorylation and

activation (Ball et al. 2005). Both ATR and ATRIP are DNA binding proteins (Unsal-Kacmaz et al. 2002; Unsal-Kacmaz and Sancar 2004), they both possess similar affinities to ssDNA or RPA-coated ssDNA, and ATR has higher affinity to UV-damaged DNA (Unsal-Kacmaz et al. 2002; Unsal-Kacmaz and Sancar 2004). Cimprich and colleagues found that inhibiting DNA polymerase before initiation of replication does not inhibit uncoupling of helicase and polymerase, but inhibits Chk1 phosphorylation (Byun et al. 2005). This suggests that another signal that depends on DNA polymerase is required for ATR's activation. Therefore, RPA-ssDNA may not be the only signal or DNA structure recognized by ATR pathway. There may be additional signals that require different protein or protein-protein interactions to activate ATR. In support of this, ATR has been found to be required for a G2 checkpoint response to incompletely decatenated DNA (Deming et al. 2001; Deming et al. 2002).

Downstream of ATR signaling pathway

Regardless of how ATR gets activated, downstream signaling of ATR has been well-characterized. In response a variety of DNA damaging agents and replication stress, ATR regulates and orchestrates several cellular responses and cell cycle progression by either directly phosphorylating downstream checkpoint effector proteins or by regulating the activity of a distal signal transducer kinase, Chk1 (**Figure 1.2**). Chk1 is a serine/threonine kinase, conserved in several different organisms from yeast, worms, and flies to mammals (Chen and Sanchez 2004). Similar to ATR, Chk1 is an essential gene. Chk1-/- mice die at embryonic days 3.5 to 7.5 (Liu et al. 2000; Takai et al. 2000) and embryonic cells generated

from these mice are not viable (Takai et al. 2000). In addition Chk1 is important for cell cycle progression in *Xenopus* and *Drosophila* (Brauchle et al. 2003; Sibon et al. 1997). This indicates that Chk1 also has essential functions in early development and/or normal cellular growth. Down-regulation of Chk1 expression in the chicken somatic DT40 cells and human cell lines does not affect cellular survival, however, these lines possess defects in checkpoint activation in response to IR, HU and UV treatments (Chen and Sanchez 2004, and references therein).

In the presence of DNA lesions and replication stress, Chk1 is phosphorylated by ATR on two conserved serine residues, S345 and S317 in humans. Phosphorylations on these SQ/TQ motifs are crucial for checkpoint activation, as mutations of these residues render the cells that express the mutant forms sensitive to several DNA damaging agents in yeast, *Xenopus* and humans (Guo et al. 2000; Jiang et al. 2003; Liu et al. 2000; Lopez-Girona et al. 2001).

In G1 phase, ATR controls two different pathways that involve Cdc25A and p53 proteins. Cdc25A is a dual specificity phosphatase that dephosphorylates and activates CyclinE/Cdk2 and Cyclin A/Cdk2 complexes (Boutros et al. 2006). Activation of CyclinE/Cdk2 and Cyclin A/Cdk2 complexes provide G1/S transition. In response to DNA damage, ATR-dependent Chk1 activation leads to phosphorylation of Cdc25A and subsequent increase in its ubiquitination and proteasome mediated degradation (Boutros et al. 2006; Molinari et al. 2000; Zhao et al. 2002). SCF complex has been shown to be the ubiquitin ligase complex that controls Cdc25A half life in response to DNA damage (Jin et al. 2003). Chk1 also regulates basal Cdc25A levels during the normal cell cycle in the absence of DNA damage (Sorensen et al. 2003) and under stressed conditions increased

Chk1 kinase activity accelerates Cdc25A degradation. As Cdc25A is transcribed late in G1 phase, this pathway is only functional late in G1 phase and it delays progression into S phase for several hours. Therefore, it constitutes the initial and fast response to DNA damage in G1.

The second pathway involves phosphorylation of p53 directly by ATR and/ or Chk1. Phosphorylation of p53 prolongs its half life and therefore increases p53-dependent transcription (Lukas et al. 2004). Accumulation of p21, one of the p53 targets inhibits of CyclinE/Cdk2. As this pathway requires transcription and translational steps, it is in effect later than the Cdc25A pathway and provides maintenance of the response (Lukas et al. 2004). The role of Cdc25A in G1 checkpoint has been challenged by an earlier study (Dulic et al. 1994). It was shown that in the absence of functional p53 ionizing radiation does not activate G1 checkpoint (Dulic et al. 1994). While this study emphasized the importance of p53 for G1 checkpoint activation, it questioned the importance of Cdc25A for G1 checkpoint activation. Therefore, there is need of further studies to establish the role of Cdc25A in G1 checkpoint.

DNA damage during S phase slows down DNA replication by a passive mechanism due to inability of DNA polymerase to bypass the lesion or by an active mechanism that involves DNA replication checkpoint. At the end of mitosis, replication origins are licensed for replication by sequential loading of Orc, Cdc6, Cdt1 and multiple copies of MCM helicase, leading to formation of pre-replicative complexes (pre-RC) (Takeda and Dutta 2005). Cdk2/Cylin E and Cdc7/Dbf4 are two S phase kinases that phosphorylate several components of pre-RC and other replication proteins to initiate DNA replication. Activities of Cdk2/Cyclin E and Cdc7/Dbf4 kinases are down-regulated by ATR in the presence of replication stress, imposed by decrease in dNTP pools or by stalled replication forks (Bendjennat et al. 2003; Costanzo et al. 2003). Cdk2 activity is regulated through checkpoint

induced Cdc25A degradation. Cdc25A removes an inhibitory phosphorylation on Cdk2 and activates it. ATR dependent Chk1 activation increases Cdc25A degradation, similar to DNA damage response in G1 phase.

The second S phase kinase regulated by ATR signaling is Cdc7/Dbf4 kinase. Cdc7 is a serine/threonine kinase that forms distinct complexes with its regulatory subunits, Dbf4 and Drf1 (Dbf4-related factor 1) (Furukohri et al. 2003). Both of the complexes are important for DNA replication (Furukohri et al. 2003; Takeda and Dutta 2005). Dbf4 is a cell cycle regulated protein that restricts Cdc7 activity to S phase. In several organisms, it has been shown that checkpoint-dependent phosphorylation of Dbf4 leads to its dissociation from chromatin and decrease in the kinase activity of the Cdc7/Dbf4 complex (Costanzo et al. 2003). Cdc7 forms an additional complex with Drf1. Although Drf1 seems to be dispensable for DNA replication in *Xenopus*, after aphidicolin treatment ATR-dependent phosphorylation increases its chromatin association (Yanow et al. 2003). Functional consequences have not been fully addressed yet, but it has been suggested that down-regulation of Cdc7/Drf1 complex prevents loading of Cdc45 onto chromatin in the presence of replication stress, thereby inhibits replication initiation.

The second branch of replication checkpoint involves ATR and Chk1 dependent phosphorylations of p53, resulting in the increase of p53 stability. Accumulation of p53 leads to increase in p21 levels, which inhibits PCNA and Cdk activities (Shechter et al. 2004b).

ATR and Chk1 have functions during normal S phase in addition to their role in replication checkpoints. Both proteins have been shown to modulate the timing of replication origin firing initiation during unperturbed replication (Shechter et al. 2004a; Syljuasen et al. 2005). In *Xenopus* egg extracts, inhibition of ATR activity either by addition of caffeine or

by addition of specific neutralizing antibodies promotes origin firing due to high Cdk2 and Cdc7 activities (Shechter et al. 2004a). Similarly, down-regulation of Chk1 in humans increases replication initiation, in addition, it leads to a rise in phosphorylation of ATR's substrates, in the amount of ssDNA and DNA breaks (Syljuasen et al. 2005). Therefore, ATR and Chk1 prevent unscheduled replication and formation of aberrant replication intermediates, which could potentially harm the stability of the genome.

Transition from G2 to mitosis is brought by the activity of Cdk1/Cyclin B complex. All three isoforms of Cdc25 cooperate to activate Cdk1/Cyclin B complex (Boutros et al. 2006). In the presence of DNA damage, ATR signaling pathway down-regulates the activities of these phosphatases to prevent progression into mitosis. Cdc25A activity is controlled by DNA damage induced degradation; this pathway is shared by all three G1, S and G2 checkpoints. The second pathway involves the phosphorylation of Cdc25A, B and C by Chk1 to create a binding site for 14-3-3 and to inhibit the interaction with CDK1-Cyclin B by sequestering these phosphatases into the cytoplasm (Chen et al. 2003; Zhao et al. 2002). Similar to G1 checkpoint, in G2 checkpoint regulation of Cdc25 activities constitutes the fast and short response to DNA damage, the longer response is provided by the up regulation of p53 targets, such as p21, GADD45 and 14-3-3F, which in turn inhibits Cdk1 activity (Taylor and Stark 2001).

Although ATR and ATM kinases are activated by different type of DNA lesions, in the presence of double strand breaks (DSB), they cooperate to phosphorylate and activate Chk1. ATM is the main PIKK3 kinase that modulates cellular responses to double strand breaks. Recent evidence placed ATM upstream of ATR in checkpoint regulation in the presence of DSBs (Jazayeri et al. 2006). ATM, Nbs and Mre11 nuclease activity are required

for RPA and ATR foci formation. ATM regulates ATR chromatin association in the presence of DSBs (Cuadrado et al. 2006). Kinetic analysis revealed that after DSB formation, ATM activation is followed by ATM-dependent NBS1 phosphorylation and this precedes ATR activation and Chk1 phosphorylation. Moreover, ATR activation in response to IR seems to be cell cycle regulated (Jazayeri et al. 2006).

Mediators of ATR signaling

Regulation of Chk1 activation in response to DNA damage depends on other proteins, namely mediators of checkpoint. Mediators of ATR-Chk1 pathway form a bridge between ATR and Chk1 and they are required for optimal Chk1 phosphorylation. None of mediator proteins has been shown to have kinase activity, but they provide specificity and efficiency of Chk1 activation. In humans BRCA1, TopBP1, Claspin and Timeless have been shown to be essential for Chk1 activation; however, the exact mechanism by which they mediate the phosphorylation and how they collaborate with each other in the activation of Chk1 is not well understood.

Human TopBP1 (topoisomerase IIβ binding protein 1) is identified in a yeast two hybrid screen to find proteins interacting with DNA topoisomerase IIβ (Yamane et al. 1997). It contains 8 BRCT (BRCA1 C terminus) repeats. BRCT repeats have been found in several checkpoint and DNA repair proteins and it is thought that they function as phosphopeptide binding domains (Yamane et al. 1997). Similar to its yeast homologs, human TopBP1 functions in DNA replication and checkpoint activation (Garcia et al. 2005; Kumagai et al. 2006) and it has been also implicated in transcriptional regulation. In *Xenopus,* TopBP1 is

required for ATR chromatin association and Chk1 activation. It has recently been shown to interact with ATR through ATRIP and induce ATR kinase activity *in vitro* (Kumagai et al. 2006). Interestingly, it stimulates phosphorylation of several ATR substrates such as Mcm2, Rad17 (Kumagai et al. 2006). This allosteric regulation of ATR kinase by TopBP1 adds another level of regulation or mechanism involved in ATR activation after DNA damage. In humans, together with BRCA1, TopBP1 regulates Chk1 activation in response to IR treatment (Yamane et al. 2003). It binds to directly to DNA ends, however, physiological role of this binding is not known (Yamane and Tsuruo 1999). It gets phosphorylated in response to IR, HU and UV treatments (Garcia et al. 2005). UV induced phosphorylation can be inhibited by caffeine treatment, suggesting ATR or ATM is the responsible kinase.

Human Tim (Timeless) exhibits a 24-hour circadian oscillation and functions in circadian clock. Recently, it has been implicated in Chk1 activation in response to DNA replication stress, providing a connection between cell cycle and circadian clock (Unsal-Kacmaz et al. 2005). It has a cell cycle regulated expression, peaking at S phase. Tim interacts both with ATR/ATRIP complex and Chk1 (Unsal-Kacmaz et al. 2005). Tim is required for the activation of Chk1 after DNA damage and replication stress.

BRCA1 (breast cancer susceptibility gene 1) is tumor suppressor gene and is mutated in 50% of familial breast cancer cases (Venkitaraman 2002). In addition to its function in DNA repair and recombination, BRCA1 has a role in checkpoint activation, distinct BRCA1 residues are phosphorylated in response to different of DNA damaging agents by ATM and ATR (Venkitaraman 2002). It co-localizes to same foci with ATR after DNA damage. It is required for optimal phosphorylation of Chk1 after IR (Tibbetts et al. 2000; Yarden et al. 2002). BRCA1 and Claspin seems to cooperate for Chk1 activation in the presence of DSBs

(Lin et al. 2004). As Claspin is the main focus of this dissertation, it will be discussed in detail in the next section.

Claspin- a mediator or a sensor in ATR signaling

Dunphy and his colleagues developed an *in vitro* system using *Xenopus* egg extracts to study the requirements of efficient checkpoint activation. Addition of poly(dA)70-poly(dT)70 DNA homopolymers into the extracts led to activation of Chk1 (Kumagai and Dunphy 2000). Using this system, they identified a novel protein, Claspin as a regulator for Chk1 activity after DNA damage in *Xenopus*. Immunodepletion of Claspin from egg extracts diminished DNA damage and replication stress induced phosphorylation of Chk1 and compromised the checkpoint activation (Kumagai and Dunphy 2000). In mammalian cells, Claspin has been also shown to be a regulator for Chk1 activation (Chini and Chen 2003).

Human Claspin is a 1339 amino acid polypeptide with a calculated mass of 151 kDa, it is highly conserved in several organisms, from *M. musculus*, *R. norvegicus*, *Xenopus* to humans. There is 50% identity between *Xenopus* and human Claspin. Although it is a well conserved protein, except SQ/TQ motifs, it does have any additional motifs that can give insights into its biochemical function.

Human Claspin is a nuclear protein with a cell cycle regulated expression profile. The protein levels peak at S phase, similar to Chk1 (Chini and Chen 2003). Recently, Claspin has been identified as an E2F1 target, E2F1 is a transcription factor that controls expression of several cell cycle regulated genes. E2F1 induces Claspin expression in response to serum stimulation (Iwanaga et al. 2006).

Functions and Regulation of Claspin

Checkpoint activation in response to replication stress depends on Claspin in both *Xenopus* and humans (Chini and Chen 2003; Kumagai and Dunphy 2000; Lee et al. 2003). Presence of stalled replication forks leads to phosphorylation of Claspin in both of the organisms and induces Claspin-Chk1 interaction. Down-regulation of Claspin expression diminishes UV, IR and HU induced Chk1 phosphorylation and as a result of this, cells can not inhibit DNA synthesis, indicating that Claspin is required for DNA replication and damage checkpoint activation. In addition, down-regulation of Claspin expression by RNAi leads to in premature chromatin condensation after HU treatment, suggesting that Claspin also functions in S-M checkpoint to prevent entry into mitosis before completion of DNA replication (Chini and Chen 2003).

Claspin has also a role in cell cycle progression under unperturbed conditions and it is essential for cell survival (Lin et al. 2004). Down-regulation of Claspin inhibits cellular proliferation and decreases DNA synthesis rate (Lin et al. 2004). Consistent with this, in *Xenopus* Claspin interacts with several replication and checkpoint proteins at the replication sites, such as Cdc45, RPA, DNA polymerase, replicative and checkpoint clamp loaders (Lee et al. 2005). Interestingly, Claspin over-expression accelerates cellular proliferation (Lin et al. 2004). In addition, down-regulation of Claspin causes Cdc25A up regulation, indicating that together with ATR and Chk1, Claspin regulates basal levels of Cdc25A under normal conditions (Sorensen et al. 2004). These data indicates that Claspin might act a tumor suppressor and an oncogene.

Although Claspin is an important component of ATR signaling, there are no studies to understand its biochemical properties to gain better insight into how Claspin functions and how it is regulated. One of the models proposed was that Claspin might provide Chk1 phosphorylation by stimulating ATR/ATRIP kinase activity towards Chk1. However, Dunphy and his colleagues showed that Claspin do not stimulate ATR/ATRIP kinase activity *in vitro*, but it provides Chk1 activation *in vivo* (Kumagai et al. 2004). Than, it was suggested that Claspin might function by recruiting Chk1 in close proximity to ATR and/or induce conformational change in Chk1 and make it a better substrate for ATR. This second model requires further studies. However, it is known that Claspin not only affects phosphorylation status of Chk1, but also increases Chk1 kinase activity and therefore, it induces Chk1 autophosphorylation activity.

Regardless of how Claspin functions, site-specific damage dependent phosphorylation of Claspin is one of the mechanisms involved in Claspin regulation. The Chk1 binding domain (CKBD) of Claspin has been mapped to conserved repeats with the ExxxLC(S/T) GxF sequence within the C terminus (Kumagai and Dunphy 2003). *Xenopus* Claspin has two repeats, whereas human Claspin has three copies. Phosphorylation of serine or threonine residues in these repeats is required for the Chk1-Claspin interaction and consequent checkpoint activation in *Xenopus* in response to replication stress (Kumagai and Dunphy 2003). Clarke and his colleagues developed a cell-free *in vitro* system to study the requirements of Chk1 activation. Similar to *Xenopus* system, addition of poly(dA)70-poly(dT)70 DNA homopolymers into HeLa cell extracts induced Claspin and Chk1 phosphorylation (Clarke and Clarke 2005). Using this *in vitro* system, they showed that phosphorylation of the two of corresponding sites in these repeats is enough for Chk1-

Claspin interaction, phosphorylation of the third site seems to be less significant for the binding of Claspin to Chk1 (Clarke and Clarke 2005).

