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Roles of Replication Fork-interacting and Chk1-activating Domains from Claspin in a DNA Replication Checkpoint Response[D]

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Claspin is essential for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing incompletely replicated DNA. Claspin associates with replication forks upon origin unwinding. We show that Claspin contains a replication fork-interacting domain (RFID, residues 265–605) that associates with Cdc45, DNA polymerase ε, replication protein A, and two replication factor C complexes on chromatin. The RFID contains two basic patches (BP1 and BP2) at amino acids 265–331 and 470–600, respectively. Deletion of either BP1 or BP2 compromises optimal binding of Claspin to chromatin. Absence of BP1 has no effect on the ability of Claspin to mediate activation of Chk1. By contrast, removal of BP2 causes a large reduction in the Chk1-activating potency of Claspin. We also find that Claspin contains a small Chk1-activating domain (residues 776–905) that does not bind stably to chromatin, but it is fully effective at high concentrations for mediating activation of Chk1. These results indicate that stable retention of Claspin on chromatin is not necessary for activation of Chk1. Instead, our findings suggest that only transient interaction of Claspin with replication forks potentiates its Chk1-activating function. Another implication of this work is that stable binding of Claspin to chromatin may play a role in other functions besides the activation of Chk1.

INTRODUCTION

Checkpoint control mechanisms ensure the integrity of the genome by preventing the transmission of incompletely replicated or damaged DNA to progeny cells (Osborn *et al*., 2002; McGowan and Russell, 2004; Sancar *et al*., 2004; O'Connell and Cimprich, 2005). For example, the DNA replication checkpoint monitors whether DNA synthesis occurs normally throughout S phase. When problems become evident, this regulatory network forestalls premature entry into mitosis and stabilizes aberrant replication forks until normal replication can resume and subsequently reach completion. The manifestation of this checkpoint is most obvious when DNA replication forks stall at sites of exogenously inflicted DNA damage. However, this pathway also operates when spontaneous errors arise during replication or the replication apparatus encounters difficult-to-replicate sequences.

Checkpoint pathways contain a variety of regulatory proteins that detect the status of the genome and that relay this information to effector enzymes that regulate downstream processes (Melo and Toczyski, 2002; Osborn *et al*., 2002; Sancar *et al*., 2004). In the DNA replication checkpoint, ATR functions at or near the top of this regulatory hierarchy (Abraham, 2001). ATR is a member of the phosphoinositide kinase-related family of protein kinases that also includes ATM. One key function of ATR involves activation of the checkpoint effector kinase Chk1 (Guo *et al*., 2000; Hekmat-Nejad *et al*., 2000; Liu *et al*., 2000). Significantly, ATR cannot carry out this function alone but must cooperate with numerous other proteins. For example, ATR possesses a conserved binding partner called ATRIP (Cortez *et al*., 2001). Other collaborating factors include the checkpoint clamp assembly of Rad9, Rad1, and Hus1 (the 9-1-1 complex) (Sancar *et al*., 2004). A checkpoint clamp loader consisting of Rad17 and the four small subunits of replication factor C (RFC) is responsible for deposition of the 9-1-1 complex onto DNA. The checkpoint clamp loader and clamp proteins most likely interact with boundaries between single-stranded and double-stranded regions of DNA that would be present in incompletely replicated or damaged DNA (You *et al*., 2002; Ellison and Stillman, 2003; Lee *et al*., 2003; Zou *et al*., 2003).

ATR as well as ATM also work together with a class of proteins known as mediators (McGowan and Russell, 2004; O'Connell and Cimprich, 2005). In vertebrates, these proteins consist of Claspin and various BRCA1 C-terminal repeat (BRCT)-containing proteins, including TopBP1, 53BP1, Mdc1, and BRCA1 itself (Kumagai and Dunphy, 2000; Canman, 2003; Chini and Chen, 2003). These mediators either have been shown to or are thought to serve as adaptors between ATR/ATM and the downstream kinases Chk1 and Chk2. In addition, accumulating evidence has indicated that mediator proteins may function as sensors of chromatin structures. For example, in response to double-stranded DNA breaks, mammalian 53BP1 and its fission yeast relative

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Crb2 recognize methylated forms of lysine 79 in histone H3 and lysine 20 in histone H4, respectively (Huyen *et al*., 2004; Sanders *et al*., 2004). These histone methylations do not vary in response to DNA damage. Instead, modified histones may become inappropriately exposed at sites of damage. Furthermore, 53BP1, Crb2, and other BRCT-containing proteins respond to the phosphorylation of histone H2AX and H2A in mammals and fission yeast, respectively (Celeste *et al*., 2003; Nakamura *et al*., 2004). This type of histone phosphorylation is not required for initial recruitment of mediator proteins to sites of damage, but it is necessary for their stable incorporation into damage-induced foci. These observations suggest that the interaction of mediator proteins with chromatin is multifaceted and may fulfill multiple functions.

