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Human Claspin Is a Ring-shaped DNA-binding Protein with High Affinity to Branched DNA Structures*

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Funda Sar, Laura A. Lindsey-Boltz, Deepa Subramanian, Deborah L. Croteau‡, Stephanie Q. Hutsell, Jack D. Griffith, and Aziz Sancar§

From the Department of Biochemistry and Biophysics and Lineberger Comprehensive Cancer Center, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

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 because of DNA damage.

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 four main groups: DNA damage sensors, mediators, signal transducers, and effectors. DNA damage sensors recognize 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 phoshoinositide kinase-like kinase family members ATM[1] and ATR and 2) the clamp loader/DNA clamp family members Rad17-RFC/9-1-1 complex (1–3).

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 (4). The yeast proteins scRad9/spCrb2 and sp/scMrc1 are the prototypical members of the mediator class of checkpoint proteins (5–7). Another example of a mediator in humans is BRCA1, which has been shown to be required for optimal Chk1 phosphorylation after ionizing radiation (8). 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 redefinition 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 215-kDa protein, Claspin (4). Activation of the replication checkpoint induces Claspin phosphorylation, which is essential for Claspin-Chk1 association and subsequent activation of Chk1 (4, 9). Subsequent work showed that similar to Xclaspin, human Claspin is also required for phosphorylation of Chk1, it interacts with Chk1, and the association between these two proteins depends on the phosphorylation of Claspin (10). In addition to Chk1, Claspin also interacts with other checkpoint proteins, ATR and Rad9 (10). As ATR is the primary phoshoinositide kinase-like kinase 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 (11).

A detailed study on Xclaspin suggests a more complex role in cell cycle regulation and replication checkpoint activation (12). 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 the 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 (12). Of particular interest, it was reported that

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‡ Current address: NIEHS, National Institutes of Health, 111 T. W. Alexander Dr., MD D3-01, Research Triangle Park, NC 27709.

§ To whom correspondence should be addressed: Dept. of Biochemistry and Biophysics, Mary Ellen Jones Bldg. CB 7260, University of North Carolina School of Medicine, Chapel Hill, NC 27599. Tel.: 919-962-0115; Fax: 919-843-8627; E-mail: Aziz_Sancar@med.unc.edu.

[1] The abbreviations used are: ATM, ataxia-telangiectasia-mutated; ATR, ataxia-telangiectasia-mutated and Rad3-related; SSB, single-stranded DNA-binding protein; RPA, replication protein A; RT, reverse transcriptase; EM, electron microscopy; PCNA, proliferating cell nuclear antigen; 9-1-1, Rad9-Rad1-Hus1 complex; MCM, minichromosome maintenance proteins.

even though Claspin associates with replicating sperm chromatin in *Xenopus* extracts, Xclaspin exhibited no intrinsic DNA binding activity (12). 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 (11), a protein with high affinity for branched DNA structures (13), 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.

EXPERIMENTAL PROCEDURES

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 the 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 a *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 h. The cells were washed with 1× phosphate-buffered saline and then lysed in 20× packed volumes of lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors (Roche Applied Science). After incubation on ice for 10 min, the cell lysate was centrifuged for 20 min at 32,000 × *g*. The supernatant was incubated with anti-FLAG agarose (Sigma) for 4 h 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, and 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 min at 4 °C. A typical yield was about 2 μg of Claspin from 10^7 cells. Concentrations 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 (14). These oligomers were annealed in appropriate combinations to obtain the DNA forms shown in Figs. 3 and 4. To prepare each substrate, oligonucleotide 1 was labeled on the 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 Biosciences) and annealed with appropriate non-radioactive 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 min and then allowed to cool to room temperature overnight. The substrates were separated on a 10% polyacrylamide gel in 1× 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 the 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 activities.