The kinase responsible for these phosphorylations and how this kinase activity is regulated are not known. These motifs are not typical ATR phosphorylation sites. It is well established that ATM and ATR kinases have strong preference to SQ/TQ motifs. Although these motifs do not contain an ATR kinase consensus target sequence, some findings place ATR upstream of these phosphorylation events. Firstly, immunodepletion of ATR from *Xenopus* egg extracts compromises Claspin phosphorylation in response to DNA damage (Kumagai and Dunphy 2003), secondly, these phosphorylations are inhibited by caffeine, ATR and ATM inhibitor (Chini and Chen 2003; Kumagai and Dunphy 2003). Lastly, ATR and Claspin interacts *in vivo* in humans (Chini and Chen 2003), however so far direct phosphorylation of Claspin by ATR has not been detected *in vitro.*

The region of Chk1 for Claspin interaction has also been mapped to N terminal kinase domain of Chk1. Four positively charged residues lying in the region responsible for catalytic activity of Chk1 are involved in interaction (Jeong et al. 2003). Although C terminus of Chk1 does not directly interact with phosphopeptide motif of Claspin, it has a regulatory role in this interaction. Time course analysis of interaction revealed that fully phosphorylated highly active Chk1 no longer associates with Claspin, this may provide several rounds of Chk1 activation and amplification of the signal (Jeong et al. 2003).

Systematic mutational analysis of all SQ/TQ motifs in Claspin led to identification of different phosphorylation sites that are important for checkpoint activation in response to DSBs. Phosphorylation of T817 or S819 in SQ/TQ motifs of Claspin is essential for Chk1 activation, when the EcoRI digested chromatin is added to *Xenopus* egg extracts (Yoo et al.

2006). Interestingly, phosphorylations of these residues are dispensable for checkpoint activation in response to replication stress. These findings indicate that specificity and efficiency of checkpoint activation in response to specific DNA lesions might be regulated by site-specific phosphorylation of Claspin. In humans, two studies showed that Claspin is required for checkpoint activation in the presence of DSBs (Chini and Chen 2003; Lin et al. 2004). Although the residues are conserved in humans, it is not known whether the phosphorylations of these residues are also required in humans.

Dunphy and his coworkers showed that Claspin associates with chromatin during S phase in *Xenopus* (Lee et al. 2003). Binding of Claspin requires the presence of the pre-replication complex (pre-RC). During S phase, incorporation of Cdc45 into the pre-RC complex results in the formation of pre-initiation complex. Cdc45 is required for the initial unwinding of DNA at replication origins, which is then followed by binding of RPA and other replication machinery proteins to chromatin. In addition to formation of pre-RC, the presence of Cdc45 is essential for Claspin binding (Lee et al. 2003). These findings suggest that Claspin binds to chromatin at the replication origins after the initial origin unwinding and in the presence of replication block its binding increases. A recent study showed that Claspin associates with undamaged chromatin and its amount on chromatin increases in the presence of DSBs, created by EcoRI digestion of chromatin (Yoo et al. 2006). DNA damage induced changes in the dynamics of Claspin chromatin association seems to be the additional mechanism to regulate Claspin.

Surprisingly, Claspin associates with chromatin independent of other DNA damage sensors such as ATR and Rad17 (Lee et al. 2003). Independent binding of all three protein complexes suggests that they may recognize the different structures or replication

intermediates formed at the stalled replication forks and more importantly, there might be another damage sensing mechanism, involving Claspin. It also raised an important question into how Claspin functions in the DNA damage pathway. Despite its chromatin association, no direct DNA binding of Claspin has been detected in *Xenopus.* Therefore, Claspin could bind to chromatin directly or may require protein/protein interactions for binding. It may also have different preferences for certain DNA damage types or aberrant DNA structures. Therefore, the characterization of the DNA binding properties of Claspin would provide better understanding into how Claspin functions and also provide more insights into DNA damage sensing problem.

Although Rad17, ATR and Claspin are recruited to chromatin independent of each other, they could be collaborating on chromatin to sense the DNA damage properly (Lee et al. 2003). This possibility correlates with the fact that Claspin interacts with ATR and Rad9 *in vivo*. It is not clear whether these proteins interact directly with each other or whether they require other proteins or DNA for the interaction. Further characterization of these interactions will increase our understanding of how DNA damage is sensed and how protein-protein interactions regulate Claspin function. Although Claspin is a Chk1 binding protein, the interaction between these proteins is very weak and transient. In immunoprecipitation experiments the signal for the interacting endogenous proteins is very low (28). This suggests that there might be other proteins that interact weakly with Claspin and remained undetected. These proteins may regulate Claspin function and/or they may be regulated by Claspin.

Claspin has a sequence homology to Mrc1 (mediator of replication checkpoint) in *S. pombe* and *S. cerevisiae.* Mrc1 is an important regulator of replication checkpoint. It is required for the activation of spCds1 by spRad3 and of scRad53 by scMec1 in the presence

of replication stress (Alcasabas et al. 2001; Tanaka and Russell 2001). In both organisms Mrc1 gets phosphorylated in response to replication blocks, depending on the spRad3 and scMec1. Like Claspin, Mrc1 is cell cycle regulated at both RNA and protein level, it expression increases at S phase. Mrc1 moves along with the replication forks and essential for replication during normal cell cycle (Osborn and Elledge 2003). Mrc1 null cells progress through S phase with slower kinetics. This replication function of Mrc1 is independent of its checkpoint function, as slow replication phenotype of Mrc1 null cells can be complemented by checkpoint mutant forms of Mrc1 (Osborn and Elledge 2003).

SUMMARY

Genomic instability is one of major cause of cancer in humans. DNA damage checkpoints act as a surveillance system to maintain the genome integrity. In the presence of DNA damage, DNA damage checkpoints arrest cell cycle until DNA damage is repaired. Depending on the nature of the damage, several signal transduction pathways are activated, one of which includes ATR, Claspin and Chk1. Claspin is indispensable for Chk1 activation in response IR, UV treatments or replication blocks.

Claspin associates with chromatin during S phase, DNA damage increases it association with chromatin. As it binds to chromatin independent of any other sensor proteins, it was suggested that Claspin may function as a sensor and a mediator. Several proteins have been classified as DNA damage sensors, however, none of them have been studied carefully in terms of their DNA binding properties. Furthermore, there is not enough evidence on the nature of DNA damage that activates the checkpoints. Experiments in this

dissertation examine the biochemical properties of Claspin to understand its role in the checkpoint activation and test whether Claspin might be a sensor protein in the checkpoint activation. Experiments in the chapter II shows that Claspin is a DNA binding protein with high affinity to branched DNA structures. In addition, the location of binding on these branched DNA structures is determined. Chapter III describes the experiments performed to investigate whether Claspin recruits Chk1 to ATR in proximity for its phosphorylation by ATR.

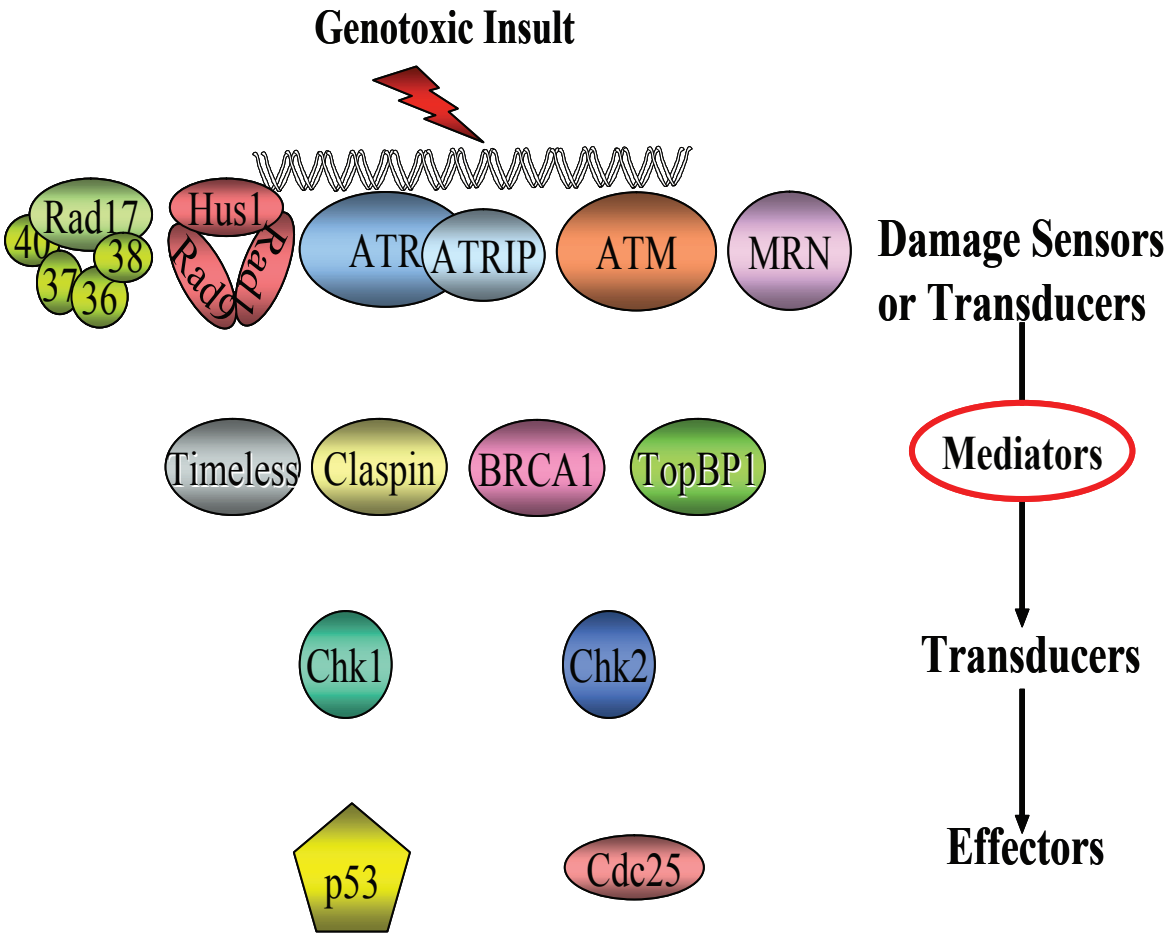


Figure 1.1: Organization of DNA damage checkpoints. A schematic representation of five major classes of checkpoints.

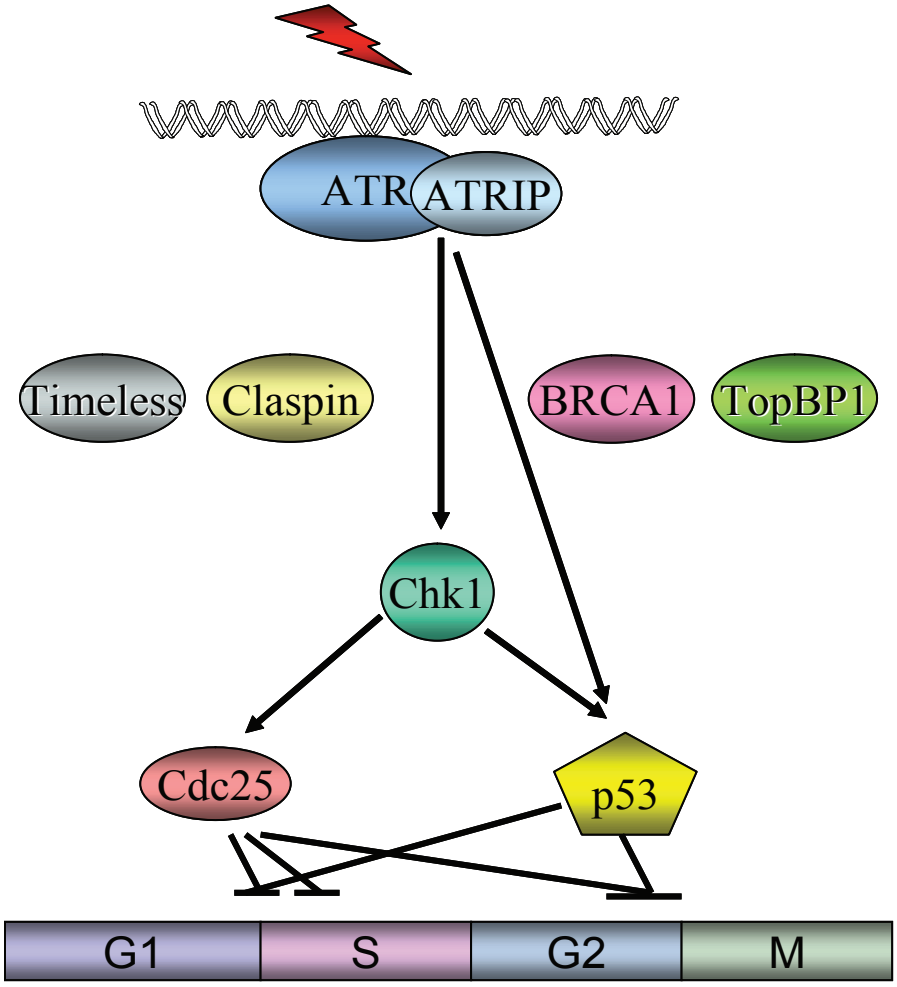


Figure 1.2: ATR signaling pathway. A schematic representation of components of ATR signaling pathway that regulates cellular responses to a variety of DNA damaging agents.

CHAPTER II

HUMAN CLASPIN IS A RING-SHAPED DNA BINDING PROTEIN WITH HIGH AFFINITY TO BRANCHED DNA STRUCTURES

ABSTRACT

Claspin is an essential protein for the ATR-dependent activation of the DNA replication checkpoint response in *Xenopus* and human cells. Here we describe the purification and characterization of human Claspin. The protein has a ring-like structure and binds with high affinity to branched DNA molecules. These findings suggest that Claspin may be a component of the replication ensemble and plays a role in the replication checkpoint by directly associating with replication forks and with the various branched DNA structures likely to form at stalled replication forks due to DNA damage.

INTRODUCTION

The replication and DNA damage checkpoints ensure genome integrity by coordinating cell division with replication and arresting the cell cycle in the presence of stalled replication forks. Several components of the checkpoints have been identified and classified into five main groups: DNA damage sensors, mediators, apical, distal signal transducers and effectors. DNA damage sensors recognize the DNA lesions or DNA structures, formed upon genotoxic stress and relay the signal to downstream signal transducers. So far, genetic and biochemical analyses have implicated two groups of proteins in sensing DNA damage or replication intermediates: (1) the phosphoinositide kinase like kinase (PIKK) family members ATM and ATR, and (2) the clamp loader/DNA clamp family members Rad17-RFC/9-1-1 complex (Abraham 2001; Melo and Toczyski 2002; Sancar et al. 2004).

Claspin has been placed in the mediator class of checkpoint proteins because it was originally shown in *Xenopus* egg extracts to be required for ATR to phosphorylate the downstream signal transducer, Chk1, upon activation of the replication checkpoint (Kumagai and Dunphy 2000). The yeast proteins, scRad9/spCrb2 and sp/scMrc1, are the prototypical members of the mediator class of checkpoint proteins (Alcasabas et al. 2001; Sun et al. 1998; Tanaka and Russell 2001). Another example of a mediator in humans is BRCA1, which has been shown to be required for optimal Chk1 phosphorylation after ionizing radiation (Lin et al. 2004). Currently, mediator proteins are thought to promote protein-protein interactions between checkpoint sensors and signal transducers, leading to phosphorylation and activation of transducer kinases. However, it is becoming apparent that this narrow definition of

mediators does not explain their functions fully. In particular, the possibility of mediators having a more active role in damage sensing has not been addressed. Such a role in damage sensing may lead to the re-definition of the checkpoint proteins initially classified as mediators, as exemplified by recent findings on Claspin.

In *Xenopus*, ATR phosphorylates and activates Chk1 in the presence of poly(dA)$_{70}$-poly(dT)$_{70}$, which activates the DNA replication checkpoint. Phosphorylation of Chk1 depends on its association with a 140 kDa protein, Claspin (Kumagai and Dunphy 2000; Kumagai and Dunphy 2003). Activation of the replication checkpoint induces Claspin phosphorylation, which is essential for Claspin-Chk1 association and subsequent activation of Chk1 (Kumagai and Dunphy 2000; Kumagai and Dunphy 2003). Subsequent work showed that similar to Xclaspin, human Claspin is also required for phosphorylation of Chk1, interacts with Chk1, and the association between these two proteins depends on the phosphorylation of Claspin (Chini and Chen 2003). In addition to Chk1, Claspin also interacts with other checkpoint proteins, ATR and Rad9 (Chini and Chen 2003). As ATR is the primary PIKK family kinase that phosphorylates Chk1, it was proposed that Claspin may work as an adaptor molecule that brings the damage sensors, ATR and the Rad9-Rad1-Hus1 (9-1-1) complex, and the signal transducer Chk1 in close proximity for checkpoint activation. In line with these findings, it was recently reported that DNA damage induces the formation of a complex between Claspin and BRCA1, another regulator of ATR-dependent Chk1 activation (Lin et al. 2004).