We have been studying the DNA replication checkpoint in *Xenopus* egg extracts. In this system, the DNA replication inhibitor aphidicolin elicits the formation of stalled replication forks, which in turn trigger the activation of *Xenopus* Chk1 (Xchk1) (Kumagai *et al*., 1998; Michael *et al*., 2000). The *Xenopus* homologue of ATR (Xatr) is responsible for the phosphorylation-dependent activation of Xchk1 (Guo *et al*., 2000; Hekmat-Nejad *et al*., 2000). This process also requires the mediator protein Claspin in *Xenopus* egg extracts and human cells (Kumagai and Dunphy, 2000; Chini and Chen, 2003; Lin *et al*., 2004). Claspin associates directly with Xchk1 and thereupon strongly enhances the ability of Xatr to phosphorylate Xchk1 (Kumagai and Dunphy, 2003; Kumagai *et al*., 2004). In addition, Claspin displays dynamic spatial localization by associating with replication forks during S phase (Lee *et al*., 2003). This binding requires Xcdc45 and Cdk2 but not replication protein A (RPA), suggesting that Claspin associates with incipient replication forks at around the time of DNA unwinding (Lee *et al*., 2003). The initial binding of Claspin occurs before Xatr-Xatrip and the replication factor C (RFC) proteins and therefore must involve, at least in part, chromatin structures that are distinct from those recognized by these proteins. The budding yeast homologue of Claspin called Mrc1 likewise associates specifically with replication forks (Katou *et al*., 2003; Osborn and Elledge, 2003). These observations imply that Claspin and its homologues may also function as checkpoint sensor proteins.

In this study, we have explored the mechanism by which Claspin associates with the DNA replication apparatus to understand the purpose of this interaction. Our results indicate that Claspin uses a conserved domain to interact with key replication and checkpoint proteins, including Cdc45, DNA polymerase ϵ (Pol ϵ), RPA, and both the replicative and Rad17-containing RFC complexes. Interestingly, although a portion of this domain is required for optimal activation of Chk1, stable retention of Claspin on chromatin is not essential for its Chk1-activating function.

MATERIALS AND METHODS

Xenopus Egg Extracts

Extracts from *Xenopus* eggs were prepared as described previously (Lee *et al*., 2003). The DNA replication checkpoint was induced by the addition of demembranated *Xenopus* sperm nuclei (3000/μl) and aphidicolin (100 μg/ml). Caffeine (5 mM) was used to override this checkpoint response. Isolation of nuclear and chromatin fractions from egg extracts was described previously (Lee *et al*., 2003).

Antibodies

Antibodies against *Xenopus* Claspin, RPA70, Xorc2, phospho-Ser864 of Claspin, Xatr, Xatrip, Xchk1, Xrad17, and Xhus1 were described previously (Kumagai and Dunphy, 2003; Lee *et al*., 2003; Kumagai *et al*., 2004). Antibodies against *Xenopus* RFC40 and proliferating cell nuclear antigen (PCNA) were raised against bacterially expressed His6-tagged proteins containing either residues 1–320 of RFC40 or full-length PCNA, respectively. The sequence of *Xenopus* RFC40 (expressed sequence tag database XGI TC34364) showed 89.6% identity at the amino acid level with human RFC40. Anti-Xcdc45 and anti-p60 of Pol ϵ antibodies were raised as described previously (Mimura and Takisawa, 1998; Waga *et al*., 2001). These antibodies were all affinity-purified with their antigens. Antisera against *Xenopus* importin α, *Xenopus* Pol δ (p125 subunit), Xsld5, *Xenopus* cyclin E, and Xmcm7 were generously supplied by D. Görlich (Universität Heidelberg, Heidelberg, Germany), S. Waga (Osaka University, Osaka, Japan), H. Takisawa (Osaka University, Osaka, Japan), P. Jackson (Stanford University, Stanford, CA), and J. Blow (University of Dundee, Dundee, Scotland, United Kingdom), respectively. Antisera against human RFC37 and *Xenopus* RFC140 were the kind gifts of J. Hurwitz (Memorial Sloan-Kettering Cancer Center, New York, NY) and S. Waga, respectively. Anti-human Chk1 phospho-Ser345 antibody was purchased from Cell Signaling Technology (Beverly, MA). Purified control rabbit IgG and anti-FLAG monoclonal antibodies were obtained from Zymed Laboratories (South San Francisco, CA) and Sigma-Aldrich (St. Louis, MO), respectively. Immunodepletion procedures for Claspin, Xcdc45, and RPA were described previously (Lee *et al*., 2003).

Recombinant Proteins

The pBluescript vector was engineered to encode a nuclear localization signal (NLS) (TPPKKKRKVEDP) (Moore *et al*., 2002) fused upstream of Claspin fragments for in vitro protein synthesis. The same NLS was inserted into pGEX-4T-3 (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) to make glutathione *S*-transferase (GST)-NLS fusion constructs of Claspin. Baculoviruses encoding full-length or truncated His6-Claspin-FLAG proteins were generated with the Bac-to-Bac system with a His6 tag and FLAG epitope (DYKDDDDK) at the N-terminal and C-terminal ends, respectively. Various Claspin mutants (internal deletions or amino acid substitutions) were produced through PCR-based mutagenesis by standard methods. Recombinant Claspin proteins were expressed and purified from baculovirus-infected insect cells or bacteria as described previously (Kumagai and Dunphy, 2003). ^{35}S-labeled proteins were synthesized in vitro with the TnT system (Promega, Madison, WI). Human GST-p27 (gift from T. Hunter, Salk Institute, La Jolla, CA) was expressed in bacteria and purified with glutathione agarose.