Gel Mobility Shift Assay—Full-length Claspin or its fragments at the indicated concentrations were incubated with 0.25 nM DNA substrates for 25 min at 30 °C in a 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 dithiothreitol, and 3 mg/ml bovine serum albumin. Glycerol was added to 6.1%, and the DNA-protein complexes were separated on a 5% polyacrylamide gel in 1× TBE. Electrophoresis was performed at 120 V for 210–240 min at 4 °C. The gels were dried and exposed to a PhosphorImager screen (Amersham Biosciences), and the data was analyzed by ImageQuant software (version 5.0, Amersham Biosciences). Bound DNA fraction was 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 min on ice before adding it to DNA and performing a gel mobility shift assay.

Electron Microscopy—Linear double-stranded DNA templates containing either an internal single-stranded or double-stranded tail (Fig. 5, *A* and *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 (15). Plasmids were nicked with N.BbvCIA followed by strand displacement with Klenow exo$^-$ fragment in reactions lacking dCTP to produce the single-stranded tail. Double-stranded tails were generated by annealing primers to the single-stranded 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 a 20-μl binding buffer containing 10 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol for 20 min at 25 °C. In the control reaction, the single-stranded tail template was incubated with 500 ng of *Escherichia coli* SSB protein under the same conditions. The samples were fixed by the addition of glutaraldehyde to a final concentration of 0.6% for 10 min at 25 °C and chromatographed on 1-ml Bio-Gel A50m columns to remove unbound proteins. Fractions containing DNA-protein complexes were prepared for electron microscopy as described previously (16). 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. Fig. 1*A* is a schematic representation of fragments that were inserted into baculovi-

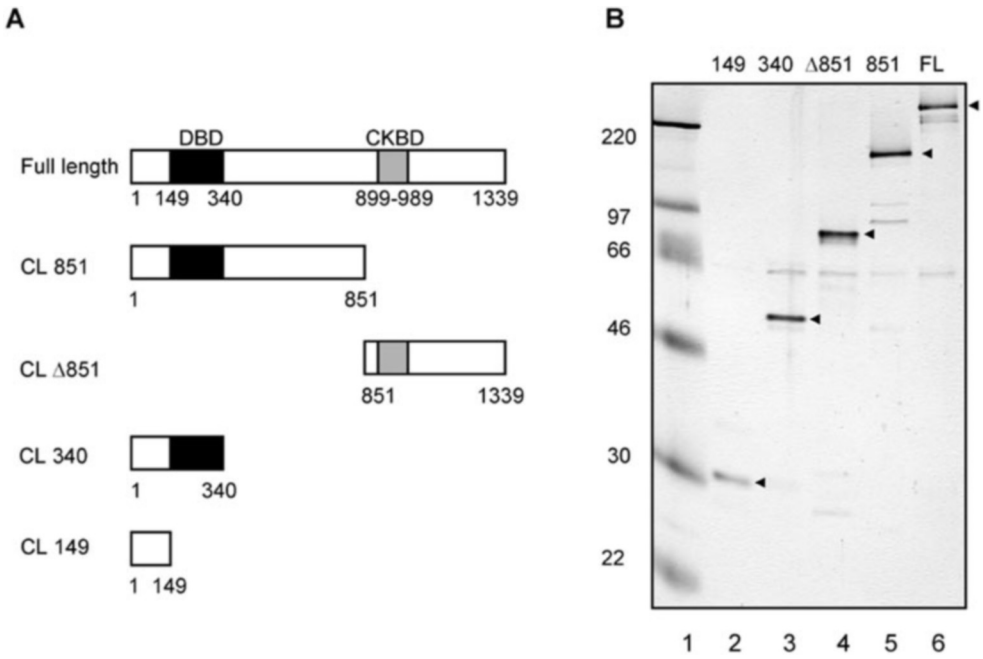

FIG. 1. **Purification of Claspin and Claspin fragments.** *A*, schematic representation of Claspin and the Claspin fragments that were expressed in the baculovirus/insect cell system. The *numbers* indicate the position of amino acids in the respective constructs. *DBD*, DNA binding domain; *CKBD*, Chk1 binding domain identified by Kumagai and Dunphy (9). *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 *A*. The *arrowheads* point to Claspin and Claspin fragments. Each *lane* contains ~0.1 μg of protein. *FL* denotes full-length protein.

rus, and Fig. 1*B* shows the Claspin domains encoded by these fragments separated on an SDS-PAGE. Human Claspin is 1339 amino acids in length with a calculated molecular mass of 151 kDa. However, as noted for the Xclaspin, the human Claspin migrates anomalously on SDS-PAGE with an apparent molecular mass of 250 kDa (Fig. 1*B*, *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 (4). 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 (Fig. 1*B*, *lanes 2–5*).