A detailed study on Xclaspin suggests a more complex role in cell cycle regulation and replication checkpoint (Lee et al. 2003). Using an *in vitro* system consisting of *Xenopus* egg extracts supplemented with demembranated sperm chromatin, it was found that Claspin

is recruited to chromatin in a manner dependent on the replication initiation proteins, MCM2-7 and Cdc45, but independent of proteins known to be essential for checkpoint signaling such as RPA, ATR, or Rad17-RFC and the 9-1-1 complex. Therefore, it was proposed that Claspin, ATR, and Rad17-RFC/9-1-1 act independently, but in concert, to recognize three distinct features of replication fork and activate the replication checkpoint. According to this model, Claspin recognizes some component of the replication fork that is associated with unwinding DNA, ATR binds RPA-covered single-stranded DNA, and Rad17-RFC loads the 9-1-1 ring onto a primer/template to provide a triple-tiered mechanism of recognizing replication forks and initiating the replication checkpoint response (Lee et al. 2003). Of particular interest, it was reported that even though Claspin associates with replicating sperm chromatin in *Xenopus* extracts, Xclaspin exhibited no intrinsic DNA binding activity (Lee et al. 2003). It was, therefore, suggested that the association of Claspin with chromatin during replication was mediated by protein-protein interactions, possibly with Xcdc45. The recent finding that Claspin binds to BRCA1 (Lin et al. 2004), a protein with high affinity for branched DNA structures (Paull et al. 2001), raised the possibility that BRCA1 may be the protein responsible for recruiting Claspin to replication forks and related replication intermediates.

To test this emerging model for the mode of action of Claspin that is largely based on *in vitro* experiments with *Xenopus* egg extracts and *in vivo* assays with human cells, we purified the human Claspin and investigated its biochemical properties. Here we report our findings on the interaction of Claspin with DNA. We find that Claspin binds to double-stranded DNA directly, but with low affinity, and to branched DNA structures, which are likely to be found at replication forks, with high affinity and specificity. Analysis by electron

microscopy reveals that Claspin is a ring-shaped protein that appears to circle DNA specifically at single-stranded branch points. Collectively, these data suggest that Claspin is a direct primary sensor of replicating DNA and of DNA structures that may form at stalled replication forks, and therefore it is a frontline replication and damage sensor in the mammalian checkpoint response.

MATERIALS AND METHODS

Cloning of Claspin into Expression Vectors. We isolated human Claspin cDNA by RT-PCR. Whole cellular RNA was isolated from HEK-293T cells using Trizol reagent (Invitrogen) according to manufacturer's directions. Attempts to amplify the entire open reading frame of Claspin (4.1 kb) by RT-PCR were unsuccessful. Therefore, the 5' and 3' halves of the cDNA were amplified separately and inserted into the pCRBlunt II Topo vector sequentially to obtain a plasmid containing full-length cDNA of Claspin. The primers used for amplifying Claspin by RT-PCR were based on Claspin GenBank sequence with the accession number NM_022111. The sequences of the primers used for the 5' and 3' termini of human Claspin were 5'-GGATCCGCCGCCACCATGACAGGCGAGGTGGGT-3' and 5'-TCTAGACTCGAGGCTCTCCAAATATTTG-3', respectively. The entire Claspin cDNA was inserted into pcDNA4 to obtain the mammalian expression vector pcDNA4-Claspin-Flag. This plasmid was used as a template to amplify Claspin cDNA and insert it into the pFastBacFlag vector to obtain a baculovirus vector that expresses a full length Claspin. The baculovirus constructs CL149, CL340, and CL851, expressing the N-terminal regions of the corresponding amino acids, and CLΔ851 lacking the N-terminal 851 amino acids, were generated by standard methods including subcloning of restriction enzyme fragments and amplifying the desired sequences by appropriate PCR primers followed by insertion into a pFastBacFlag vector. All of the plasmids containing Claspin and Claspin fragments were sequenced to ensure that no mutations were introduced into the gene during *in vitro* manipulation.

Expression and Purification of Recombinant Proteins. We exclusively used Claspin and Claspin fragments made in the baculovirus/insect cell system for our studies. Baculoviruses were generated using the Bac-to-Bac system (Invitrogen). Monolayer High Five cells (Invitrogen) were infected with the appropriate baculovirus and then harvested after 48 hrs. The cells were washed with 1X PBS and then lysed in 20X packed volumes of lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40 (NP-40), and protease inhibitors (Roche Molecular Biochemicals). After incubation on ice for 10 min, the cell lysate was centrifuged for 20 min at 32,000g. The supernatant was incubated with anti-Flag agarose (Sigma) for 4 hrs at 4˚C. The resin was washed once with 10 volumes of lysis buffer, once with buffer containing 50 mM Tris-HCl, pH 7.5, 1 M NaCl, once with elution buffer (40 mM Tris-HCl, pH 7.5, 80 mM NaCl, 10% glycerol), and then eluted with two volumes of elution buffer containing 0.2 mg/ml flag peptide (Sigma) for 30 minutes at 4˚C. A typical yield was about 20-30 μg of Claspin from 10^7 cells. Concentration of Claspin and its fragments were determined by Bradford assay. The proteins were stored at -80˚C.

DNA Substrates. The sequences of oligonucleotides used in preparation of various substrates for gel mobility shift assays have been described elsewhere (Ozsoy et al. 2003). These oligomers were annealed in appropriate combinations to obtain the DNA forms shown in **Figure 2.5** and **2.6**. To prepare each substrate, oligonucleotide 1 was labeled on 5' terminus using T4 polynucleotide kinase (Biolabs) and [γ^{32}P]-ATP (ICN). The labeled oligomer was purified on a Probe Quant G-50 Micro column (Amersham) and annealed with appropriate non-radioactive (partially) complementary oligomers at 1 to 4 molar ratios. The mixture, which contained the radiolabeled oligomer at 25 nM and the complementary

oligomer at 100 nM concentrations, in a buffer containing 50 mM NaCl and 50 mM Tris-HCl, pH 7.5, was heated at 95˚C for 20 minutes and then allowed to cool to room temperature overnight. The substrates were separated on a 10% polyacrylamide gel in 1X TBE (50 mM Tris-borate, pH 7.9, 1.2 mM EDTA). Gel slices containing the substrates were excised and incubated in 0.3 M sodium acetate and 10 mM EDTA at 25˚C overnight. The liquid phase was then separated from gel pieces and the DNA was precipitated with 70% ethanol and dissolved in a buffer with 10 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM EGTA. Specific activities of substrates were determined and concentrations were based on specific activity.

Gel Mobility Shift Assay. Full length Claspin or its fragments at the indicated concentrations were incubated with 0.25 nM DNA substrates for 25 minutes at 30˚C in 25 µl reaction mixture containing 11 mM HEPES, pH 7.9, 12 mM Tris, pH 7.5, 70 mM NaCl, 1.1 mM EGTA, 1 mM DTT, and 3 mg/ml BSA. Glycerol was added to 6.1%, and the DNA-protein complexes were separated on a 5% polyacrylamide gel in 1X TBE. Electrophoresis was performed at 120 V for 210-240 minutes at 4˚C. The gels were dried and exposed to a PhoshoImager screen (Molecular dynamics), and the data was analyzed by ImageQuant software (version 5.0, Molecular dynamics). Bound DNA fraction is determined by subtracting the free DNA from the total DNA in the lane. Percentage of bound DNA was calculated and plotted against Claspin concentration to generate binding isotherms for Claspin binding to various substrates. For the antibody supershift assay, full length Claspin or UvrA (bacterial DNA binding protein used as a control) was preincubated with anti-flag

M2 monoclonal antibody (Sigma, F-3165) for 20 minutes on ice before adding to DNA and performing gel mobility shift assay.

Electron Microscopy. Linear double-stranded DNA templates containing either an internal single-stranded or double-stranded tail (**Figure 2.7A, B**) were generated to mimic a replication fork junction using the plasmid pGLGAP12, which contains a site for the nicking endonuclease, N.BbvCIA, on the sense strand immediately followed by a 400 bp G-less cassette in which the complementary strand contains no guanines (Sawadogo and Roeder 1985). Plasmids were nicked with N.BbvCIA followed by strand displacement with Klenow exo$^-$ fragment in reactions lacking dCTP to produce the single strand tail. Double strand tails were generated by annealing primers to the single strand tail near the junction site followed by synthesis using Klenow exo$^-$ fragment.

The linear replication fork templates (100 ng) were incubated with Claspin (200 ng) in 20 µl binding buffer containing 10 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM EGTA, 1 mM DTT for 20 min at 25˚C. In the control reaction, the single-stranded tail template was incubated with 500 ng *E. coli* SSB protein under the same conditions. The samples were fixed by the addition of glutaralaldehyde to a final concentration of 0.6% for 10 min at 25˚C and chromatographed on 1-ml Biogel A50m columns to remove unbound proteins. Fractions containing DNA-protein complexes were prepared for electron microscopy as described before (Griffith and Christiansen 1978). The samples were examined on a Phillips CM12 instrument, and images were scanned using Nikon-4500 film. Length measurements were made using Digital Micrograph software.

RESULTS

Purification of Claspin. We cloned the human Claspin gene by RT-PCR and inserted it into both mammalian and insect expression vectors and purified the protein from both sources by affinity chromatography. Claspin purified from transiently transfected human cells was of low yield and moderate purity. In contrast, High Five insect cells infected with recombinant baculovirus containing the Claspin gene expressed the protein at high levels and were used as our main source of Claspin. Early on in our study on Claspin, we found that it is a DNA binding protein and therefore made baculovirus constructs expressing various regions of the protein with the aim of identifying the DNA binding domain. **Figure 2.1A** is a schematic representation of fragments that were inserted into baculovirus, and **Figure 2.1B** shows the Claspin domains encoded by these fragments separated on an SDS-PAGE gel. Human Claspin is 1339 amino acids in length with a calculated molecular weight of 151 kDa. However, as noted for the Xclaspin, the human Claspin migrates anomalously on SDS-PAGE with an apparent molecular weight of 250 kDa (**Figure 2.1B**, lane 6). Both *Xenopus* and human Claspins are acidic proteins with pI 4.5, and it was suggested that this anomalous migration might be the consequence of this low pI (Kumagai and Dunphy 2000). The same is true for all of the Claspin fragments we expressed; all have pIs in the range of 4.4 to 5.7 and migrate on SDS-PAGE considerably slower than expected from their calculated molecular weights (**Figure 2.1B**, lanes 2-5).

Binding of Claspin to DNA. Since it has been reported that Xclaspin associates with chromatin in *Xenopus* extracts, we wished to determine whether Claspin directly binds to

DNA without a protein intermediary. We tested DNA binding by gel mobility shift assays using a 30-bp duplex. Claspin forms a DNA-protein complex that is supershifted by anti-flag antibodies (**Figure 2.2A).** We concluded that Claspin is a DNA binding protein. To identify the DNA binding domain, we tested the Claspin fragments shown in Figure 1 in the gel mobility shift assay. The results are shown in **Figure 2.2B**. Based on the data in **Figure 2.2B** (lanes 1-8), the main DNA binding domain (DBD) is identified as the region spanning amino acids 149 to 340. However, the C-terminal CLΔ851 fragment also exhibited some weak DNA binding activity (**Figure 2.2B,** lane 6). Indeed, with high concentrations of this fragment a substantial amount of the DNA exhibits slower migration, albeit without forming a distinct DNA-protein band (**Figure 2.2B,** lanes 11-15). This behavior is commonly observed with DNA-protein complexes with rapid dissociation rates. Thus, it appears that Claspin has a primary DBD in the N-terminal region and a secondary DNA binding site in the C-terminal region.

To further characterize the primary DBD of Claspin, mutational analysis has been performed. Yeast homolog of Claspin, Mrc1 is also a DNA binding protein. Its DBD has been mapped to N terminus of the protein. Claspin and Mrc1 share sequence similarity in the DBD (**Figure 2.3**). This region is predicted to have helix-loop-helix motif. Mutational analysis surrounding this motif revealed the physiological importance of DBD of Mrc1. They identified the mutant Mrc1 with diminished DNA binding activity. The cells expressing the mutant Mrc1 have increased sensitivity to HU and defects in checkpoint activation. In order to examine the physiological importance of DNA binding activity of Claspin and further characterize DBD of Claspin, we deleted either helix A (denoted as ClpDBD1) or Helix B (denoted as ClpDBD2) in Claspin baculovirus contructs (**Figure 2.3**). Mutant Claspin and

wild type Claspin were purified from insect cells and DNA binding activities of each protein was determined by gel mobility shift assay. Binding isotherms were generated and K_d values were determined. Wild type and mutant forms of Claspin bind to branched DNA structures with similar affinities (**Figure 2.4**). Although we could not identify a DBD mutant of Claspin to examine the role of DNA binding, we narrowed DBD to a region spanning between 149 to 292 amino acids (**Figure 2.4**).

Binding of Claspin to Branched DNA. In *Xenopus* extracts, it was found that Claspin requires Xcdc45 to bind to chromatin. Cdc45 is necessary for ATP-dependent unwinding of DNA at replication origins (Bell and Dutta 2002). Hence, it was hypothesized that Claspin may associate with a special structure formed at replication origins as a consequence of DNA unwinding. The specific DNA structures formed at unwound origins that can activate the checkpoint is not known. However, it was previously shown that hydroxyurea treatment, which leads to replication block, increases the number of hemi-replicated DNAs and also single strandedness of the replicons (Sogo et al. 2002). Therefore, we decided to test the following branched DNA structures for binding as a potential model system for studying Claspin-replicating DNA interactions: "Y structure", "3'-Flap", "Replication Fork", and "Holliday Junction" (**Figure 2.5,** top panel**)**. Claspin binds to all of these structures with higher affinity than double-stranded DNA (**Figure 2.5**). To assess the relative affinity of Claspin to various DNA forms, we generated binding isotherms with gel mobility shift assays (**Figure 2.6A-G**) and K_d values were determined as the concentration at which 50% of the DNA is bound by Claspin (**Figure 2.6**). We find that Claspin binds to all branched DNAs with 10-20 fold higher affinity than double-stranded DNA, and with even much higher

affinity than single-stranded DNA (**Figure 2.6G**). These findings suggest that when the replication origin is unwound, Claspin binds directly to the branched DNA at the origin without the intermediacy of another protein. Furthermore, this high affinity is not due to the single-stranded DNA per se that is associated with replicating DNA.

Structure of Claspin-DNA Complexes. Our data combined with previous data on Xclaspin (Lee et al. 2003), suggest that Claspin may specifically bind to the regions of replication forks not covered by other replication and checkpoint proteins (Lee et al. 2003) and may even travel with the replication fork as a replication sensor protein even though it is not essential for replication (Lee et al. 2003). Hence, we prepared a 3.2 kb linear double-stranded DNA template containing a 400 nt single strand tail or a circular double strand template containing a 400 bp double-strand tail as model substrates for structures associated with leading and lagging strand synthesis (**Figure 2.7A, B**). We incubated these substrates with Claspin, and then the samples were prepared for electron microscopy (EM) as described elsewhere (Griffith and Christiansen 1978).

To verify the position of the single-stranded tail, DNA templates (**Figure 2.7A**) containing a single-stranded tail at an asymmetric location with respect to the termini of the linear duplex were first treated with *E.coli* single strand DNA binding protein (SSB). The results show that 95% of the molecules (N=30) contained a single strand tail that had at least 3-5 SSB particles bound (19), indicating a tail length of approximately 400 nucleotides (**Figure 2.8A**). Additionally, the shorter double-stranded arm was measured and found to be 18.6±1% of the total DNA length, which matches the expected position of single-stranded tail.

When Claspin was incubated with these templates and the products examined by EM, 15±2.3% (N=66) of the replication fork templates were found to be bound by Claspin (**Figure 2.8B, C**). Claspin appears slightly oblong, and had a size consistent with a protein particle of ~150-200 kDa mass, based on previous visualization of other proteins by these EM methods (Makhov et al. 1996). The location of the protein was determined by measuring the short arm and found to be 19.9±0.5% of the full duplex length, which closely corresponds with the position of the single-stranded tail as determined by SSB binding, indicating that Claspin binds at the junction of the single-stranded tail with the double-stranded linear DNA. Unlike SSB, where multiple protein molecules were seen along the tail, only one particle of Claspin was seen per DNA molecule. This suggests that Claspin binding is most likely at the junction as a monomer, and not along the single-stranded tail. Frequently (**Figure 2.8B, C, insets**), Claspin exhibits a ring-like structure that may be circling the DNA. Preliminary examination of the protein alone using negative staining provided additional images of ring-like particles, and will be described later.

The junction-specific binding was confirmed by performing binding experiments with a template containing a double-stranded tail. Circular templates containing an internal double-stranded tail (**Figure 2.7B**) which could be considered a model for replication forks undergoing leading and lagging strand synthesis, were mixed with Claspin, examined by EM and scored for protein binding at the junction of double-stranded tail with the circle, along the tail or on the double-stranded circle. The results show that 38% (N=156) of the double-stranded templates were bound by Claspin. Of the bound molecules, 53% were bound at the junction (**Figure 2.8D-F**), 21% were bound along the tail and 26% bound somewhere on the circle other than the junction point. The overall fraction of molecules bound to the junction

point was lower with this template than the single-stranded tail template, consistent with the *in vivo* data suggesting Claspin may prefer single-stranded fork structures that are not covered by other proteins such as RPA (Lee et al. 2003). In the double-stranded tailed templates, there is a 25 nt gap at the base of the junction. However this may not provide a sufficient single-stranded region for efficient Claspin recognition. Regardless of the details of the requirement for high specificity binding, clearly, Claspin binds to branched DNA structures preferentially. Collectively, these data indicate that Claspin is a checkpoint sensor that binds directly and with high affinity to unwound DNA at replication origins and replication forks.

DISCUSSION

Here we describe the purification and characterization of human Claspin. Our main findings are that Claspin appears to be a ring-shaped protein with high affinity for branched DNA structure and low affinity to single- or double-stranded DNAs. The low affinity of Claspin to single- and double-stranded DNAs relative to branched DNA structures may explain why a previous study concluded that Claspin had no intrinsic DNA binding activity ((Lee et al. 2003). In light of our finding of high affinity for branched DNAs and of the report demonstrating that Claspin associates with replicating chromatin in *Xenopus* egg extracts (Lee et al. 2003), we conclude that Claspin is not a mediator, but actually is a sensor in the replication checkpoint.