Identification of Claspin-binding Proteins from Egg Extracts

Claspin was immunoprecipitated from 5 ml of interphase egg extract with 200 μg of anti-Claspin antibodies cross-linked to Affiprep protein-A beads (Bio-Rad, Hercules, CA). The beads were washed four times with immunoprecipitation (IP) buffer B (10 mM HEPES-KOH, pH 7.6, 0.5 M NaCl, 0.1% NP-40, and 20 mM β-glycerolphosphate). Samples were boiled in SDS buffer, concentrated, separated by SDS-PAGE, transferred to nitrocellulose, and stained with Ponceau S. Protein bands containing p55 and p90 were excised and subjected to chemical sequencing and nanoelectrospray tandem mass spectrometry in the Howard Hughes Medical Institute protein sequencing facility at University of California (Berkeley, CA). Sequences from p55 (KXTQH/AP and KYFXGEEA) and p90 (KFYAK and KTLATWATK) corresponded to importin α and β, respectively.

Identification of Claspin-binding Proteins in Chromatin Eluates

For analytical experiments, sperm nuclei reconstituted in interphase egg extract (1 ml) were collected through a sucrose cushion (Lee *et al*., 2003). Nuclei were resuspended in 100 μl of HEPES-buffered saline (10 mM HEPES-KOH, pH 7.6, and 150 mM NaCl) supplemented with 10% dimethyl sulfoxide and 5 mM caffeine, incubated at room temperature for 30 min, and cooled on ice for 20 min. An equal volume of elution buffer (10 mM HEPES-KOH, pH 7.6, 1 M NaCl, and 1% NP-40) was added, and the incubation was continued on ice for 20 min. After dilution with 200 μl of 10 mM HEPES-KOH, pH 7.6, soluble proteins were recovered by centrifugation at 14,000 rpm in an Eppendorf centrifuge for 10 min. Antibodies (2.5 μg) cross-linked to protein A beads were incubated with these preparations for 1 h at 4°C, washed twice with IP buffer C (10 mM HEPES-KOH, pH 7.6, 150 mM NaCl, 0.1% CHAPS, 2.5 mM EGTA, and 20 mM β-glycerolphosphate), and subjected to SDS-PAGE.

For identification by protein sequencing, four batches of egg extract totaling 50 ml were used to reconstitute nuclei (3000/μl) in the presence of aphidicolin and caffeine. Proteins were removed from chromatin by elution with 0.5 M NaCl and immunoprecipitated with anti-Claspin antibodies as described above. Bound proteins were concentrated, separated by SDS-PAGE, stained with Coomassie Blue, and subjected to in-gel trypsin digestion as described previously (Shevchenko *et al*., 1996). Methods for nanoelectrospray tandem mass spectrometry of tryptic peptides and analysis of the sequencing data were carried out exactly as described previously (Shevchenko *et al*., 2001, 2003; Yoo *et al*., 2004). The following protein identifications were obtained: p180 (Claspin, 10 peptides), p150 (RFC140, 3 peptides), and p65 (RPA70, 3 peptides). p36-40 contained RFC36 (2 peptides), RFC37 (5 peptides), RFC38 (2 peptides), and RFC40 (5 peptides).

Oligonucleotide Binding Assay

Preparation of magnetic beads coated with DNA oligonucleotides and assay conditions for binding of proteins in egg extracts to these beads were described previously (Lee *et al.*, 2003). For the double-stranded template, annealed $(dA)_{70}$-$(dT)_{70}$ was prepared as described previously (Kumagai and Dunphy, 2000). For the 30d-40s branched DNA, the following oligonucleotides were annealed: 5′-ACTGATTACGGTGCTGCTTATCGATGGTTTGCAGTGCTCGCATGGAGCTGGTTTCCGGCCTTGCTAATGG and 5′-biotin-CCATTAGCAAGGCCGGAAACCAGCTCCATGATCATTGGCAATCATTGGCACAACGATCAGCCAACTAAAC. Oligonucleotides were added to egg extracts at a final concentration of 5.5 nM.

RESULTS

Search for Claspin-interacting Proteins in Cytoplasmic Egg Extracts

To probe the mechanism by which Claspin promotes the phosphorylation of Xchk1 in *Xenopus* egg extracts that contain incompletely replicated DNA, we attempted to find Claspin-interacting proteins. In particular, we wished to evaluate whether Claspin could recognize specific components of the DNA replication apparatus. We first searched for Claspin-interacting proteins by immunoprecipitating Claspin from whole cytoplasmic egg extracts (Figure 1A). We identified two prominent binding proteins at 55 and 90 kDa as *Xenopus* importins α and β, respectively (see *Materials and Methods*). The binding of importin α was verified by immunoblotting with anti-importin α antibodies (Figure 1A). These proteins could not be found in anti-Claspin immunoprecipitates from nuclear extracts (see below), which is consistent with the fact that importins dissociate from their cargo upon nuclear entry. In further studies, we found that importin α binds well to a GST peptide containing the C-terminal 56 amino acids of Claspin (residues 1230–1285), which contains sequences that closely resemble a NLS (our unpublished data).