Binding of Claspin to DNA—Because 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 (Fig. 2*A*). We concluded that Claspin is a DNA-binding protein. To identify the DNA binding domain, we tested the Claspin fragments shown in Fig. 1 in the gel mobility shift assay. The results are shown in Fig. 2*B*. Based on the data in Fig. 2*B* (*lanes 1–8*), the main DNA binding domain is identified as the region spanning amino acids 149 to 340. However, the C-terminal CLΔ851 fragment also exhibited some weak DNA binding activity (Fig. 2*B*, *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 (Fig. 2*B*, *lanes 11–15*). This behavior is commonly observed with DNA-protein complexes with rapid dissociation rates. Thus, it appears that Claspin has a primary DNA binding domain in the N-terminal region and a secondary DNA binding site in the C-terminal region.

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 (17). 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 are 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 (18). 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" (Fig. 3, *top panel*). Claspin binds to all of these structures with higher affinity than double-stranded DNA (Fig. 3). To assess the relative affinity of Claspin to various DNA forms, we generated binding isotherms with gel mobility shift assays (Fig. 4, *A–G*), and K_d values were determined as the concentration at which 50% of the DNA is bound by Claspin (Table I). 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 (Fig. 4*G*, Table I). 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 because of the single-stranded DNA *per se*, which are associated with replicating DNA.

Structure of Claspin-DNA Complexes—Our data combined with previous data on Xclaspin (12), suggest that Claspin may specifically bind to the regions of replication forks not covered by other replication and checkpoint proteins (12) and may even travel with the replication fork as a replication sensor protein even though it is not essential for replication (12). Hence, we prepared a 3.2-kb linear double-stranded DNA template containing a 400-nucleotide single-stranded tail or a circular double strand template containing a 400-bp double-stranded tail as model substrates for structures associated with leading and lagging strand synthesis (Fig. 5, *A* and *B*). We incubated these

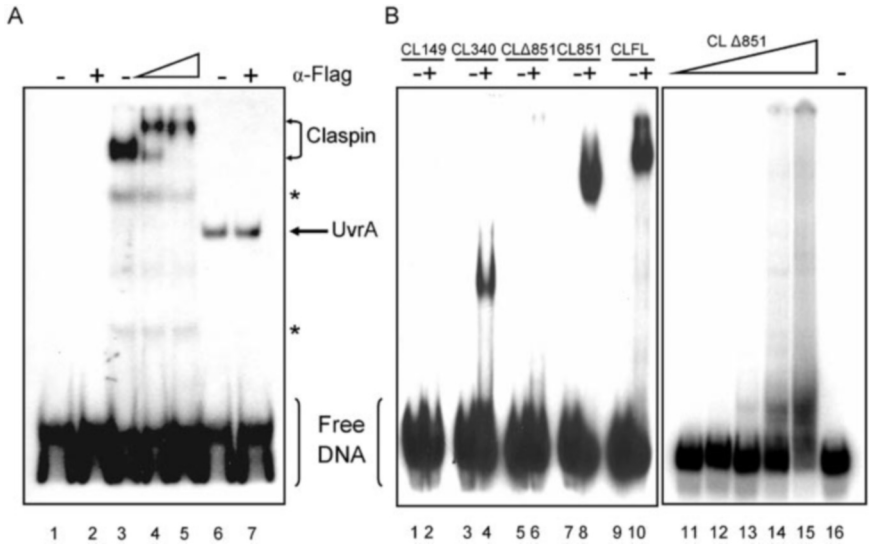

FIG. 2. **Binding of Claspin to DNA.** *A*, direct binding to double-stranded DNA. A terminally labeled 30-bp duplex (0.1 nM) was incubated with Flag-Claspin (100 nM) or *UvrA* (2 nM) as indicated, and the DNA-protein complexes were separated on a 5% polyacrylamide gel. Anti-FLAG (*α-FLAG*) antibodies were added to samples in *lanes 2*, *4*, *5*, and *7* before loading onto the gel. *Lanes 2*, *4*, and *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 nonspecific 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*), and 210 nM (*lane 15*) *CLΔ851* and separated on a 5% polyacrylamide gel.