Currently, a key question in the field of DNA damage and replication checkpoint response is the mechanisms by which the DNA damage or replication intermediates are detected. Genetic and biochemical analyses, including the data in this chapter have implicated three groups of proteins in sensing DNA damage or replication intermediates (1-3): (1) the phoshoinositide kinase like kinase (PIKK) family members ATM and ATR, (2) the clamp loader/DNA clamp family members Rad17-RFC/9-1-1 complex, and (3) Claspin. Claspin was first thought to function as a mediator or an adaptor in the ATR-Chk1 signaling pathway (Chini and Chen 2003; Kumagai and Dunphy 2000; Lee et al. 2003) in a manner similar to the two known mediators in yeast, scRad9 and Mrc1 proteins (Alcasabas et al. 2001; Tanaka and Russell 2001). However, recent work clearly demonstrated that Claspin is one of the first checkpoint proteins to associate with replicating chromatin (Lee et al. 2003) and hence has the potential to function as a replication fork sensor.

In the checkpoint response, the nature of DNA structures recognized by checkpoint sensors is of particular interest. It has been suggested that the ATR/ATRIP heterodimer binds to RPA-covered single stranded DNA (Zou and Elledge 2003). Similarly, it was reported that Rad17-RFC loads the 9-1-1 complex onto RPA-covered template/primer-like structures (Ellison and Stillman 2003; Zou et al. 2003). Finally, it has been found that association of Claspin with replicating sperm chromatin in *Xenopus* egg extracts was independent of RPA, but dependent on the Cdc45 pre-RC protein, which raised the possibility that Claspin may be recruited to replicating DNA through its interaction with Cdc45 (Lee et al. 2003). These findings would suggest that checkpoint sensors do not actually recognize specific DNA lesions or structures but instead recognize specific nucleoprotein complexes that form at such sites. However, there are other findings that either is not consistent with these data or the specific model alluded to above. First, ATR, with and without ATRIP, binds with moderate affinity to single- and double-stranded DNA and with a somewhat higher affinity to a UV lesion in DNA (Unsal-Kacmaz et al. 2002; Unsal-Kacmaz and Sancar 2004). Under the reaction conditions used in those studies, RPA had only a minor effect on binding of ATR and the ATR/ATRIP complex to DNA. Second, it has been reported that Rad17-RFC can load the 9-1-1 complex onto template/primer structures in the absence of RPA (Bermudez et al. 2003) and that the loading of the yeast equivalent of the 9-1-1 complex by the yeast Rad17-RFC counterpart onto a model primer/template is not stimulated, but actually inhibited, by RPA (Majka and Burgers 2003). Finally, in this study we show that Claspin binds with high affinity and specificity to unwound DNA–like structures in the absence of Cdc45 or any other protein.

These apparently contradictory results may be reconciled by assuming that Cdc45 and RPA act as facilitators and stabilizers of the checkpoint protein-DNA complexes rather than recruiters of checkpoint proteins. In fact, RPA is known to function as a facilitator and stabilizer in transcription and DNA repair by either facilitating protein-protein interactions or removing secondary structures to stabilize specific complexes. In the light of our results, the requirement for Cdc45 for Claspin binding is most likely due to its indispensable role in firing of replication origins and formation of a replication fork rather than its recruitment of Claspin by a protein-protein interaction. With these assumptions, we propose a revised version of the three-tiered checkpoint activation model (Lee et al. 2003) (**Figure 2.9**): Cdc45 and other pre-RC proteins unwind DNA at replication origins. This unwound DNA attracts Claspin, Polα and RPA. Primer synthesis by Polα creates a substrate for Rad17-RFC, and the naked DNA in the replication bubble becomes a binding site for ATR/ATRIP. Thus, the three sensors (Claspin, ATR, Rad17-RFC) are recruited to a replication fork independent of one another, and the 9-1-1 complex is loaded onto DNA by the Rad17-RFC complex. This complex, as well as the ATR/ATRIP complex, at the replication fork may be stabilized by RPA.

Eventually, the interactions between the three sensors and possibly phosphorylation of Rad17 (Bao et al. 2001) and possibly Claspin by ATR or ATR-dependent kinase (Kumagai and Dunphy 2003) promote interactions with mediators such as BRCA1 and signal transducers such as Chk1, resulting in basal replication checkpoint response. Replication block by hydroxyurea or DNA damage increases the number of partially replicated replicons and the level of single strandedness in these replicons (Sogo et al. 2002) and causes more

extensive binding of checkpoint sensors (Lee et al. 2003) and amplification of the checkpoint signal.

An unexpected finding of our study was the ring-shaped structure of Claspin. Two ring-like structures are known to be associated with the replication fork: the MCM helicase, that unwinds the helix in front of a replication fork, and PCNA that functions as a polymerase clamp. Our data indicate that Claspin is the third ring-like molecule associated with the replication fork. In some of the electron micrographs, the DNA seems to be threading through the hole in the Claspin ring. However, further biochemical work is required to formally prove that DNA threads through Claspin. It should be noted that in the case of MCM and PCNA, ATP hydrolysis by MCM2-7 and RFC respectively, is required for opening the corresponding rings and clamping them onto DNA. Similarly, the checkpoint dedicated PCNA ortholog, the 9-1-1 complex, is clamped onto DNA by the checkpoint counterpart of the RFC complex, Rad17-RFC in an ATP-dependent manner (Bermudez et al. 2003; Ellison and Stillman 2003; Majka and Burgers 2003). Claspin is not an ATPase and it does not depend on another protein to slide onto DNA. It is possible that Claspin binds to DNA ends non-specifically, and slides along DNA by diffusion, and when it encounters a branched DNA, binds at the site with high affinity. Preliminary analyses of EM data as well as velocity sedimentation analysis suggest that Claspin is a monomeric protein (data not shown). However, further work is required to determine the quaternary structure of Claspin on and off DNA.

In conclusion, our data, in combination with recent data on Claspin association with replicating chromatin in *Xenopus* (Lee et al. 2003) and its apparent requirement for cell viability in human cells (Chini and Chen 2003; Lin et al. 2004), suggest that Claspin is

loaded at the replication origin onto unwound DNA and travels with the replication fork as a part of replication monitoring machinery for maintaining the checkpoint response. When replication is blocked, the accumulation of replication/damage sensors, ATR/ATRIP, Rad17-RFC complex, Claspin, and RPA at the blockage site initiate a checkpoint signaling cascade that arrests cell cycle progression.

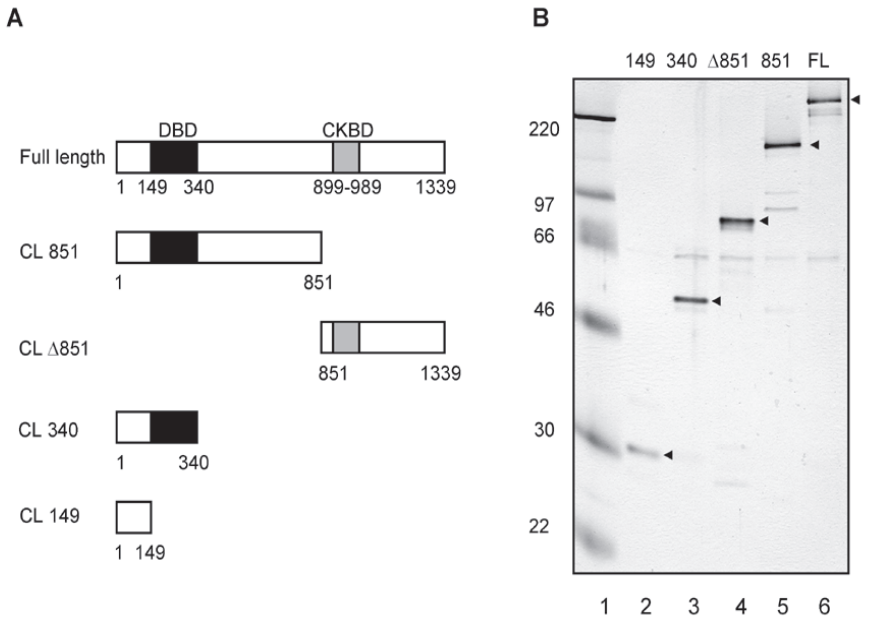


Figure 2.1: Purification of Claspin and Claspin fragments. (A) Schematic representation of Claspin and the Claspin fragments that were expressed in the baculovirus/insect cell system. The numbers indicate the position of amino acids in the respective constructs. DBD, DNA binding domain; CKBD, Chk1 binding domain identified by Kumagai and Dunphy (2003). **(B)** Analysis of purified proteins by SDS-PAGE followed by Silver staining. The notations on top indicate the identity of the fragments as defined in Panel A. The arrowheads point to Claspin and Claspin fragments. Each lane contains approximately 0.1 μg of protein.

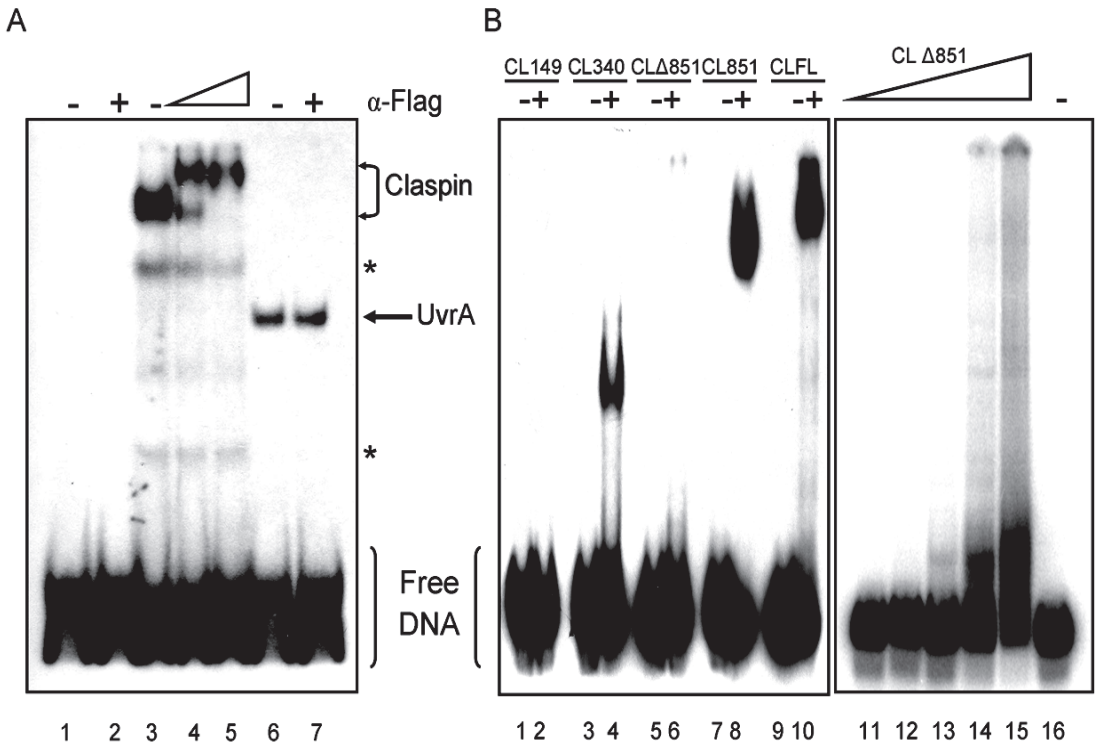


Figure 2.2: Binding of Claspin to DNA. (A) Direct binding to double-stranded DNA. A terminally labeled 30 bp duplex (0.1 nM) was incubated with Flag-Claspin (100 nM) or UvrA (2 nM) as indicated, and the DNA-protein complexes were separated on a 5% polyacrylamide gel. Anti-Flag (α-flag) antibodies were added to samples in lanes 2, 4, 5 and 7 before loading onto the gel. Lanes 2, 4, 7 contained 90 ng and lane 5 contained 180 ng of antibodies. Note that the antibodies supershift the Flag-Claspin-DNA band (lanes 4-5), but not DNA alone (lane 2) or the UvrA-DNA band (lane 7). Asterisks denote non-specific bands. **(B)** Identification of DNA binding domains of Claspin. Claspin or the Claspin fragments at 50-65 nM (lanes 2, 4, 6, 8, and 10) were incubated with the 3' Flap DNA structure (0.25 nM). The DNA-protein complexes were separated on a 5% polyacrylamide gel. In lanes 11-15, the 3' Flap structure (0.25 nM) was incubated with 10 nM (lane 11), 21 nM (lane 12), 52.5 nM (lane 13), 105 nM (lane 14), 210 nM (lane 15) of CL851 and separated on a 5% polyacrylamide gel.

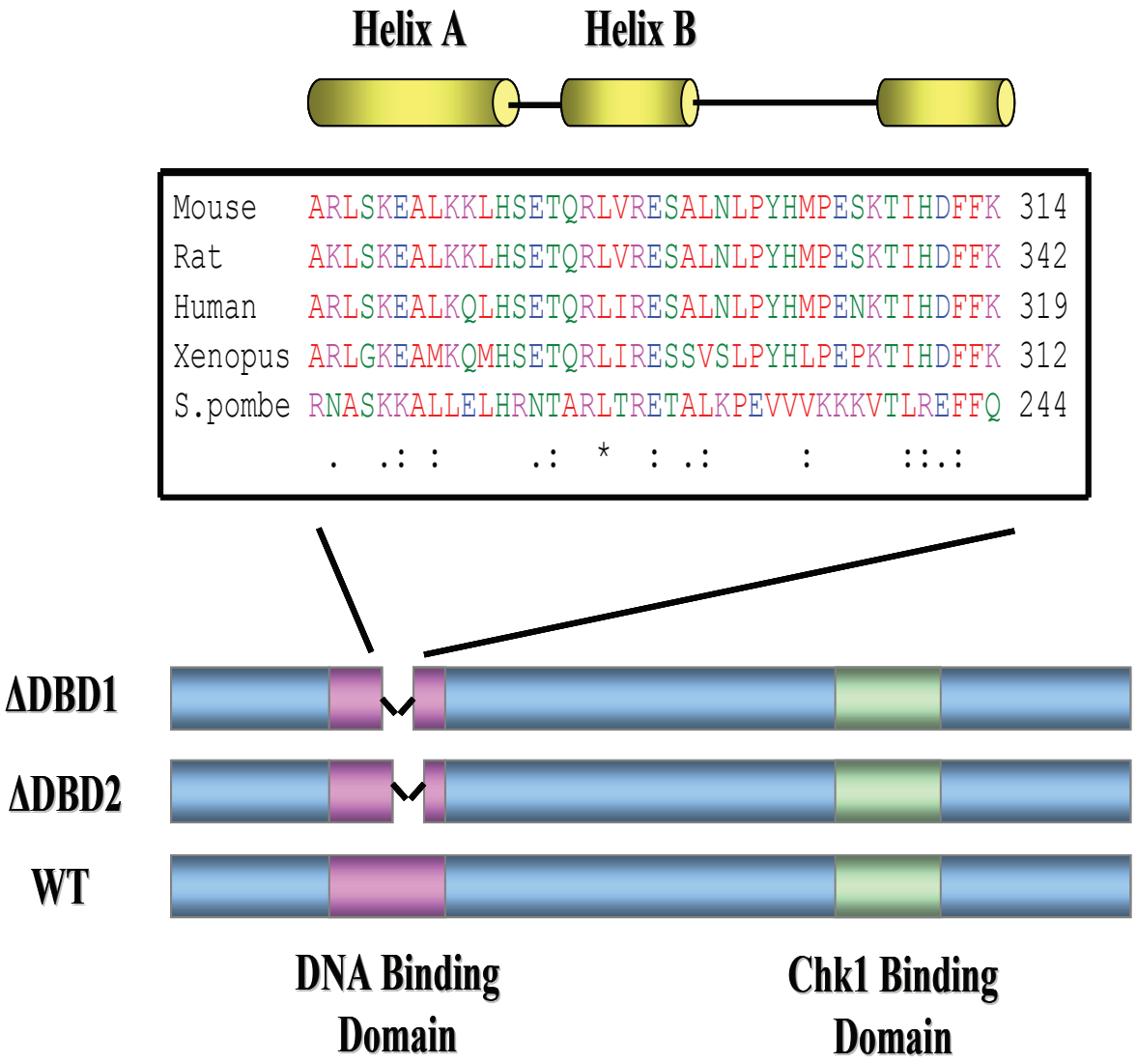


Figure 2.3: Alignment of DNA binding domain of Claspin from various organisms. (A) Multiple sequence alignment of DNA binding domain of Claspin from different organisms (mouse, rat, *Xenopus*, human and *S. pombe* Mrc1). Positions of predicted helix-loop-helix are indicated by the cartoons. **(B)** Schematic representation of mutant and wild type Claspin.