Claspin Interacts with Key Replication Proteins on Chromatin

Because we could not identify any additional Claspin-binding proteins in cytoplasmic extracts, we examined whether Claspin associates specifically with other proteins upon binding to chromatin. To pursue these experiments, we first considered the richest potential source of Claspin-interacting, chromatin-binding proteins. Previously, we observed that Claspin accumulates on chromatin in aphidicolin-treated extracts. The binding of Claspin increases dramatically upon the further addition of caffeine. Recent studies have indicated that caffeine stimulates the firing of inhibited replication origins in aphidicolin-treated chromatin (Yanow *et al.*, 2003; Marheineke and Hyrien, 2004; Shechter *et al.*, 2004). Therefore, the increased binding of Claspin to chromatin in such extracts seems to reflect binding to numerous additional origins of replication. Under this condition, >80% of the nuclear Claspin is bound to chromatin. Hence, for the experiments described below, we have searched for Claspin-binding proteins on chromatin in extracts containing both aphidicolin and caffeine.

Next, we assessed different methods for immunoprecipitation of the chromatin-derived form of Claspin. Some commonly used techniques, such as nuclease digestion or sonication of the chromatin before immunoprecipitation, resulted in anti-Claspin immunoprecipitates that were contaminated by general DNA binding proteins. We concluded that these methods yielded chromatin fragments that were large enough to bridge Claspin nonspecifically with other proteins during immunoprecipitation. As an alternative, we attempted to extract Claspin from chromatin by mild salt treatment under conditions that would maintain certain protein–protein interactions that had initially formed on the DNA. For this purpose, we exposed chromatin to increasing concentrations of NaCl (i.e., 0.25, 0.5, 0.75, and 1 M). As shown in Figure 1B, treatment with 0.25 M NaCl led to a substantial decrease in chromatin-bound Claspin, and 0.5 M NaCl removed Claspin almost completely. Various replication and checkpoint regulatory proteins, including Xorc2, Xmcm7, Xcdc45, RPA70, RFC40, PCNA, Xatr, and Xhus1, each displayed their own characteristic salt elution profile.

We immunoprecipitated Claspin from the salt eluates and immunoblotted for various key replication and checkpoint proteins. We could readily detect Xcdc45, RPA70, and RFC40 in the anti-Claspin immunoprecipitates from the 0.5 M NaCl eluate, and, to a lesser extent, in the 0.75 M eluate (Figure 1B). The interaction between Claspin and RFC40 was very strong, remaining even in the presence of 1 M NaCl. It has been established that Xcdc45, Claspin, and Pol ϵ load onto replication origins around the same time (Mimura *et al.*, 2000; Lee *et al.*, 2003). Therefore, we also immunoblotted the anti-Claspin immunoprecipitates with anti-Pol ϵ antibodies, but we could detect only a very faint signal for Pol ϵ in the 0.5 and 0.75 M NaCl eluates (our unpublished data). However, we were able to demonstrate an interaction between Claspin and Pol ϵ by a different method (see below). We could not detect the specific presence of Pol α or Pol δ in anti-Claspin immunoprecipitates by immunoblotting with anti-Pol α or anti-Pol δ antibodies (our unpublished data). Finally, we could not find Xatr, Xhus1, Xorc2, Xmcm7, or PCNA in anti-Claspin immunoprecipitates from any of the salt eluates. These observations argue that our procedure detects specific protein–protein interactions that become established on chromatin. Furthermore, the absence of abundant DNA binding proteins such as Xorc2 indicates that these immunoprecipitates do not contain DNA fragments with contaminating proteins.

To identify Claspin-binding proteins in a more general manner, we analyzed anti-Claspin immunoprecipitates by silver staining (Figure 1C). We observed specifically associated bands at 150, 140, and 65 kDa as well as a cluster of bands at 36–40 kDa. Analysis by nanoelectrospray tandem mass spectrometry indicated that p150 and p65 correspond to the largest subunits of RFC (RFC140) and RPA (RPA70), respectively. In addition, p36-40 contained all four small subunits of RFC (e.g., RFC36, RFC37, RFC38, and RFC40). p140 is still under investigation. We verified these associations by immunoblotting anti-Claspin immunoprecipitates with specific antibodies that recognize the *Xenopus* versions of RFC140 and RPA70 (Figure 1D). We could also detect the presence of RFC37 and RFC40 by immunoblotting with antibodies against these proteins. Because the small RFC subunits are also present in other clamp loader complexes, such as the one containing Rad17, we asked whether Claspin could also associate with Rad17. As shown in Figure 1D, Xrad17 could also be found in anti-Claspin immunoprecipitates from chromatin by immunoblotting with anti-Xrad17 antibodies.

Claspin Interacts Successively with Xcdc45 and RFC Complexes

To corroborate these results, we carried out various reciprocal immunoprecipitation experiments. First, we asked whether complexes containing Xcdc45 and Claspin could also be identified by immunoprecipitation with anti-Xcdc45 antibodies. As shown in Figure 2A, we could clearly detect Claspin as well as RPA70, RFC40, and Xrad17 in anti-Xcdc45 immunoprecipitates from 0.5 M NaCl eluates of chromatin.