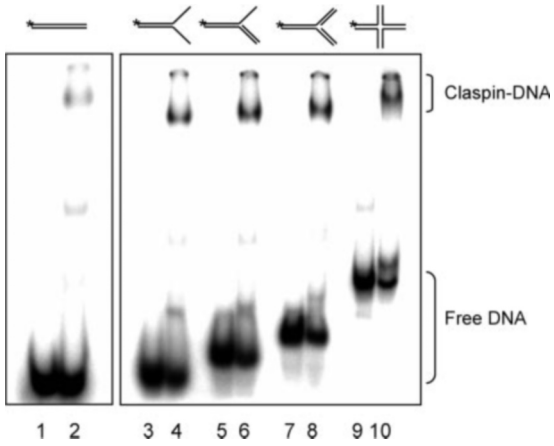

FIG. 3. **Preferential binding of Claspin to branched DNA structures.** The indicated DNA structures (0.25 nM) were incubated with 26.5 nM Claspin and separated on 5% polyacrylamide gels. Because the double-stranded DNA migrates much faster than the branched DNA, the Claspin-double-stranded DNA complex was resolved on a separate gel. *Lanes 1* and *2*, double-stranded DNA; *lanes 3* and *4*, Y structure; *lanes 5* and *6*, 3′-flap; *lanes 7* and *8*, replication fork; *lanes 9* and *10*, Holliday junction. *Asterisks* indicate the position of the radioactive label.

substrates with Claspin, and then the samples were prepared for electron microscopy (EM) as described elsewhere (16).

To verify the position of the single-stranded tail, DNA templates (Fig. 5*A*) 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-stranded tail that had at least 3–5 SSB particles bound (19), indicating a tail length of ~400 nucleotides (Fig. 6*A*). 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 a 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 (Fig. 6, *B* and *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 (20). 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 (Fig. 6, *B* and *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 (Fig. 5*B*), 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 the 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 (Fig. 6, *D–F*), 21% were bound along the tail, and 26% were 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 with the single-stranded tail template, which is consistent with the *in vivo* data suggesting that Claspin may prefer single-stranded fork structures that are not covered by other proteins such as RPA (12). In the double-stranded tail templates, there is a 25-nucleotide gap at the base of the junction. However, this may not provide a sufficient single-stranded region for efficient Claspin recognition. Re-