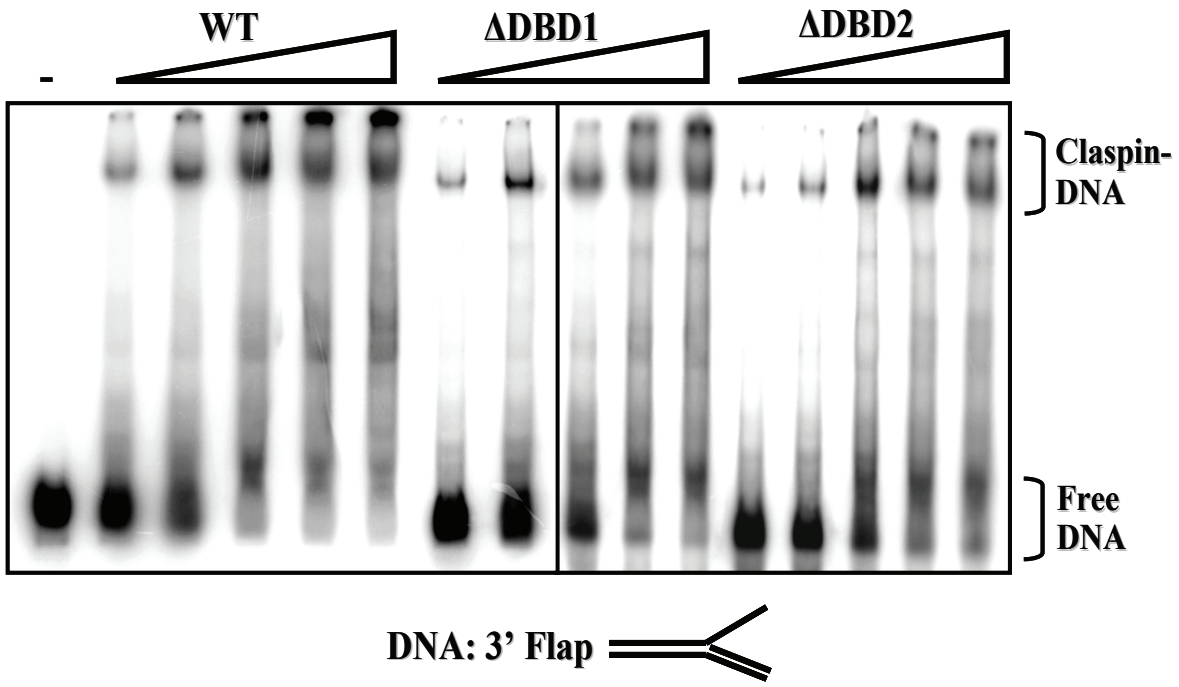


Figure 2.4: Analysis of DNA binding domain of Claspin. 0.75 nM of 3'flap structure was incubated with increasing concentrations of wild type and mutant Claspin (5.3 nM-232 nM) and separated on 5% polyacrylamide gels.

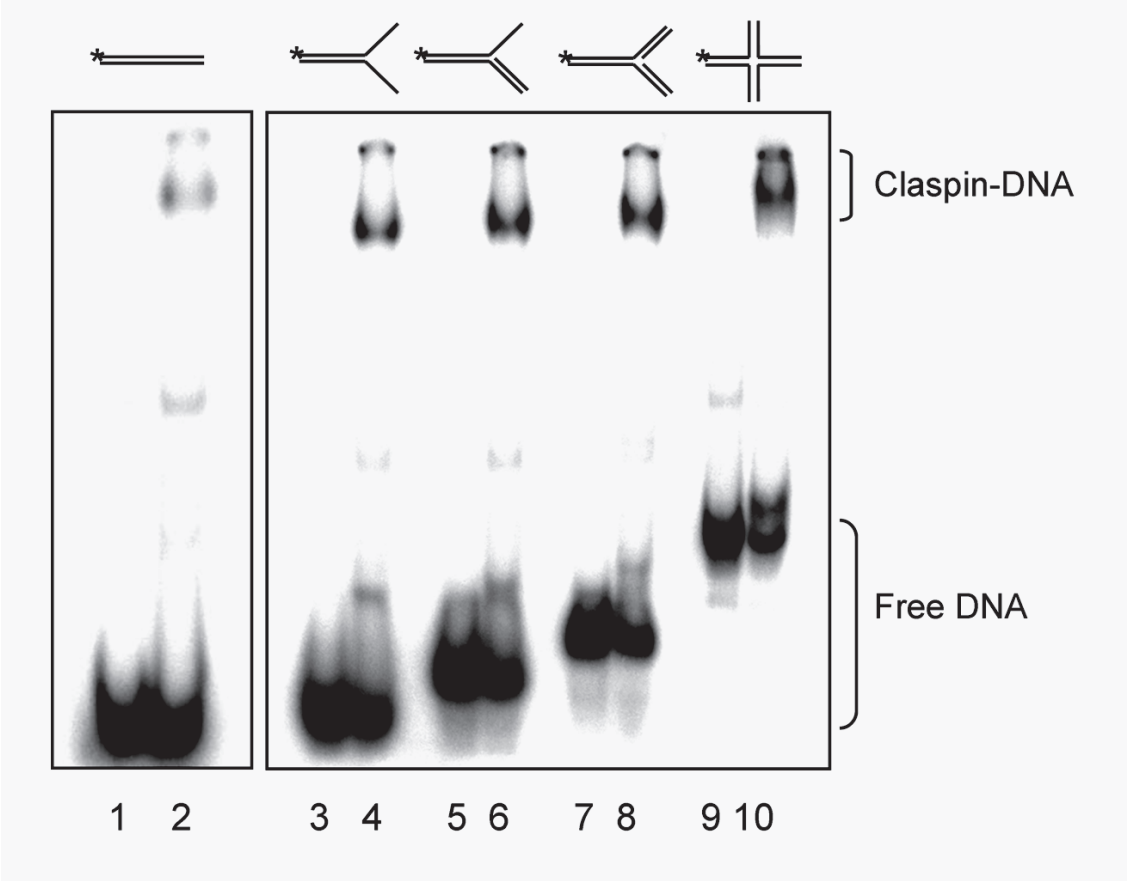
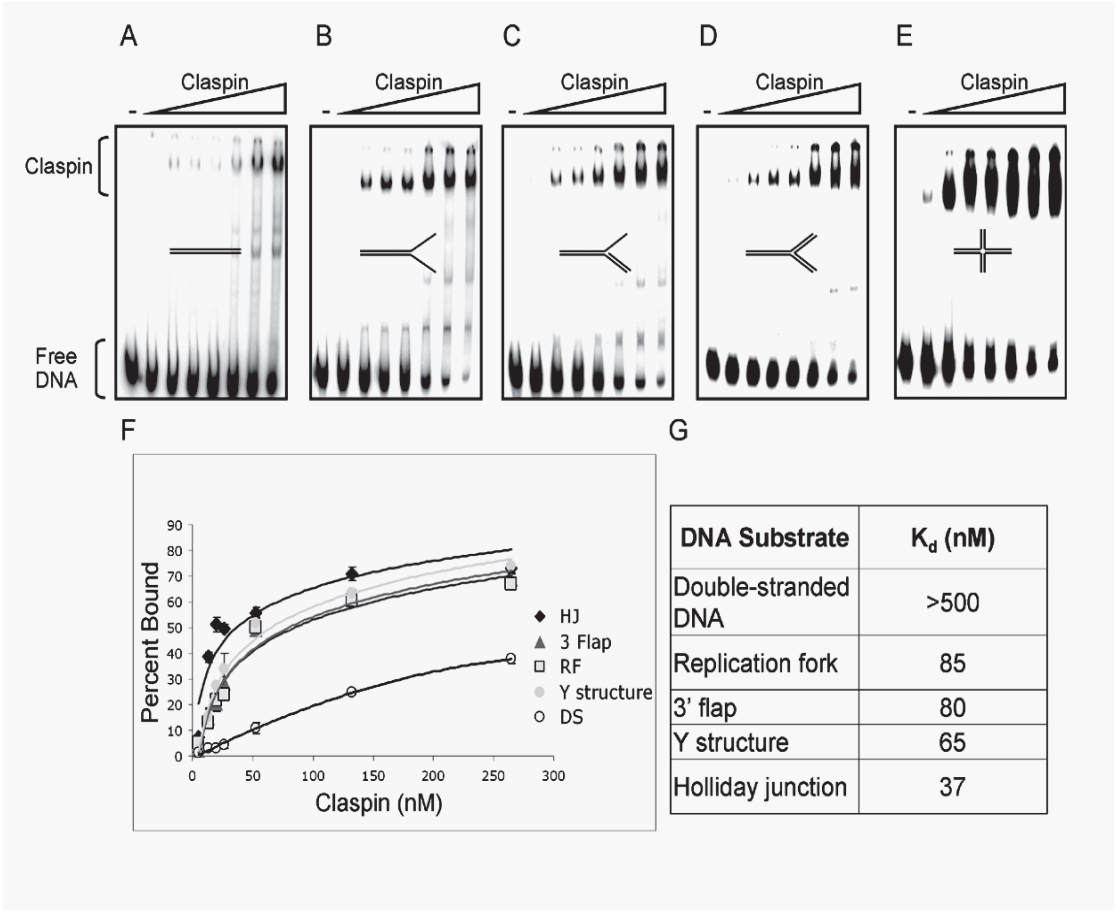


Figure 2.5: Preferential Binding of Claspin to Branched DNA structures. 0.25 nM of indicated DNA structures were incubated with 26.5 nM of Claspin and separated on 5% polyacrylamide gels. Because the double-stranded DNA migrates much faster than branched DNA, the Claspin-double-stranded DNA complex was resolved on a separate gel. Asterisks indicate the position of the radioactive label.



DNA Substrate	K_d (nM)
Double-stranded DNA	>500
Replication fork	85
3' flap	80
Y structure	65
Holliday junction	37

Figure 2.6: Claspin-DNA Binding Isotherms with Various DNA Forms. (A-F) The indicated DNAs at 0.25 nM were incubated with increasing concentrations of Claspin (5.3 nM-232 nM) and analyzed by gel mobility shift assays. Representative gels are shown for each of the DNAs. **(G)** Binding isotherms were obtained from two gel mobility shift assays, including the ones shown in Panel A-F. The error bars are the standard error values for two experiments.

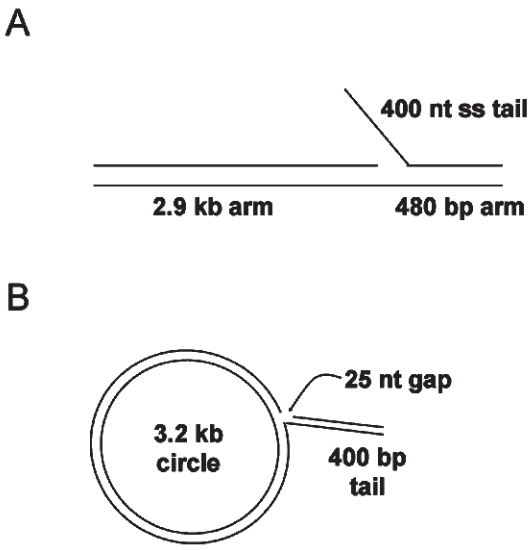


Figure 2.7: Substrates for Electron Microscopy. (A) The substrates were generated by nicking the pGLGAP12 plasmid with N.BbvcIA enzyme and strand displacement and restriction enzyme digestion to generate arms of the indicated sizes or **(B)** strand displacement followed by primer extension to generate a circle with double-stranded tail.

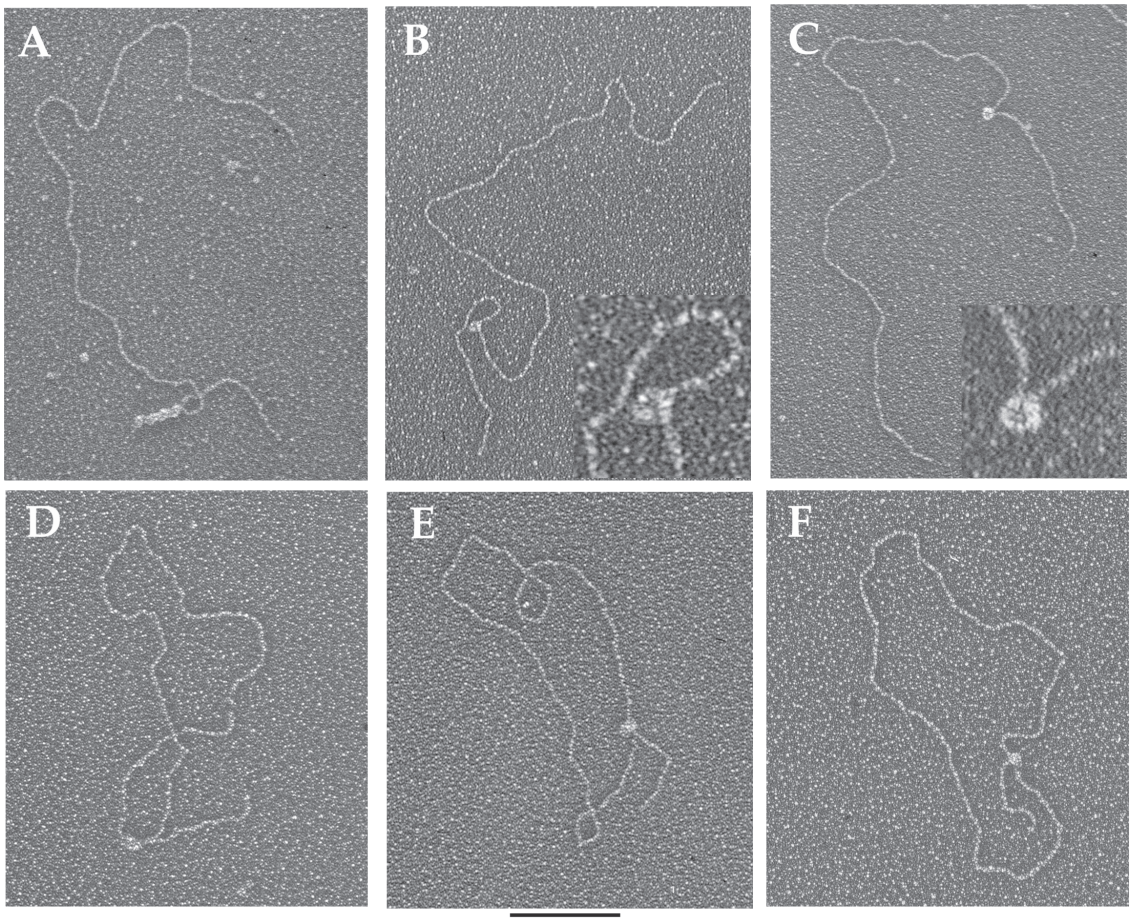


Figure 2.8: Claspin binds to the junction of single- or double-stranded tails on duplex DNA templates. (A) Visualization of E.coli SSB protein to single-stranded tails of replication fork templates by electron microscopy. Samples were directly mounted onto thin carbon-coated copper grids and rotary shadowcasted with tungsten. Arrows point to the single-stranded tail coated with SSB protein. **(B-C)** Replication fork templates containing single-stranded tails were incubated with Claspin and prepared for electron microscopy. The complexes shown are representatives of the population of protein-DNA complexes observed. **(D-F)** Claspin binding to replication fork templates containing double-stranded tails. Bar represents 500 bp.

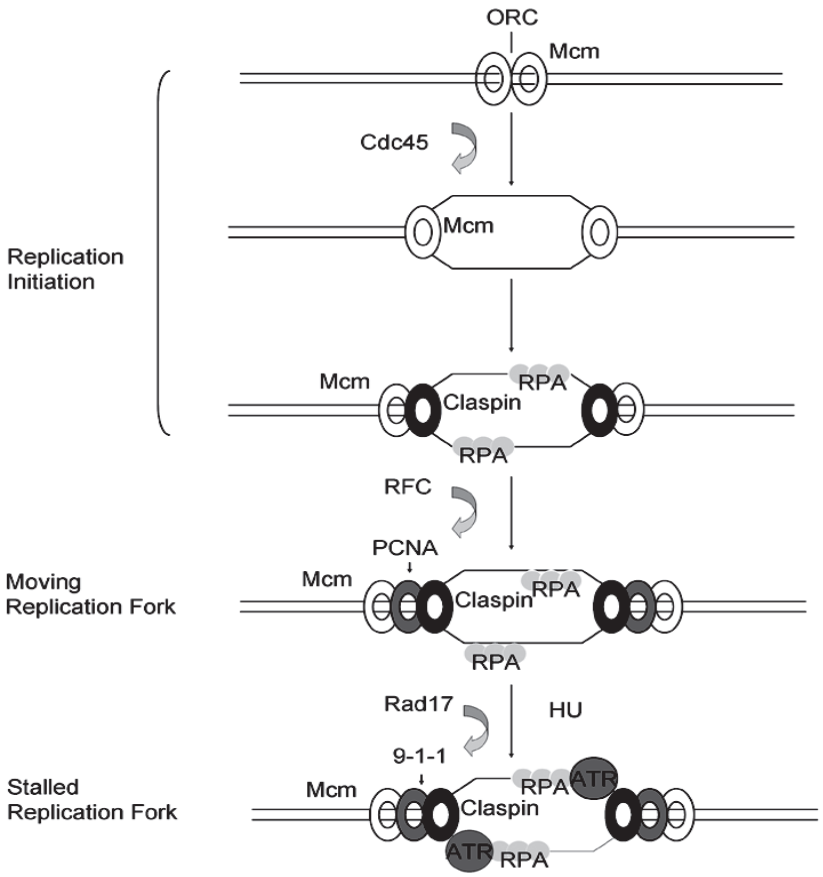


Figure 2.9: Model for Replication Checkpoint Sensors Association with DNA. The Cdc6, ORC, Cdt1 and MCM proteins open the duplex at the replication origin. MCM, which has a ring-like structure, remains associated with the replication fork. Once the two strands are separated Claspin, also binds to the fork. During elongation, a third ring-like molecule PCNA becomes associated with the fork. In addition, during replication RPA and ATR bind to replicating DNA. Stalling of the replication fork by HU or DNA damage leads to loading of the 9-1-1 ring onto DNA by Rad17-RFC. For clarity the polymerases involved in initiation and elongation are not shown.