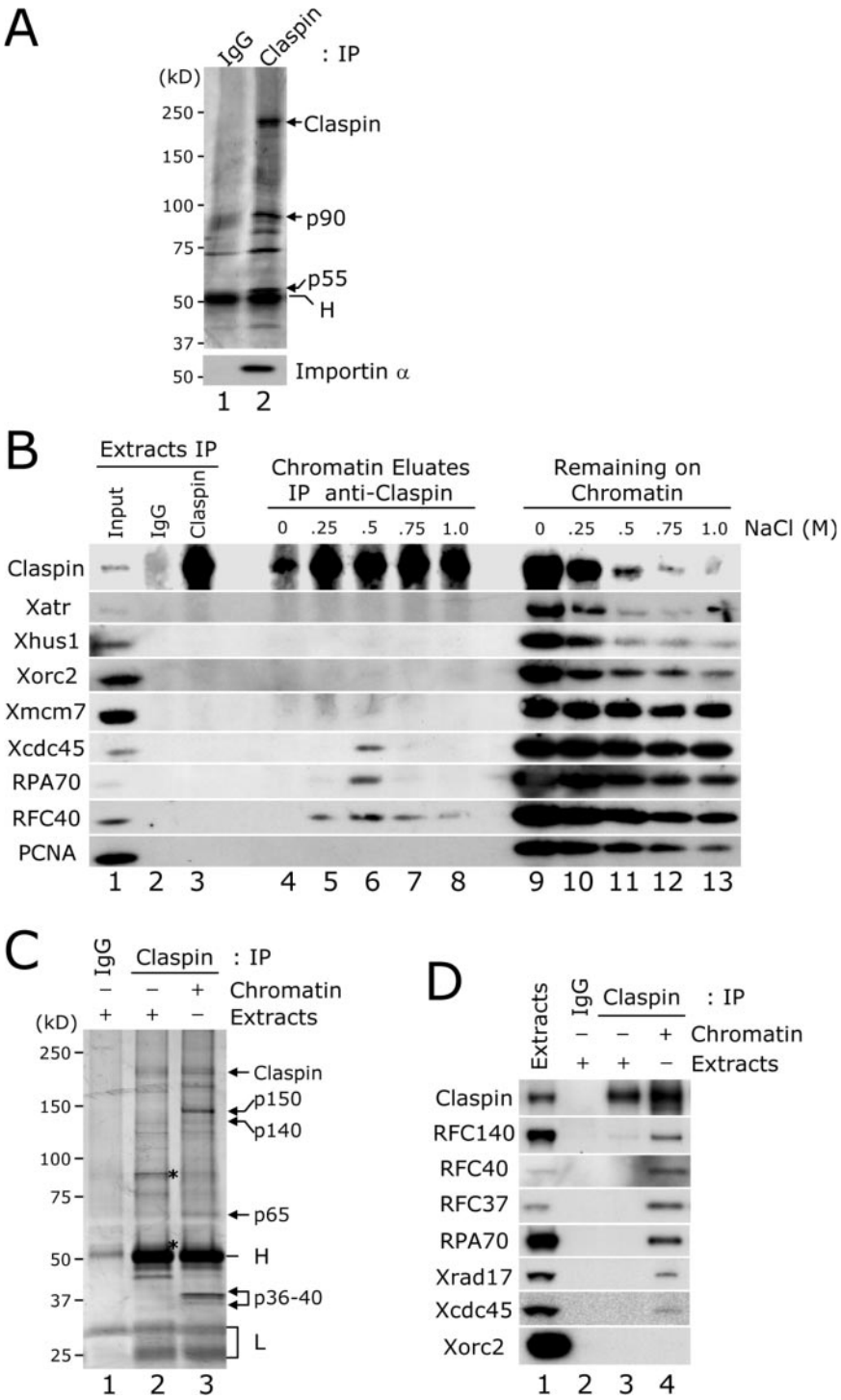

Figure 1. Identification of Claspin-interacting proteins in cytoplasmic and chromatin fractions from *Xenopus* egg extracts. (A) Claspin-binding proteins from egg extracts. IP was performed with control (lane 1) or anti-Claspin antibodies (lane 2) from interphase egg extracts. Samples were analyzed by silver staining (top) and immunoblotted for *Xenopus* importin α (bottom). H, IgG heavy chain. (B) Interaction of Claspin with proteins on chromatin. Chromatin-binding proteins were eluted from reconstituted nuclei (derived from 250 μl of egg extract) with various concentrations of NaCl and immunoprecipitated with anti-Claspin antibodies (lanes 4–8). Chromatin fractions after salt elution were loaded in lanes 9–13. In parallel, whole egg extracts (50 μl) were immunoprecipitated with control (lane 2) and anti-Claspin antibodies (lane 3). Lane 1 depicts 1 μl of egg extract. Samples were immunoblotted for the indicated proteins. (C) Silver staining of Claspin-binding proteins on chromatin. Interphase egg extracts were immunoprecipitated with control (lane 1) and anti-Claspin antibodies (lane 2). For lane 3, chromatin proteins from nuclei reconstituted in 1 ml of egg extract were eluted with 0.5 M NaCl and immunoprecipitated with anti-Claspin antibodies. Samples were subjected to SDS-PAGE and silver staining. Bands designated as Claspin (p180), p150, p140, p65, and p36–40 were prepared on a large scale for identification by mass spectrometry (see *Materials and Methods*). Asterisks in lane 2 denote importin α and β. H and L, IgG heavy and light chains. (D) Confirmation of Claspin-binding proteins by immunoblotting. Samples from C were immunoblotted for the indicated proteins (lanes 2–4). Lane 1 depicts 1 μl of egg extract.