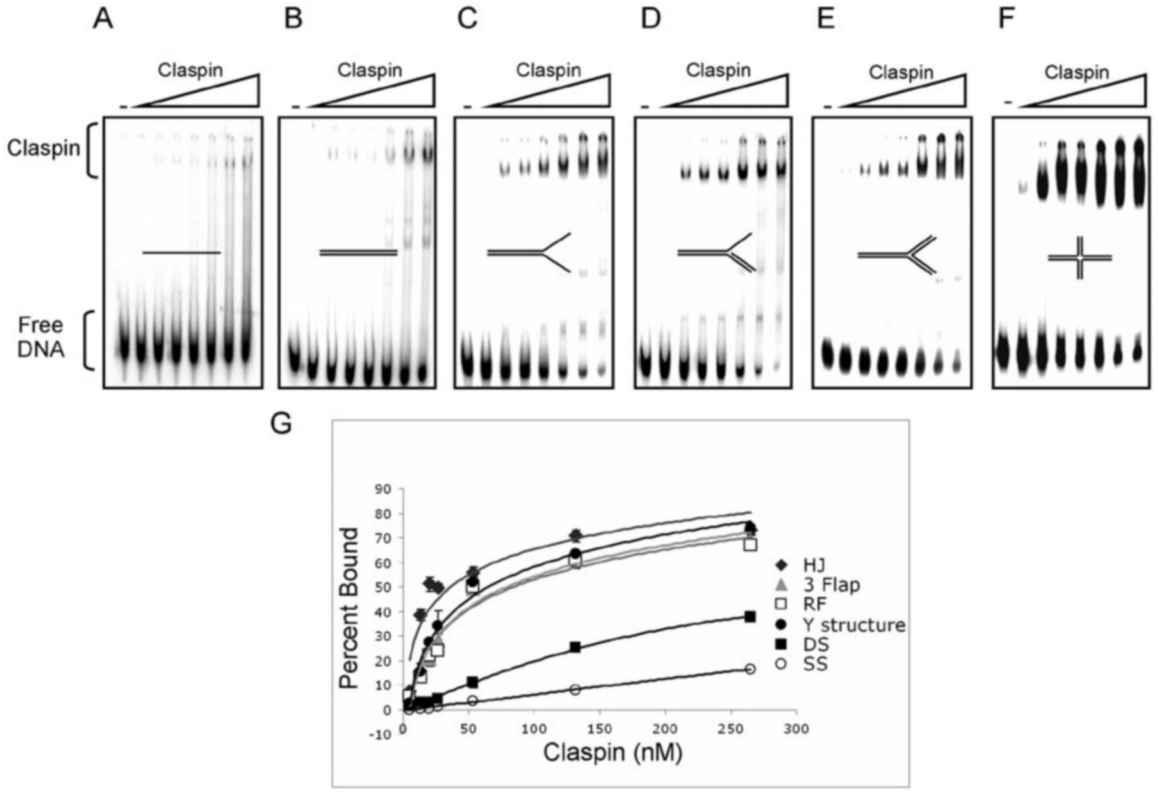

FIG. 4. **Claspin-DNA binding isotherms with various DNA forms.** *A–F*, the indicated DNAs at 0.25 nM were incubated with increasing concentrations of Claspin (5.3–232 nM) and analyzed by gel mobility shift assays. Representative gels are shown for each of the DNAs (single-stranded DNA (*A*), double-stranded DNA (*B*), Y structure (*C*), 3′-flap (*D*), replication fork (*E*), and Holliday junction (*F*)). *G*, binding isotherms were obtained from two gel mobility shift assays, including the ones shown in *A–F*. The *error bars* are the standard error values for two experiments. *HJ*, Holliday junction; *RF*, replication fork; *DS*, double-stranded DNA; *SS*, single-stranded DNA.

TABLE I
Affinities of Claspin for different DNA structures

DNA structures	K_d[a]
	nM
Single-stranded DNA[b]	>1000
Double-stranded DNA[b]	>500
Replication fork	85
3′-Flap	80
Y structure	65
Holliday junction	37

[a] Estimated from the concentration required for 50% binding.
[b] Extrapolated from the binding curves to the amount required for 50% binding.

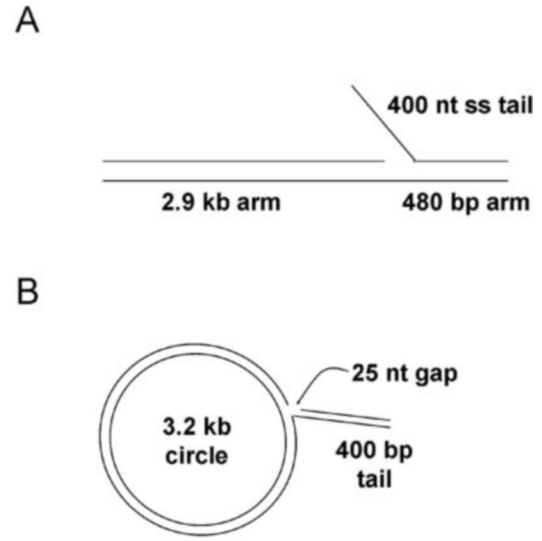

FIG. 5. **Substrates for electron microscopy.** The substrates were generated by nicking the pGLGAP12 plasmid with N.BbvcIA enzyme and strand displacement and restriction enzyme digestion to generate arms of the indicated sizes (*A*) or by strand displacement followed by primer extension to generate a circle with double-stranded tail (*B*). *ss*, single-stranded.