CHAPTER III

THE ROLE OF CLASPIN IN CHK1 ACTIVATION

ABSTRACT

Chk1 is an essential component of ATR signaling pathway. Its activity is tightly regulated by several mechanisms. The requirements of mediator proteins for Chk1 activation adds an additional level to complex network events, leading to checkpoint activation. Exact mechanism by which mediators regulate Chk1 activation is not well understood. The necessity of different mediator proteins in response to different genotoxic insults, together with PIKK dependent site-specific phosphorylations of the mediators provide specificity to checkpoint activation. Claspin is required for Chk1 phosphorylation and activation in response DNA damage and replication stress. Similar to ATR, both Claspin and Chk1 associate with chromatin. Claspin and ATR has been shown to have DNA binding activities, therefore it was suggested that Claspin might recruit Chk1 to chromatin in close proximity to ATR, thereby facilitate its phosphorylation. To test whether Chk1 is recruited to DNA through its interaction with Claspin, gel mobility shift assays were performed. We determined that Chk1 binds to DNA independent of Claspin or any other protein. We further characterized its DNA binding properties.

INTRODUCTION

Chk1 has an essential role in normal cell cycle progression and DNA damage response (Bartek and Lukas 2003; Chen and Sanchez 2004). Human Chk1 is a 476 amino acid polypeptide, sharing 30-40% identity with its homologs in other organism (Flaggs et al. 1997; Sanchez et al. 1997). Chk1 is serine/threonine kinase. Its kinase domain lies in between amino acid residue 1 to 265 in the N terminus (Bartek and Lukas 2003; Chen and Sanchez 2004).

The activity of this key component of ATR pathway is tightly regulated by different mechanisms. Understanding the mechanisms of Chk1 regulation has some practical implications in developing anti cancer treatments. Usage of Chk1 inhibitors makes tumor cells more susceptible to anti-tumor agents due to a compromise in checkpoint activation.

First efforts to understand the regulation of Chk1 activity involved the structural analysis of human Chk1. The X-ray structure of kinase domain has been determined to 1.7 Å resolution (Chen et al. 2000). Interestingly, catalytic residues, the ATP binding site and the activation loop of Chk1 are well ordered and Chk1 kinase domain adopts an active conformation with slight misalignment of its catalytic residues. Although phosphorylation of the activation loop is necessary for the activation of many kinases, Chk1 does not seem to be regulated through phosphorylation within its kinase domain. In relation to this, autophosphorylation in the kinase domain does not change the kinase activity of the full-length Chk1(Chen et al. 2000). Furthermore, this study revealed a regulatory role for C terminal domain of Chk1 in the kinase activity. Chk1 kinase domain has 20-fold more activity that the full-length Chk1 toward its substrates, suggesting that C terminal of the

protein negatively controls the kinase activity. Other studies with human Chk1 and *Xenopus* Chk1 confirmed that C terminal of Chk1 suppresses the basal activity of Chk1 in unperturbed cells (Katsuragi and Sagata 2004; Ng et al. 2004; Oe et al. 2001). The exact mechanism is not understood well. However, the interaction between kinase domain and C terminal domain has been determined with *Xenopus* and rat Chk1 (Katsuragi and Sagata 2004; Shann and Hsu 2001). This suggested that C terminal might be acting directly on the kinase domain; however, it did not eliminate possibility of additional mechanism underlying the inhibition the basal activity by C terminus, such as steric hindrance of ATP or cofactor bindings.

The C terminal of Chk1 has several conserved SQ/TQ motifs. Two serine residues, serine 317 and serine 345 in these SQ/TQ motifs, are the main phosphorylation sites of Chk1 in response to DNA damage in humans and corresponding residues in other organisms are phosphorylated in response to genotoxic stress. DNA damage induced phosphorylation of Chk1 has been suggested to modulate Chk1 activity. Including humans, in several different organisms phosphorylated Chk1 has higher kinase activity (Katsuragi and Sagata 2004; Mailand et al. 2000). Katsugari and Sagata have shown that *Xenopus* Chk1 has an auto-inhibitory region (AIR) in the C terminus and this region interacts with the kinase domain (Katsuragi and Sagata 2004). Phosphorylation of SQ/TQ motif near the AIR disrupts this interaction, suggesting that DNA damage induced phosphorylation of Chk1 increase its kinase activity by relieving the inhibitory role of C terminal domain (Katsuragi and Sagata 2004).

Regulation of basal kinase activity of Chk1 after DNA damage constitutes only one of the mechanisms involved in activation of Chk1. It has been demonstrated that DNA damage induced phosphorylation of Chk1 changes in the protein-protein interactions. 14-3-3

proteins interact preferentially with the phosphorylated form of Chk1 in *S. pombe* and humans (Jiang et al. 2003). By redecorating the protein-protein interaction network of Chk1, phosphorylation of Chk1 leads to a change in its subcellular localization. Chk1 is found both in nucleus and in cytoplasm under normal conditions. In the presence of DNA damage phosphorylation of Chk1 prevents Crm1 mediated nuclear export of Chk1, leading the accumulation of Chk1 in the nucleus (Jiang et al. 2003).

The requirements of mediator proteins for Chk1 activation adds an additional level to complex network events, leading to checkpoint activation. In humans, BRCA1, TopBP1, Claspin and Timeless have been shown to be essential for optimal Chk1 phosphorylation and activation. All the mediator proteins get phosphorylated in response to DNA damage by an ATR-dependent mechanism. The necessity of different mediator proteins in response to different genotoxic insults, together with PIKK dependent site-specific phosphorylation of the mediators provides specificity to checkpoint activation.

Exact mechanism by which mediator regulate Chk1 activation is not well understood. It has been suggested that mediator proteins may act as a scaffold to bring ATR and Chk1 in close proximity. This might take place by forming complexes between ATR, Chk1 and the mediator protein. However, such a complex between Chk1, Claspin and ATR has not been detected in humans or in *Xenopus*. This might be due to a transient interaction between these proteins. In addition, mediators may allosterically activate the ATR kinase, either directly or by changing Chk1 conformation, thereby making it a better substrate for ATR. This has been tested in *Xenopus* and an effect of *Xenopus* Claspin on ATR/ATRIP complex kinase activity has not been detected (Jeong et al. 2003). Although Claspin do not stimulate ATR/ATRIP complex activity, Claspin may allosterically regulate Chk1 kinase activity, as supported by

the fact that Claspin stimulates Chk1 autophosphorylation activity *in vitro* (Jeong et al. 2003). In contrast to Claspin, *Xenopus* TopBP1 has been shown to stimulate ATR's kinase activity *in vitro* (Bartek and Mailand 2006). This finding showed that mediator proteins may utilize different mechanism(s) to provide Chk1 activation.

Like ATR and Claspin, Chk1 associate with chromatin during cell cycle. Chk1 gets phosphorylated near to damage sites and phosphorylated form of Chk1 dissociates from chromatin, this dissociation is required for checkpoint response, probably to provide proper phosphorylation of effector proteins (Smits et al. 2006). Another study confirmed that Chk1 associates with chromatin, however, this study contradicted with the findings that showed checkpoint mediated dissociation of Chk1 from chromatin (Jiang et al. 2003). Nonetheless, both of the groups failed to detect DNA binding activity of Chk1 (Jiang et al. 2003; Smits et al. 2006). Recently, studies in our laboratory showed that both ATR and Claspin have DNA binding activities. ATR binds to single- and double-stranded DNA, it has higher affinity towards UV damaged substrates, Claspin possesses higher affinity towards branched DNA structures than single- or double-stranded DNA. Both ATR and Claspin are essential for Chk1 activation in response to DNA damage and replication stress. Therefore, it has been suggested that one of the mechanism that Claspin employs in the activation of Chk1 might involve the recruitment of Chk1 to chromatin in close proximity to ATR, thereby facilitating the Chk1 phosphorylation. To test whether Chk1 is recruited to chromatin through its interaction with Claspin, gel mobility shift assays were performed. As we determined Chk1 is a DNA binding protein, we further characterized its DNA binding properties.

MATERIALS AND METHODS

Expression and purification of recombinant proteins. We exclusively used Claspin, Chk1, Chk2 and GST made in the baculovirus/insect cell system for our studies. Baculoviruses were generated using the Bac-to-Bac system (Invitrogen). Monolayer High Five cells (Invitrogen) were infected with the appropriate baculovirus and then harvested after 48 hrs. The cells were washed with 1X PBS and then lysed in 20X packed volumes of lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40 (NP-40), and protease inhibitors (Roche Molecular Biochemicals). After incubation on ice for 30 min, the cell lysate was centrifuged for 20 min at 32,000g. The supernatant was incubated with Glutathione-Sepharose 4B resin (Amersham) for 30 min at 4˚C. The resin was washed once with 10 volumes of lysis buffer, once with buffer containing 10 mM HEPES, pH 7.9, 0.5 M NaCl, once with elution buffer (10 mM HEPES, pH 7.9, 50 mM NaCl, 10% glycerol), and then eluted with two volumes of elution buffer containing 10 mM glutathione, pH 8.0 for 20 minutes at room temperature. Concentrations of the proteins were determined by Bradford assay. The proteins were stored at -80˚C.

Preparation of DNA substrates. DNA substrates were prepared as described in materials and method section in Chapter II.

Gel mobility shift assay. Claspin, Chk1, Chk2 or GST proteins at the indicated concentrations were incubated with 0.75 nM DNA substrates for 30 minutes at 30˚C in 25 µl

reaction mixture containing 10 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM DTT, and 0.04 mg/ml BSA. Glycerol was added to 5%, and the DNA-protein complexes were separated on a 5% polyacrylamide gel in 0.5X TBE. Electrophoresis was performed at 0.25mA for 120 minutes at room temperature. The gels were dried and exposed to a PhoshoImager screen (Molecular dynamics), and the data was analyzed by ImageQuant software (version 5.0, Molecular dynamics). Bound DNA fraction is determined by subtracting the free DNA from the total DNA in the lane. Percentage of bound DNA was calculated and plotted against Chk1 concentration to generate binding isotherms for Chk1 binding to various substrates.

In vitro kinase assay combined with gel mobility shift assay. GST-Chk1-WT or GST-Chk1-KD was incubated in kinase reaction buffer (20 mM Tris pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT) in the presence of 25 μM cold ATP and 10 μCi [(α-^{32}P] ATP. Into the kinase reactions DNA substrate was added to a final concentration of 0.75 nM and the mixtures were incubated at 30˚C for 30 min. The protein-DNA complexes were resolved on a 5% polyacrylamide gel in 0.5X TBE. Electrophoresis was performed at 0.25mA for 120 minutes at room temperature. The gels were dried and exposed to a PhoshoImager screen (Molecular dynamics), and the data was analyzed by ImageQuant software (version 5.0, Molecular dynamics).

RESULTS

Purification of GST, GST-Chk1 and Chk2. To obtain Chk1 at high quantities, High Five or SF21 insect cells were infected with baculoviruses encoding GST tagged human Chk1. GST-Chk1 has been purified from both sources by affinity chromatography. In addition, GST protein and GST tagged Chk2 proteins were purified by affinity chromatography from insect cells in order to use as negative controls in DNA binding analysis. The purified GST-Chk1 and GST-Chk2 proteins revealed a single band of expected molecular weight as analyzed by SDS-PAGE followed by silver staining analysis (**Figure 3.1**). Purified GST protein had minor contaminants (**Figure 3.1**). Purification of Claspin has been described in Chapter II (**Figure 2.1**).

Claspin does not recruit Chk1 to branched DNA structures. Previously we determined that Claspin binds to branched DNA structures that resemble the replication intermediates that can serve as signal(s) to activate replication checkpoint. Due to high affinity of Claspin to 3' flap DNA structure, DNA binding assays were performed with this structure. In order to test whether Claspin recruits Chk1 to DNA *in vitro*, Chk1 DNA binding was examined in the presence and absence of Claspin. Chk1, Claspin, or combinations of the two proteins were incubated with a ^{32}P-labeled 50-bp 3' flap DNA structure (**Figure 3.2**). In the reactions that contained both proteins, Chk1 and Claspin were pre-incubated on ice to allow them to interact before DNA binding reaction. Protein-DNA complexes were resolved by non-denaturing native gel electrophoresis. To be able to detect the effect of Claspin on

Chk1 DNA binding, concentrations of the proteins were chosen as such only 20-30 % of DNA substrate would be bound.

Surprisingly, in the absence of Claspin, Chk1 was able to bind to 3' flap structure, suggesting that it might be a DNA binding protein. As expected Claspin alone displayed a gel mobility shift. In the presence of Chk1, two distinct protein-DNA complexes were observed (**Figure 3.2**). Faster migrating protein-DNA complexes were running similar to Claspin-DNA complexes, indicating that this complex has only Claspin bound to DNA. The slower migration of the second complex suggests that this complex contains both Claspin and Chk1 proteins. However, when both of the proteins were present in the binding reaction, we could not detect Chk1-DNA complexes. This suggests that all Chk1-DNA complexes were bound by Claspin. Furthermore, pre-incubation with Claspin did not increase percent of DNA bound by Chk1. These observations prompted us to conclude that Claspin does not recruit Chk1 to DNA and Chk1 might be a DNA binding protein.

Chk1 is a DNA binding protein. During our attempts to understand the role of Claspin in Chk1 activation, we detected that binding of Chk1 to a branched DNA structure. However, two other groups failed to detect DNA binding activity of Chk1 *in vitro* (Jiang et al. 2003; Smits et al. 2006), therefore, it was necessary to show that the protein-DNA complexes, observed previously, is not due to a contaminant in the GST-Chk1 preparation. For this purpose, gel mobility shift assays were performed in the presence of antibodies against Chk1 or GST with aim of super shifting the protein-DNA complexes. Although the antibody supershift assay is an important qualitative method, the use of this assay for our studies has been hindered by the nonspecific binding of antibodies to DNA substrate and/or

to an unrelated protein (data not shown). Therefore, we decided to test the stringency of our DNA binding conditions to detect specific DNA binding and to test whether Chk1 DNA binding might be facilitated by GST tag. GST, GST-Chk1 and GST-Chk2 were purified from insect cells by affinity chromatography. Comparable amounts of all three proteins were used in gel mobility shift assay (**Figure 3.3A**). Titration of all three proteins revealed that only GST-Chk1 binds to 3' flap structure under our conditions. GST or GST-Chk2 did not bind to DNA, showing that the conditions do not allow nonspecific DNA binding and more importantly Chk1 binding is not mediated by its GST tag (**Figure 3.3A**).

Chk1 is a serine/threonine kinase and it phosphorylates itself. In order to confirm Chk1 is a DNA binding protein, we took the advantage of Chk1 autophosphorylation activity. Kinase assays were carried out before DNA binding assays to label Chk1. For this purpose, Chk1 was incubated under the conditions, that is permissive for kinase activity, autophosphorylation of Chk1 in the presence of [γ^{32}P]-ATP allowed labeling of Chk1. 3' flap DNA structure was added into the kinase mixture and gel mobility shift assay was performed (**Figure 3.3B**). In parallel, Chk1 was incubated with labeled DNA in DNA binding assay. This unlabeled Chk1-labeled DNA complex served as a positive control. Mobility of two complexes containing either labeled or unlabeled-Chk1 together with DNA complex was compared. Similar migration of these complexes on the native gel provided additional evidence to confirm that Chk1 is a DNA binding protein.

Chk1 binds preferentially to certain branched DNA structures. Initial observation of Chk1 DNA binding activity has prompted us to characterize DNA binding activity. Binding of GST-Chk1 to single- and double-stranded DNA as well as to branched DNA

structures, namely Y structure, 3' flap, 5' flap, replication fork and holiday junction was tested by gel mobility shift assays (**Figure 3.4**). Chk1 binds to Y structure and 3' flap with higher affinities compared to other DNA substrates and it forms distinct protein-DNA complexes. Chk1 binds to single-stranded DNA, 5' flap, replication fork and holiday junction without forming distinct protein-DNA complexes. Binding of Chk1 results in slower migration of DNA. However, due to rapid dissociation of Chk1 from DNA smearing of DNA substrate has been observed.

Chk1 forms distinct protein-DNA complexes only with 3' flap and Y structure. Chk1 was titrated and binding isotherms were generated (**Figure 3.5**). K_{id} values were determined as the concentration of Chk1 at which 50% of DNA is bound by Chk1 (data not shown). Chk1 binds to branched DNA structures with binding affinities in the range of 0.5 μM concentrations.

DISCUSSION

The fact that Chk1 associates with chromatin during cell cycle proved not only be the additional level to the regulation of Chk1 activation, but also provided key questions about spatio-temporal regulation of checkpoint signaling. The possibility of requirement of this supposedly proximal downstream kinase for the recognition of DNA damage brought the need of further biochemical and genetic analysis of Chk1 regulation. Several key components of ATR signaling have been detected on chromatin. The presence of DNA damage seems to regulate the dynamics of chromatin association of these components. In addition, ATR, Claspin, BRCA1 and TopBP1 are all DNA binding proteins. It has been well established that all these proteins are essential for Chk1 activation. Detecting Chk1's chromatin association has brought the question whether one of the mechanisms of mediator proteins to control Chk1 phosphorylation is to regulate its association with chromatin.

Claspin has been identified in *Xenopus* as an important regulator of Chk1 phosphorylation. It is required for checkpoint activation in response to DNA damage and replication blocks. These functions of Claspin have also been conserved in mammalian cells. The mechanism underlying the role of Claspin in Chk1 activation has not been elucidated. Previously, we showed that Claspin is a DNA binding protein with high affinity to branched DNA structures. In *Xenopus* Claspin binds to chromatin and its binding increases with the DNA damage. Interestingly, it does not require other DNA damage sensors for the chromatin association. Its independent chromatin association together with the fact that Claspin is a DNA binding protein suggested that Claspin might function as a sensor protein. However, at this point we can not eliminate the possibility of Claspin acting as a mediator protein,

therefore, its DNA binding activity might be required to recruit Chk1 to chromatin rather than directly sensing the DNA damage.