In addition, we could find Xmcm7 and Xsld5, components of the MCM and GINS complexes, respectively, in anti-Xcdc45 immunoprecipitates of both 0.5 and 1 M NaCl chromatin eluates. Consistent with the results described above, we found Xcdc45, RPA70, RFC40, and Xrad17 in anti-Claspin immunoprecipitates that were prepared in parallel. However, we could not find either Xmcm7 or Xsld5 in anti-Claspin immunoprecipitates. Therefore, Claspin does not associate with Xcdc45 indirectly through either the MCM or GINS complexes.

Next, we carried out immunoprecipitations with anti-RPA70, anti-RFC40, and anti-Xrad17 antibodies. We could find Claspin in anti-RFC40 and anti-Xrad17, but not in anti-RPA70 immunoprecipitates (Figure 2B). We also performed immunoprecipitations of the chromatin eluates with antibodies against Xhus1, a component of the 9-1-1 complex. Consistent with the results obtained in the anti-Claspin immunoprecipitation studies, we could not detect any Claspin in anti-Xhus1 immunoprecipitates from chromatin eluates (Figure 2B). On the other hand, we could observe RPA70,

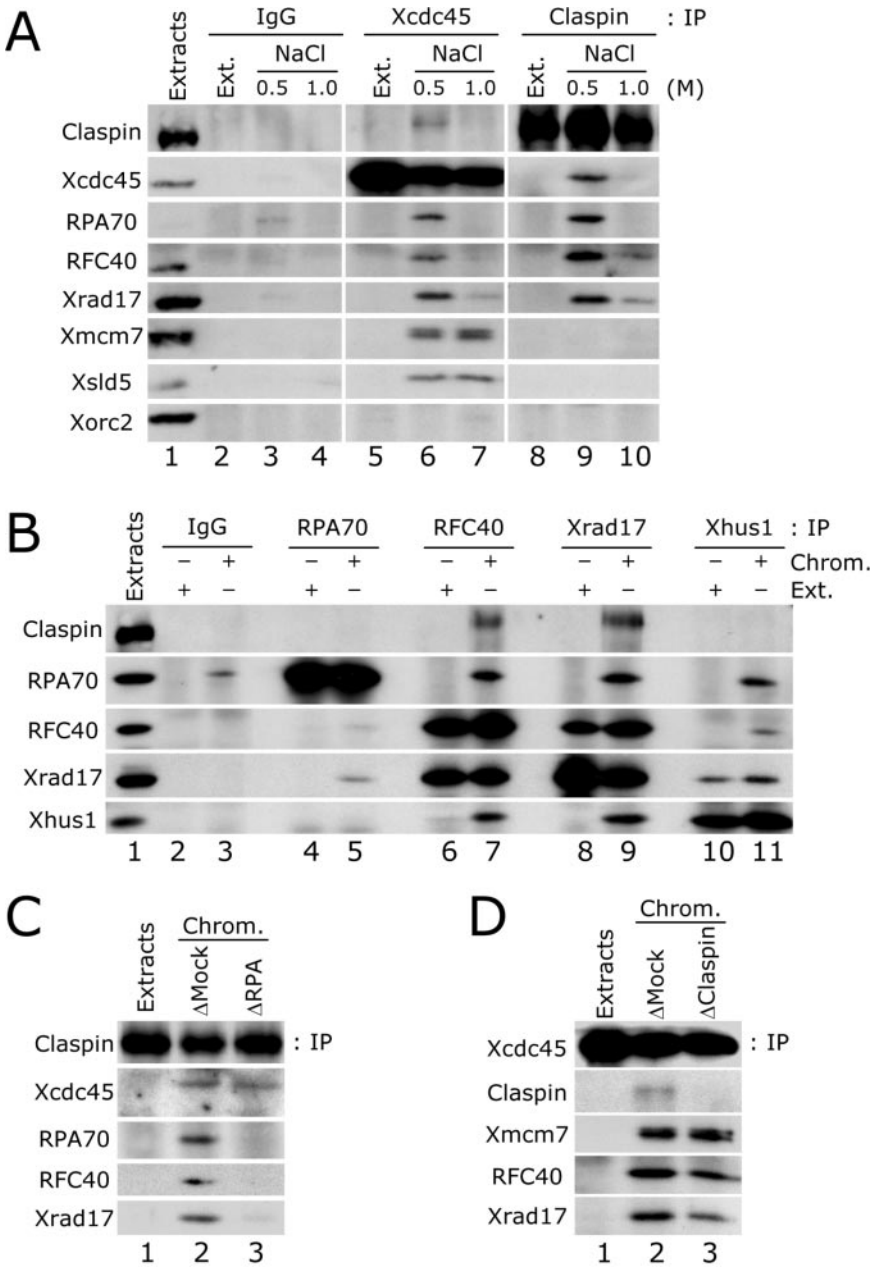

Figure 2. Binding relationships between Claspin and other proteins on chromatin. (A) Immunoprecipitation with anti-Xcdc45 antibodies. Control (lanes 2–4), anti-Xcdc45 (lanes 5–7), and anti-Claspin antibodies (lanes 8–10) were used for immunoprecipitation from egg extracts (lanes 2, 5, and 8) or chromatin eluates prepared with either 0.5 M (lanes 3, 6, and 9) or 1 M NaCl (lanes 4, 7, and 10). The samples were immunoblotted for the indicated proteins. Lane 1 depicts whole egg extract. (B) Association of Claspin with RFC40 and Xrad17. Egg extracts (lanes 2, 4, 6, 8, and 10) or 0.5 M NaCl chromatin eluates (lanes 3, 5, 7, 9, and 11) were immunoprecipitated with control (lanes 2 and 3), anti-*Xenopus* RPA70 (lanes 4 and 5), anti-*Xenopus* RFC40 (lanes 6 and 7), anti-Xrad17 (lanes 8 and 9), and anti-Xhus1 antibodies (lanes 10 and 11). Samples were immunoblotted for various proteins as indicated. (C) RPA-dependent association of RFC complexes with Claspin. Claspin was immunoprecipitated from egg extracts (lane 1) and from chromatin eluates obtained from either mock-depleted (lane 2) or RPA-depleted extracts (lane 3). Samples were immunoblotted for the indicated proteins. (D) Xcdc45 interacts with RFC independently of Claspin. Xcdc45 was immunoprecipitated from either egg extracts (lane 1) or chromatin eluates prepared from either mock-depleted (lane 2) or Claspin-depleted extracts (lane 3). Samples were immunoblotted for the indicated proteins.

RFC40, and Xrad17 in these immunoprecipitates. It should be noted that these results do not rule out an interaction of Claspin with the 9-1-1 complex, because it is possible that the chromatin elution procedure could disrupt such an association.

Previously, we reported that binding of Claspin to chromatin requires Xcdc45 but not RPA (Lee *et al*., 2003). Moreover, it is well established that the replicative and Rad17-containing RFC complexes associate with replication forks after RPA-stabilized unwinding of the DNA (You *et al*., 2002; Ellison and Stillman, 2003; Lee *et al*., 2003; Zou *et al*., 2003). Therefore, we examined whether the association of Claspin with RFC proteins also requires RPA. We immunodepleted RPA from egg extracts, prepared salt eluates of chromatin fractions from these extracts, and then immunoprecipitated Claspin from these eluates. As depicted in Figure 2C, we could detect Xcdc45 but not RFC40 or Xrad17 in anti-Claspin immunoprecipitates from RPA-depleted chromatin.