gardless 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

In this paper 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 structures 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 (12). 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 (12), 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 to determine the mechanisms by which the DNA damage or replication intermediates are detected. Genetic and biochemical analyses, including the data in this paper, have implicated three groups of proteins in sensing DNA damage or replication intermediates: 1) the phoshoinositide kinase-like kinase family members ATM and ATR, 2) the clamp loader/DNA clamp family members Rad17-RFC/9-1-1 complex, and 3) Claspin (1–3). Claspin was first

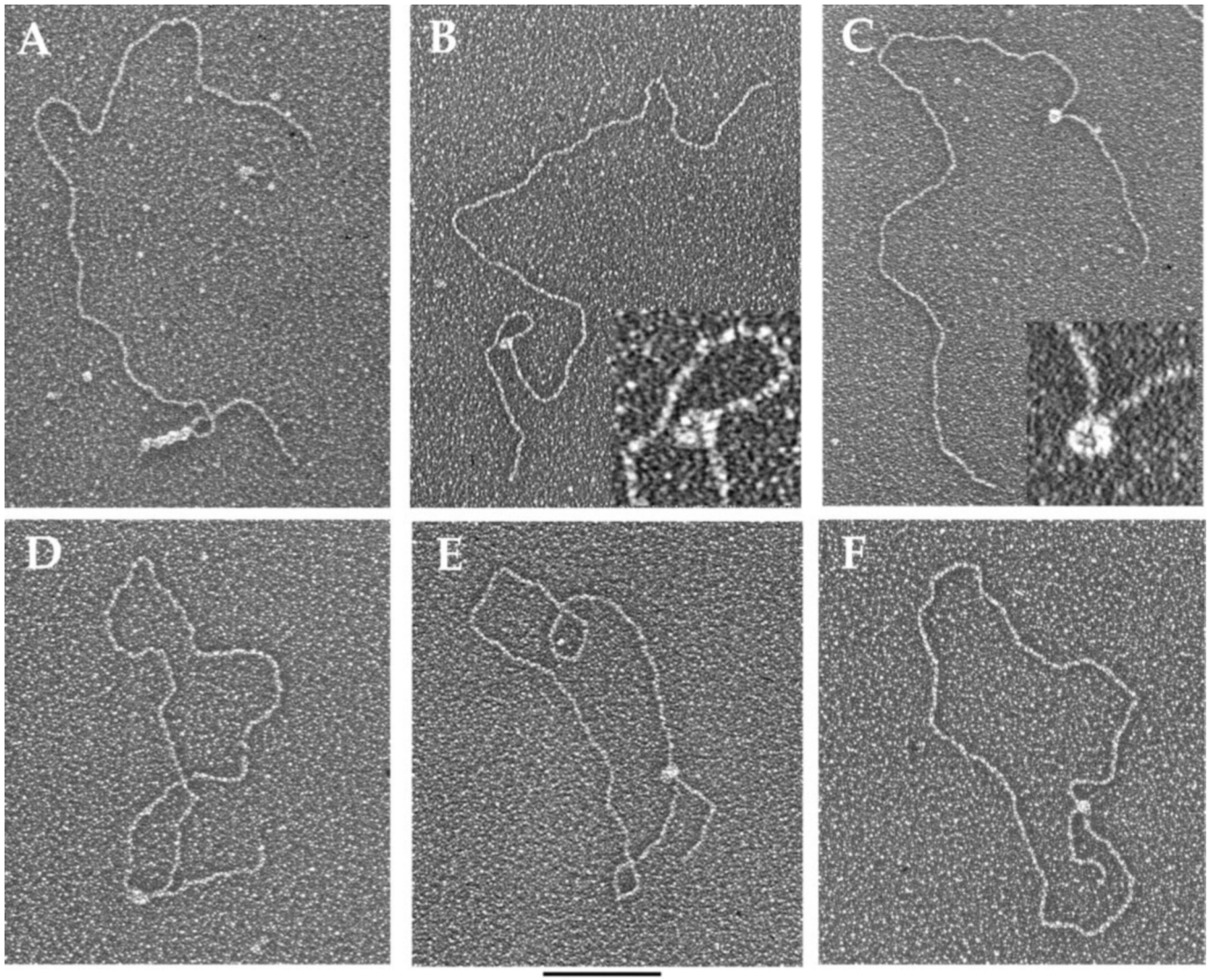

FIG. 6. **Claspin binds to the junction of single- or double-stranded tails on duplex DNA templates.** *A*, visualization of *E. coli* SSB protein to single-stranded tails of replication fork templates by electron microscopy. Samples were directly mounted onto thin carbon-coated copper grids and rotary shadow-casted with tungsten. *B* and *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. *Insets* show the protein-DNA complexes at a higher magnification. *D–F*, Claspin binding to replication fork templates containing double-stranded tails. *Bar* represents 500 bp.

thought to function as a mediator or an adaptor in the ATR-Chk1 signaling pathway (4, 10, 12) in a manner similar to the two known mediators in yeast, scRad9 and Mrc1 proteins (6, 7). However, recent work clearly demonstrated that Claspin is one of the first checkpoint proteins to associate with replicating chromatin (12) 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 (21). Similarly, it was reported that Rad17-RFC loads the 9-1-1 complex onto RPA-covered template/primer-like structures (22, 23). 