In this chapter, we tested whether Claspin recruits Chk1 to DNA. We purified Chk1 and Claspin from insect cells to obtain both proteins at high concentrations, so that we could perform DNA binding analysis. As expected we detected Claspin DNA binding activity, however, we failed to detect Claspin-dependent DNA binding of Chk1; on the contrary, we observed that Chk1 binds to DNA independent of Claspin or any other protein. More importantly, the amount of Chk1 on DNA did not change in the presence or absence of Claspin. As Claspin does not stimulate Chk1's DNA binding, we concluded that Claspin does not recruit Chk1 to DNA. Interestingly, in reactions that contained both of the proteins, we were able to detect Claspin-DNA complexes, in addition to Claspin-Chk1-DNA complexes, suggesting that Claspin might have higher affinity to DNA substrates that are already bound by Chk1. However, these experiments were performed in molar excess proteins, it would be more informative to repeat the binding assays in the molar excess DNA.

To confirm that Chk1 is a DNA binding protein, we included two negative controls to our binding assays, GST and GST-Chk2. While Chk1, neither GST nor GST-Chk2 bound to DNA. However, at high concentrations of Chk2, smearing of DNA was observed; this could be due to change in Chk2 DNA binding activity due to increase in its concentration. Combination of kinase assay with DNA binding assay provided further proof that Chk1 is indeed a DNA binding protein. Further characterization of Chk1 DNA binding revealed that it possesses higher affinity towards certain branched DNA structures. Chk1 binds to 3' flap and Y structure with high affinity. Although Chk1 binds to other substrates we tested, we could not detect distinct Chk1-DNA complexes. This is a typical behavior of proteins with

high dissociation rates. DNA binding properties of Chk1 is somewhat different than Claspin, Claspin binds to all branched DNA structures used in this study with similar affinities and has similar dissociation rates, judged by the detectable protein-DNA complexes that it forms.

Both Claspin and Chk1 are DNA binding proteins. Although Chk1 has lower affinity towards DNA compared to Claspin, independent DNA binding of Chk1 is in correlation to a recent study that investigated the role of mediator proteins in Chk1 chromatin association. RNAi mediated down-regulation of Claspin did not change the chromatin association of Chk1 in undamaged cells, Sanchez and her colleagues confirmed that Chk1 binds to chromatin independent of Claspin (Jiang et al. 2003). TopBP1 is another mediator protein required for Chk1 phosphorylation in response to DNA damage (Smits et al. 2006). Similar to Claspin, RNAi mediated down-regulation of TopBP1 or ATR expression did not change the amount of Chk1 on chromatin in unperturbed cells (Smits et al. 2006). In the light of this evidence, our findings suggest that Chk1 binds to chromatin directly. Therefore, mediator proteins are not required for Chk1 chromatin association. However, it would be interesting to examine how other components of ATR signaling cooperate to sense the DNA damage and activate Chk1.

It has been well documented that DNA damage induced phosphorylation of Chk1 increases the catalytic activity of Chk1 to both its substrates and to itself. Therefore, it is plausible to investigate the affect of these phosphorylations on DNA binding of Chk1. Jackson and his colleagues have investigated the effect Chk1 phosphorylation on chromatin binding. Treatment of the cells with UV or HU led to phosphorylation of Chk1 and rapid dissociation of Chk1 from chromatin. Phosphorylation status of Chk1 might have a direct role in regulation of DNA binding properties by changing the affinity of Chk1 to DNA or it

may have an indirect affect by changing the protein-protein interactions that might modulate the DNA binding properties. Regardless how phosphorylation-dependent dissociation is achieved, it has been shown that this rapid dissociation is required for checkpoint activation, replacing endogenous Chk1 with H2B fused immobile Chk1 can not restore checkpoint function of Chk1 (Smits et al. 2006). Although this immobile form of Chk1 gets phosphorylated, it can not activate checkpoint. Therefore, it has been suggested that in unperturbed cells Chk1 binds to chromatin, in the presence of genotoxic stress it gets phosphorylated, this phosphorylation increase its kinase activity and also releases Chk1 from chromatin, thereby it can phosphorylate downstream components of checkpoint.

Although Claspin is an essential component of ATR signaling, the exact role of Claspin is not well understood. However, it has been suggested that Claspin might function to recruit Chk1 to chromatin in close proximity to ATR, thereby facilitates activation of Chk1. However, we showed that Claspin does not recruit Chk1 to DNA, and Chk1 is a DNA binding protein with high affinity towards branched DNA structures. This provided evidence that Claspin does not simply regulate chromatin association of Chk1. In addition, Dunphy and his colleagues showed that Claspin do not stimulate ATR's kinase activity, these findings suggest that Claspin do not allosterically regulate ATR kinase activity. These findings suggest that Claspin has different roles in Chk1 activation, rather than controlling Chk1 chromatin localization and allosteric regulation of ATR kinase activity. It is still possible that Claspin-Chk1 interaction might be inducing a conformational change in Chk1 and makes it a better substrate for ATR. In addition, it might allosterically regulate Chk1 activity. Therefore, it is important to perform further biochemical analysis to understand the role of Claspin in Chk1 activation.

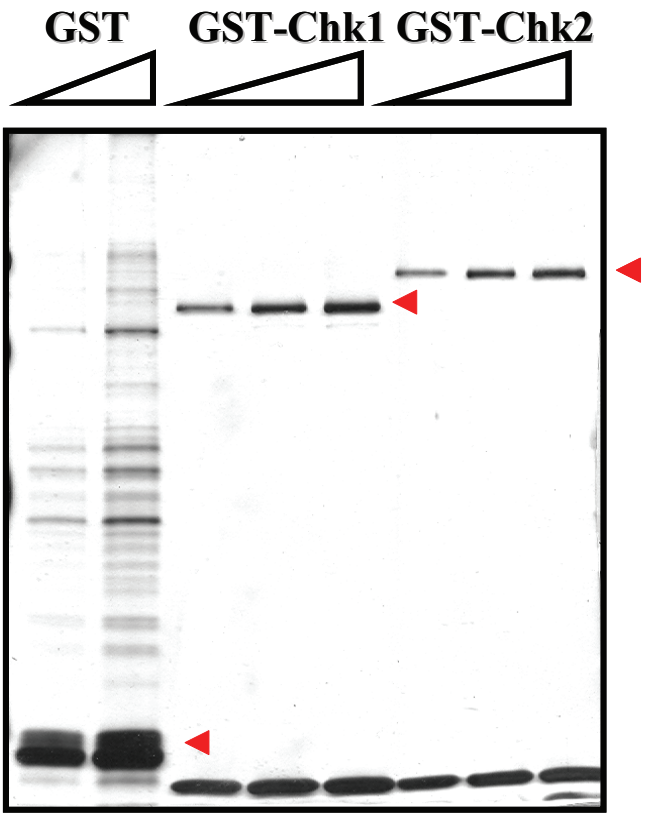


Figure 3.1: Purification of GST, GST-Chk1 and GST-Chk2. Analysis of purified proteins by SDS-PAGE followed by silver staining. The arrowheads point to each protein. GST-Chk1 and GST-Chk2 have been titrated to have 0.7 to 2.3 μg of total protein; GST was loaded to have 0.5 to 1 mg of total protein.

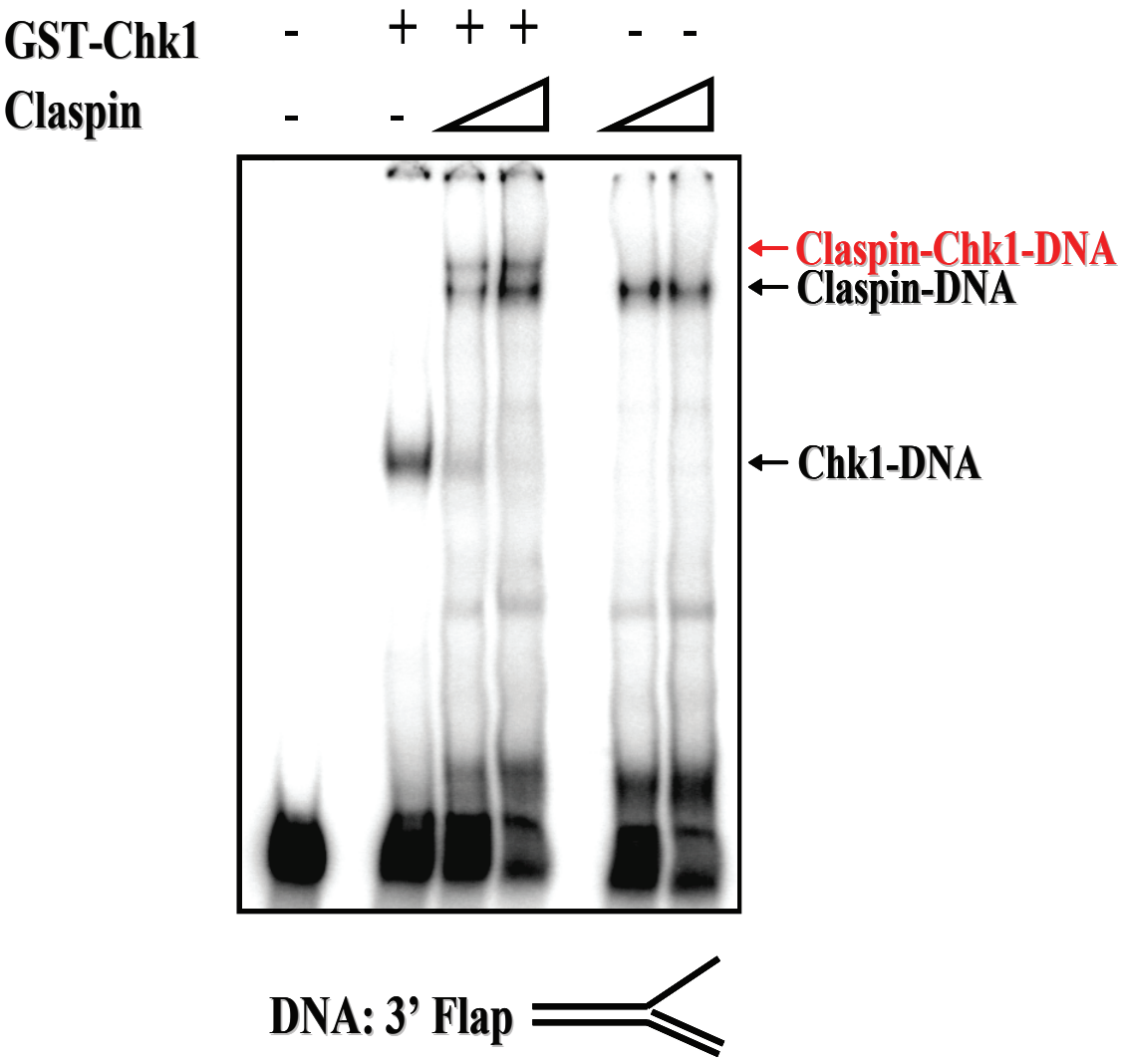


Figure 3.2: Claspin does not recruit Chk1 to DNA. A terminally labeled 3' flap structure (0.75 nM) was incubated with Flag-Claspin (13.25 and 26.5nM) in lanes 5 and 6 or GST-Chk1 (100 nM) in lane 2 or both of the proteins in lanes 3 and 4. The DNA-protein complexes were separated on a 5% polyacrylamide gel. The arrows point to Chk1-DNA, Claspin-DNA or Chk1-Claspin-DNA complexes.

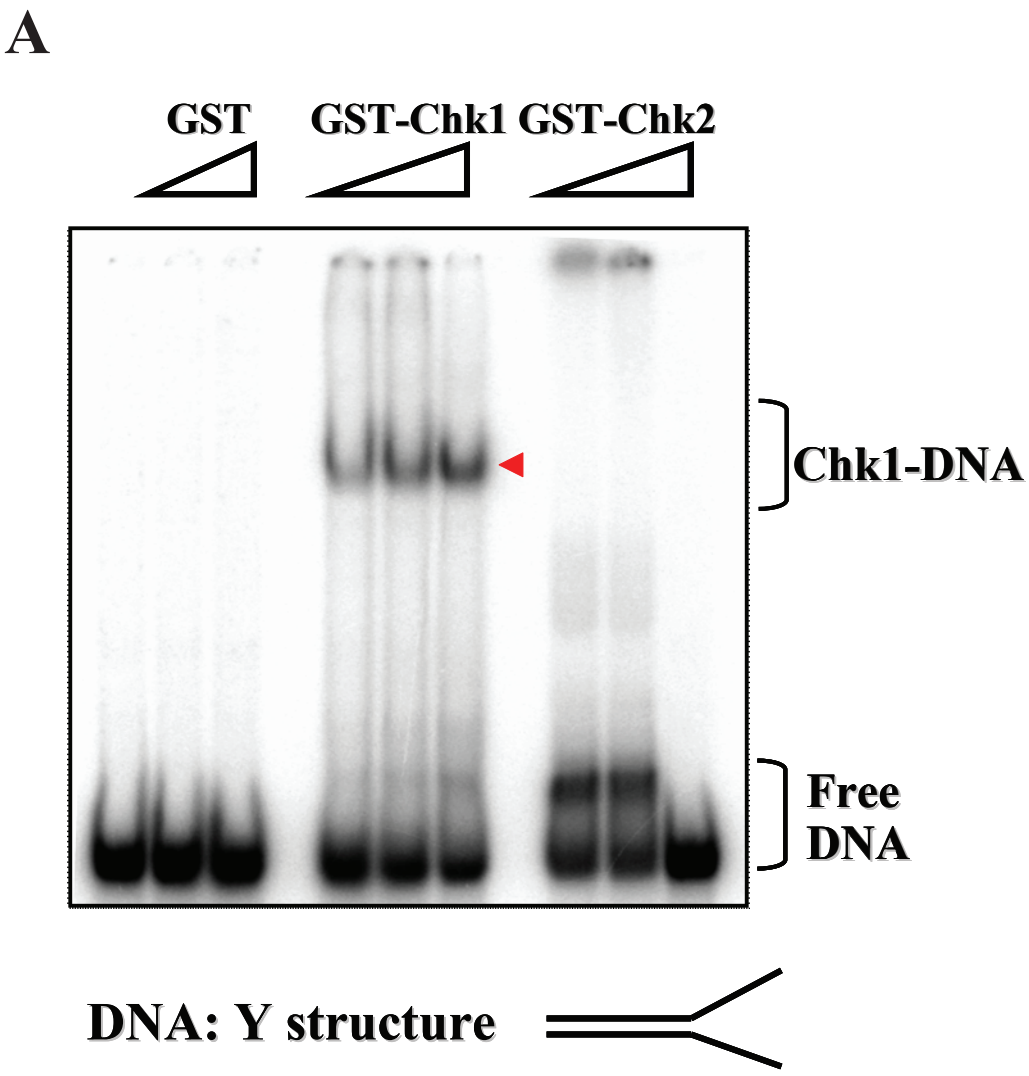


Figure 3.3: Chk1 is a DNA binding protein. (A) A terminally labeled 3' flap structure (0.75 nM) was incubated with GST, GST-Chk1or with GST-Chk2. The DNA-protein complexes were separated on a 5% polyacrylamide gel. The arrowheads show Chk1-DNA complexes.

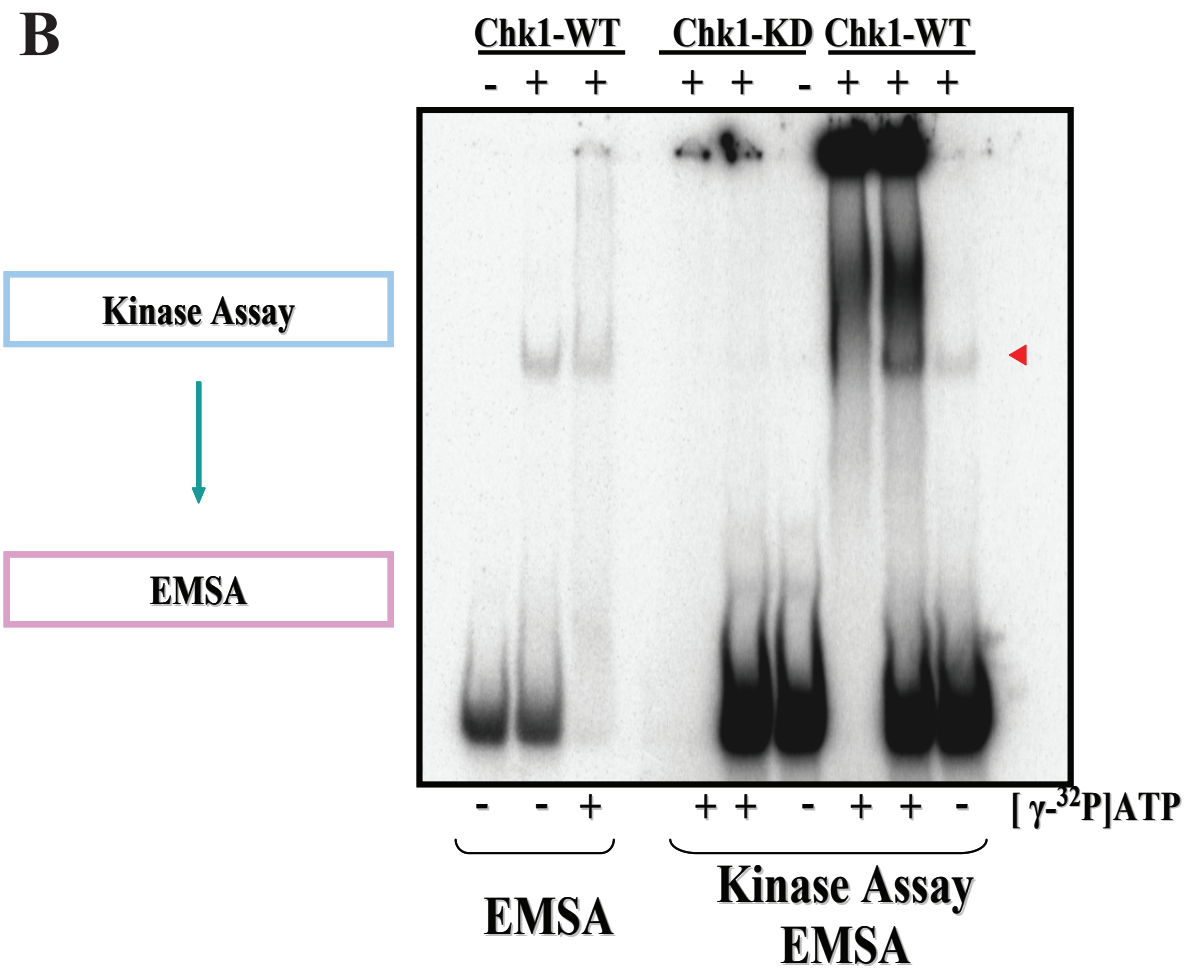


Figure 3.3: Chk1 is a DNA binding protein. (B) Chk1 was labeled by autophosphorylation in a kinase assay. This labeled Chk1 was incubated with unlabeled DNA substrate in lane 3 (in left panel indicated with "EMSA") or labeled DNA substrate in lanes 8-9. Kinase dead Chk1 was used as a negative control. In lane 2, Chk1 was incubated with labeled DNA substrate as a positive control for DNA binding. The DNA-protein complexes were separated on a 5% polyacrylamide gel. The arrowhead shows Chk1-DNA complexes.