To characterize these interactions further, we analyzed the binding partners of Xcdc45 in the absence of Claspin. As shown in Figure 2D, Xcdc45 could bind well to RFC40 and Xrad17 even in Claspin-depleted extracts. From these results, we conclude that Claspin interacts first with Xcdc45 at replication forks and later associates with RFC complexes after RPA-stabilized unwinding of the DNA. At this juncture, Xcdc45 also forms connections with RFC proteins, but these interactions do not require the presence of Claspin. These results, along with the fact that the interactions of Claspin with Xcdc45 versus RFC40 display different salt sensitivities, imply that Xcdc45 and RFC complexes associate with Claspin independently.

A Conserved N-Terminal Domain Mediates Interaction of Claspin with Chromatin

To assess the functional significance of the interactions with other proteins on chromatin, we attempted to map the chro-

matin-binding region of Claspin. Because in vitro translated ^{35}S-Claspin bound to chromatin as efficiently as endogenous Claspin, we used ^{35}S-labeled fragments of Claspin for the initial phase of these experiments. We incubated various truncated forms of Claspin in extracts containing aphidicolin and caffeine and compared their ability to associate with chromatin. For any fragment that did not contain the last 56 amino acids of Claspin, which are essential for nuclear uptake, we incorporated an ectopic NLS into the polypeptide chain. Because expression of individual fragments was variable, we normalized the data by examining what percentage of each fragment in nuclear fractions from the extracts could associate with chromatin.

These studies indicated that a fragment containing residues 1–605 of Claspin binds exceptionally well to chromatin (Figure 3A). By contrast, various fragments from the C-terminal end of Claspin (e.g., 606-1285) showed little, if any, stable interaction with chromatin. The binding of the 1–605 fragment to chromatin was sensitive to the Cdk inhibitor p27, which inhibits firing of replication origins and thus prevents binding of full-length Claspin to replication forks. Furthermore, the interaction of this fragment with chromatin depended on Xcdc45 but not on RPA (Figure 3B), as for full-length Claspin.

Next, we asked whether the 1–605 fragment could associate with the same replication and checkpoint proteins as full-length Claspin. As shown in Figure 3C, we could immunoprecipitate the 1–605 fragment from chromatin fractions with antibodies against Xcdc45, RPA70, RFC40, and Xrad17. For these experiments, we also examined binding to three major eukaryotic DNA polymerases, namely, Pol α, Pol δ, and Pol ϵ. We could observe strong interaction of the 1–605 fragment with Pol ϵ, but no binding to either Pol α or Pol δ. Finally, we could detect little or no binding of this fragment to Xorc2, Xcut5, Xmcm7, PCNA, and Xhus1 in immunoprecipitations with antibodies against these respective proteins. From these observations, we conclude that the ability of Claspin to interact stably with chromatin resides in the 1–605 fragment.