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 (12). 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 are not consistent with either 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 (24, 25). 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 (26) 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 (27). 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 (28). In the light of our results, the requirement for Cdc45 for

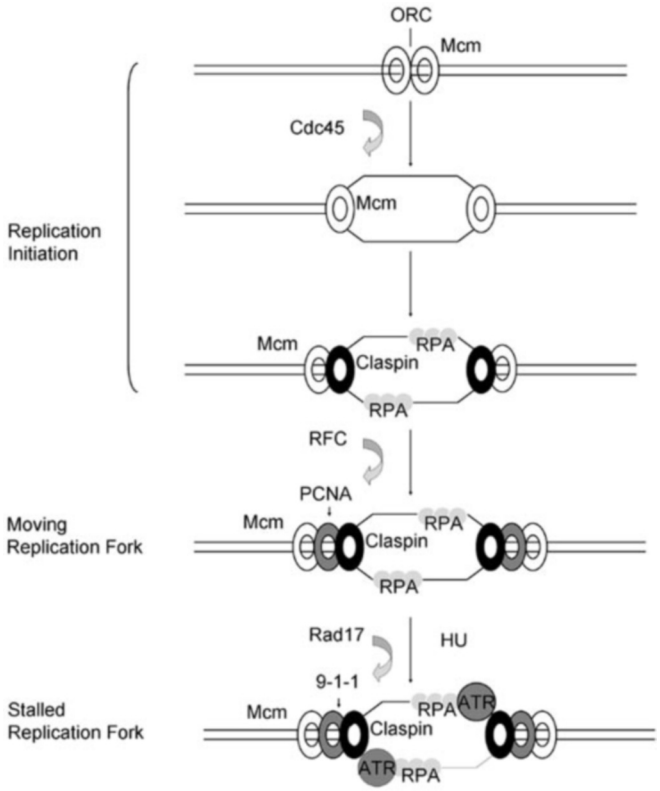

FIG. 7. **Model for replication checkpoint sensor association with DNA.** The Cdc6, ORC, Cdt1, and MCM proteins open the duplex at the replication origin. MCM, which has a ring-like structure, remains associated with the replication fork. Once the two strands are separated, Claspin also binds to the 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 hydroxyurea 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.

Claspin binding is most likely because of its indispensable role in the 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 (12) (Fig. 7). 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, and 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 (29) and Claspin by ATR (9), 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 (18) and causes more extensive binding of checkpoint sensors (12) 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 (23, 26, 27). 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 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* (12) and its apparent requirement for cell viability in human cells (10, 11), suggest that Claspin is loaded at the replication origin onto unwound DNA and travels with the replication fork as a part of the 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.

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