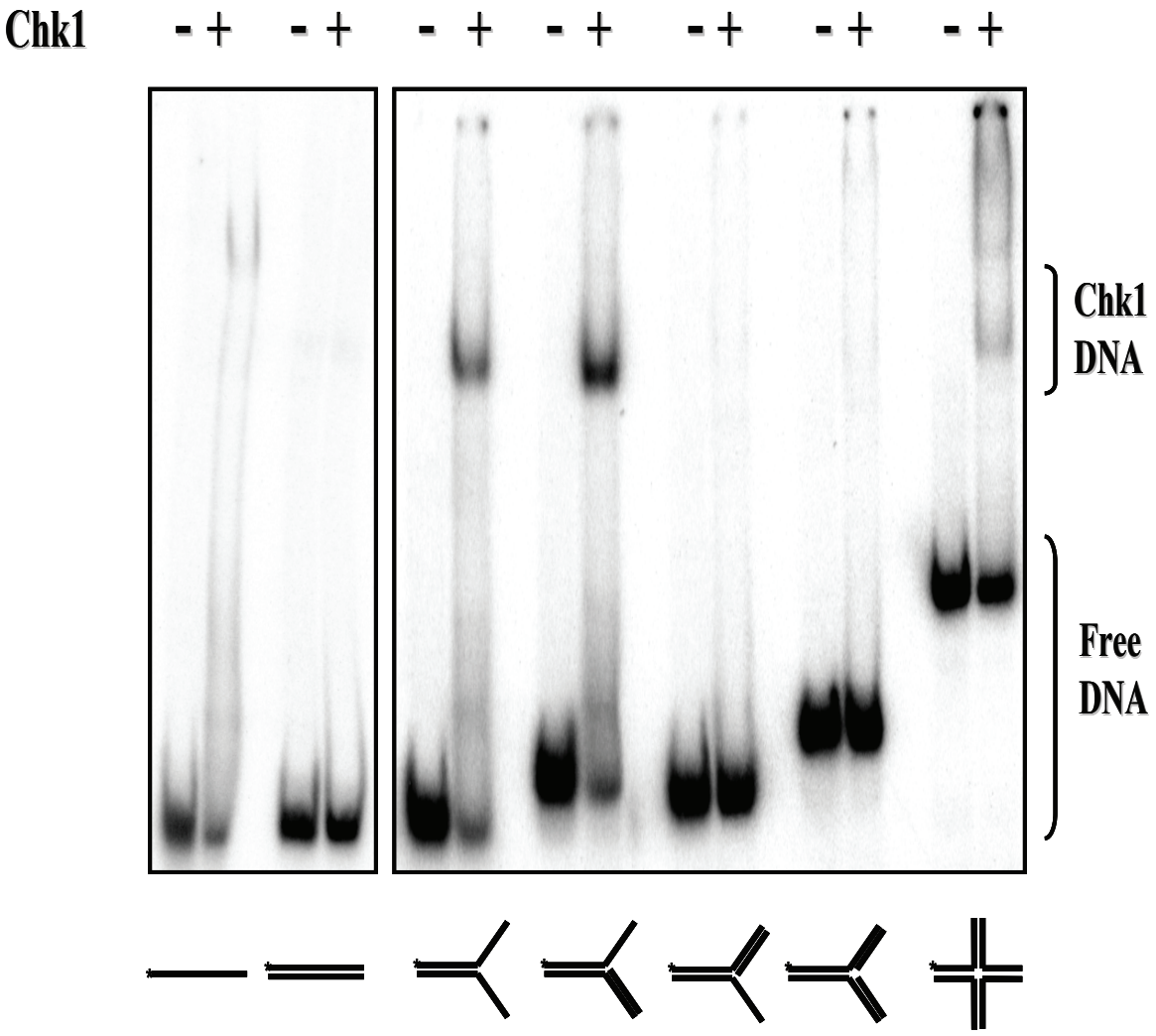


Figure 3.4: Preferential binding of Chk1 to branched DNA structures. GST-Chk1 (1.5 μM) was incubated with 0.75 nM of single-stranded DNA, double-stranded DNA, Y structure, 3' flap, 5' flap, replication fork, holiday junction. DNA substrates without protein were loaded on the same gel. Because the single- and double-stranded DNA migrate much faster than branched DNA, these reactions were resolved on a separate gel. Asterisks indicate the position of the radioactive label.

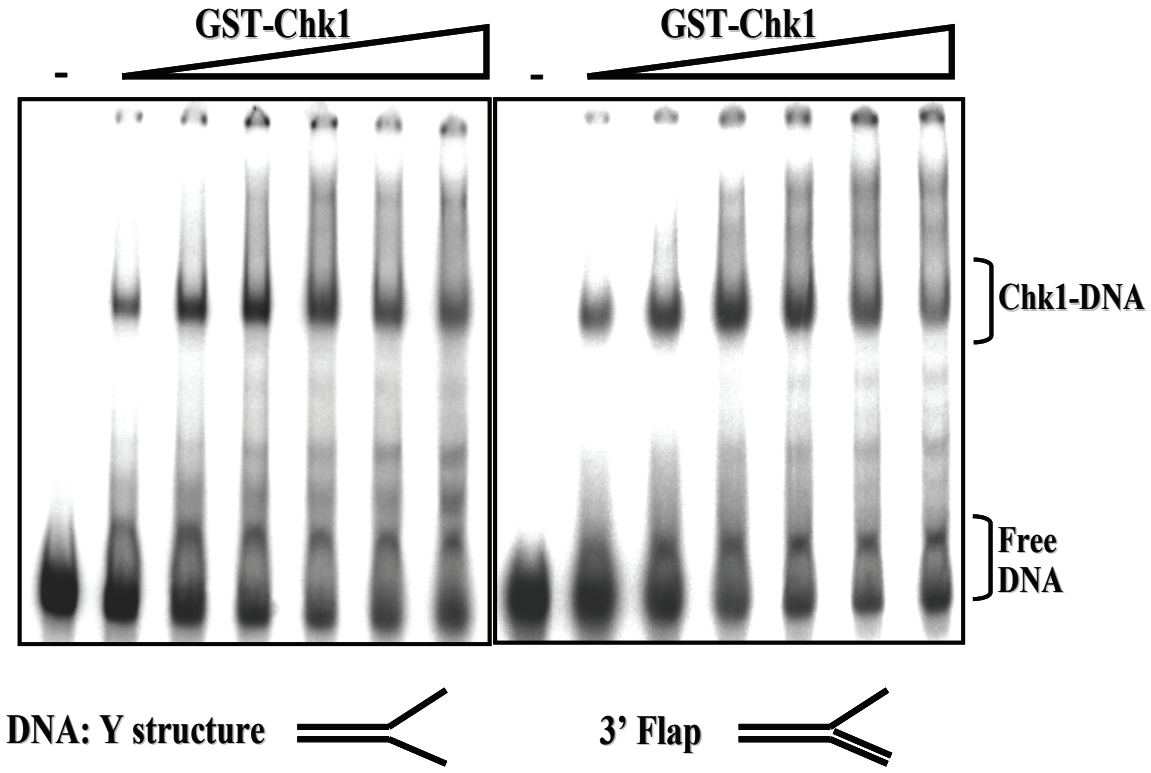


Figure 3.5: Chk1-DNA Binding Isotherms with Y and 3'flap structures. The indicated DNAs at 0.75 nM were incubated with increasing concentrations of Chk1 (0.1 μM-1μM) and analyzed by gel mobility shift assays. Representative gels are shown for each of the DNAs.

CHAPTER IV

DISCUSSION

Checkpoints are surveillance mechanisms to maintain genome integrity in all eukaryotes. They inhibit progression of cell cycle in the presence of DNA lesions or in the presence of stalled replication forks. Checkpoints operate in every cell cycle phase in both exponentially growing and quiescent cells. Ensuring the DNA damage is repaired or DNA replication is completed before progression into the next phase of the cell cycle reduces the mutation rates or chromosomal rearrangements in the cell induced by DNA damage; therefore this constant surveillance prevents cancer formation.

Cell cycle checkpoints are signal transduction pathways composed of five groups: DNA damage sensors, apical signal transducers, mediators, distal signal transducers and effector proteins. DNA damage sensors are the least well characterized group of proteins in checkpoints. Broadly, they detect the presence of DNA damage and relay this signal to downstream proteins. Apical signal transducers are activated in the presence of DNA damage either by phosphorylating proximal signal transducer kinases or directly phosphorylating effector proteins they amplify the signal and lead to cell cycle arrest. Mediator proteins facilitate the phosphorylation and activation of proximal signal transducers. Although this outlines the function of each class, checkpoints do not function as linear pathways. Several of its components function in a way that they can be classified in more than one group. This

property of checkpoints points out the need for further genetic and biochemical analysis of its components to understand how checkpoints operate.

In this dissertation, we examined the biochemical properties of one of the component ATR signaling pathway, namely Claspin to gain better understanding of how it functions in the checkpoint activation. A search for factors important for Chk1 activation led to identification of Claspin in *Xenopus* (Kumagai and Dunphy 2000) and classification of Claspin as a mediator protein, due to its requirement for Chk1 phosphorylation and activation. However, the biochemical evidence presented in Chapter II suggested that Claspin might function as a DNA damage sensor. We determined that Claspin is a ring-shaped DNA binding protein with high affinity to branched DNA structures. DNA binding activity is consistent with the fact that Claspin associates with chromatin in both humans and *Xenopus* (Jiang et al. 2003; Lee et al. 2003; Smits et al. 2006). The chromatin association of Claspin is regulated by DNA damage or replication stress, presence of genotoxic insult increases the amount of Claspin on chromatin. Interestingly, Claspin binds to chromatin independent of other damage sensor proteins, ATR and RPA (Lee et al. 2003). Therefore, the findings in *Xenopus* together with our results provided preliminary evidence to classify Claspin as a sensor protein. Yeast homolog of Claspin, Mrc1 is essential for replication checkpoint activation (Alcasabas et al. 2001; Szyjka et al. 2005; Tanaka and Russell 2001). Mrc1 has been found to be a DNA binding protein and similar to Claspin, it has higher affinity towards the branched DNA structures (Zhao and Russell 2004)(). Mrc1 moves along with replication forks during S phase (Grandin et al. 2005; Osborn and Elledge 2003; Szyjka et al. 2005; Tourriere et al. 2005). DNA binding properties, DNA damage regulated chromatin association and importance for replication checkpoint activation suggest that both of the

proteins might be a part of the replisome to monitor proper progression of replication apparatus.

Despite its importance DNA damage recognition remains as the least characterized step of the DNA damage checkpoints. Identity of the DNA damage sensors and the type of structures that activates the checkpoints are active areas of research. Currently, two main criteria are used to define a protein as a sensor. First, the sensor protein should have a DNA binding activity and secondly, it should be absolutely required for checkpoint activation. The fact that several key components of ATR signaling pathway are DNA binding proteins, such as TopBP1, BRCA1 and Claspin has challenged the current criterion to define damage sensors (Paull et al. 2001; Yamane and Tsuruo 1999). This creates a need to identify the additional determinants to classify a protein as a sensor. One of example to such requirement would be absolute necessity of DNA binding activity for the checkpoint activation. Therefore, mutational analysis of DNA binding domain in parallel to biochemical characterization DNA binding activity should be performed and this analysis should be followed by the investigation of the effect of this mutant form on checkpoint activation to classify a protein as a damage sensor.

In order to determine whether Claspin meets these criteria, we attempted to identify DNA binding mutant of Claspin. One similar approach has been followed by Russell and his colleagues. Functional importance of DNA binding of Mrc1 has been addressed by the mutational analysis in the DNA binding domain (DBD) of Mrc1. Replacing endogenous protein with DBD mutant led to defects in replication checkpoint activation and showed the importance of DNA binding activity (Zhao and Russell 2004). Although Mrc1 and Claspin do not share overall sequence similarity, they share a sequence similarity in the DBD regions

of each protein. This region is predicted to have a helix-loop-helix motif that is potentially important for DNA binding. Individual deletions of helixes in this putative helix-loop-helix motif of Claspin did no change K_d values of mutant Claspin towards branched DNA structure. Therefore, further mutational analysis that diminish Claspin DNA binding activity without significant effect on its interaction with Chk1 will prove useful to determine the exact role of Claspin in checkpoint activation.

The second approach we followed to address the role of DNA binding activity of Claspin was to test the importance of DNA binding for Chk1 activation. Therefore, in Chapter III we investigated whether Claspin recruits Chk1 to DNA. Analysis of DNA binding of Chk1 in the presence or absence of Claspin revealed that Chk1 is a DNA binding protein and it binds to DNA independent of Claspin or any other protein. Interestingly, it possesses higher affinity to certain branched DNA structures. These findings are consistent with the fact that chromatin association of Chk1 is independent of ATR or the mediators of ATR signaling (Smits et al. 2006). Chk1 chromatin association does not depend on Claspin. Therefore, we conclude that Chk1 chromatin association is not mediated by other checkpoint components.

Currently, we do not have enough data to understand the physiological role of DNA binding activity of Chk1. However, its DNA binding is consistent with the role of Chk1 in normal cellular growth and several checkpoint responses (Abraham 2001; Bartek and Lukas 2003; Chen and Sanchez 2004). In the presence of replication stress it inhibits late origin firing and it is required for the stability of the replication forks (Chen and Sanchez 2004). Recently, Chk1 has been shown to be required to maintain global fork rate at normal levels in unperturbed cells (Petermann et.al., 2006), therefore, it has been suggested that Chk1 is

important for the stability of replication forks during normal S phase (Petermann et.al., 2006). In addition, Chk1 regulates the rate of replication initiation during normal S phase (Syljuasen et al. 2005). Due to its affinity to branched DNA structures, Chk1 may associate to chromatin after initial unwinding of origins and regulate the activities of replication or checkpoint proteins.

Dynamics of Chk1 chromatin association changes in the presence of genotoxic stress. DNA induced phosphorylation of Chk1 leads to its dissociation from chromatin and dissociation of Chk1 is required for the maintenance of the checkpoint (Smits et al. 2006). Therefore, it would be interesting to analyze the effect of phosphorylation on Chk1 DNA binding properties to address whether dynamics of chromatin association of Chk1 is regulated by the phosphorylation induced changes in Chk1's affinity to DNA.

Further characterization of Chk1 DNA binding is required to understand the role of Chk1 DNA binding. Chk1 does not have any motifs that might involve in DNA binding. Therefore, systematic mutational analysis is essential to determine DNA binding domain of Chk1. This should be followed by the identification of mutant forms of Chk1 with diminished DNA binding activity. Replacing endogenous Chk1 with DNA binding mutants of Chk1 will provide the data to address the importance of Chk1 DNA binding activity in both checkpoint and DNA replication.

Presence of this supposedly downstream component of checkpoint on chromatin and its DNA binding activity pose important questions to the mechanism of damage recognition. One such question would be whether Chk1 might have a role in DNA damage sensing. As components of ATR signaling pathway associates with chromatin and possess DNA binding activities. Another interesting question would be whether checkpoint proteins preassemble as

a complex for the recognition of the DNA damage or each checkpoint protein involved in damage recognition assembles at DNA damage. It would be interesting to examine how components of ATR pathway cooperate for the activation of checkpoint. More comprehensive research about the biochemical properties of the sensors combined with analysis of spatio-temporal regulation of these proteins will reveal key aspects of mechanism(s) underlying damage recognition.

Although the main question about the exact role of Claspin remains unanswered in full, our studies determined several key aspects of Claspin. In summary, our biochemical analysis provided the preliminary evidence that Claspin might be a sensor protein; we showed that Claspin does not recruit Chk1 to DNA. Another interesting finding was to show that Chk1 is also a DNA binding protein.

CHAPTER V

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