In further mapping studies, we found that removal of the N-terminal 264 residues from the 1–605 fragment had no effect on chromatin-binding efficiency (Figure 3A). However, additional deletion of residues 265–331 abrogated binding. Similarly, removal of residues 566–605 from the opposite C-terminal end of this fragment also abolished interaction with chromatin. From these studies, we conclude that a region of Claspin stretching from residues 265–605 is involved in the interaction with chromatin. To corroborate these findings, we prepared a GST fusion protein containing residues 265–605 of Claspin as well as an ectopic NLS. The resulting GST-NLS-Claspin(265-605) protein could bind well to chromatin (Figure 3D). The binding was much higher in Claspin-depleted extracts. However, binding also occurred in mock-depleted extracts, where inclusion of the fragment also reduced the binding of endogenous Claspin to chromatin. Therefore, the isolated 265–605 fragment of Claspin fragment seems to compete with endogenous Claspin for a finite number of binding sites in chromatin.

We named the 265–605 region of Claspin the replication fork-interacting domain (RFID). Two recent studies have shown that human Claspin and its fission yeast homologue Mrc1 possess an in vitro DNA binding activity that is mediated by a DNA binding domain (DBD) (Sar *et al*., 2004; Zhao and Russell, 2004). The DBD in human Claspin (residues 149–340) corresponds closely to residues 150–331 of *Xenopus* Claspin, which overlap partially with the RFID. During our studies, we noticed an interesting structural feature of Claspin. Overall, Claspin is a very acidic protein (pI = 4.5), but it does contain four patches of basic amino acids (residues 265–331, 470–600, 721–783, and 1157–1285) with a pI value >10 (Figure 3A and Supplemental Figure S1). These segments, which we denoted BP1, BP2, BP3, and BP4, are highly conserved in metazoan Claspin, and the yeast Mrc1 proteins also possess similar basic segments. Notably, BP1 and BP2 define the boundaries of the RFID.

To evaluate directly whether the RFID can account for the chromatin-binding ability of full-length Claspin, we prepared various forms of full-length ^{35}S-labeled Claspin with mutations in this domain. We first examined relatively large deletions in the protein. Initially, we deleted BP1 (ΔBP1, residues 265–331) and 40 amino acids encompassing the C-terminal end of BP2 (ΔBP2′, residues 566–605). Moreover, we also produced a mutant (6A) in which six residues within BP1 that are highly conserved in metazoan Claspin proteins and conserved to some extent in the yeast Mrc1 proteins were changed to alanine. As shown in Figure 3E, when we added ^{35}S-labeled forms of the ΔBP1, ΔBP2′, and 6A mutants to undepleted egg extracts that contain their full complement of endogenous Claspin, binding to chromatin was abrogated or greatly reduced. By contrast, deletion of a poorly conserved segment (residues 376–425) from the center of the RFID, which we named the linker region (LK), had negligible effect on binding to chromatin (Figure 3E). Consistent with this observation, a bacterially expressed form of GST-NLS-Claspin(265-605)ΔLK bound well to chromatin (our unpublished data) and associated specifically with Xcdc45, Pol ϵ, RPA, and RFC40 in chromatin immunoprecipitation experiments (Figure 3F).

The BP1 Region of Claspin Is Not Required for Activation of Chk1

Next, we sought to replace endogenous Claspin in egg extracts with various RFID mutants to assess their abilities to function in checkpoint regulation. Toward this end, we created mutations in a baculovirus-expressed version of Claspin that contains His6 and FLAG tags at the N- and C-terminal ends, respectively (His6-Claspin-FLAG). First, we focused on the BP1 region. We produced ΔBP1 and 6A mutants of the baculovirus-expressed protein. We also prepared a larger deletion (residues 150–331) that removes all of BP1 and a block of upstream conserved sequences. The deleted region in this latter mutant corresponds to the whole DBD that was identified in human Claspin (Sar *et al*., 2004). When we examined binding to chromatin in undepleted egg extracts, we observed that none of the ΔBP1, 6A, and Δ(150-331) mutants of baculovirus-expressed His6-Claspin-FLAG could bind to chromatin (Figure 4A).

We proceeded to immunodeplete endogenous Claspin from the extracts and to replace it with equivalent amounts of these three mutants. First, we monitored the binding of these mutants to chromatin in extracts that now lacked endogenous Claspin. Unexpectedly, we observed that these three mutants could now bind very well to chromatin (Figure 4B). This observation indicates that, in the absence of competition from endogenous Claspin, these three mutants retain significant ability to interact with chromatin. Furthermore, binding of the ΔBP1 mutant to chromatin displayed the same sensitivity to p27 as wild type Claspin (Figure 4C), which argues this mutant binds specifically to replication forks. We next examined the ability of these mutants to mediate the activation of Xchk1 in response to treatment with aphidicolin. As shown in Figure 4B, all three mutants did not show any obvious difference from wild-type Claspin in the ability to promote the phosphorylation of Xchk1.

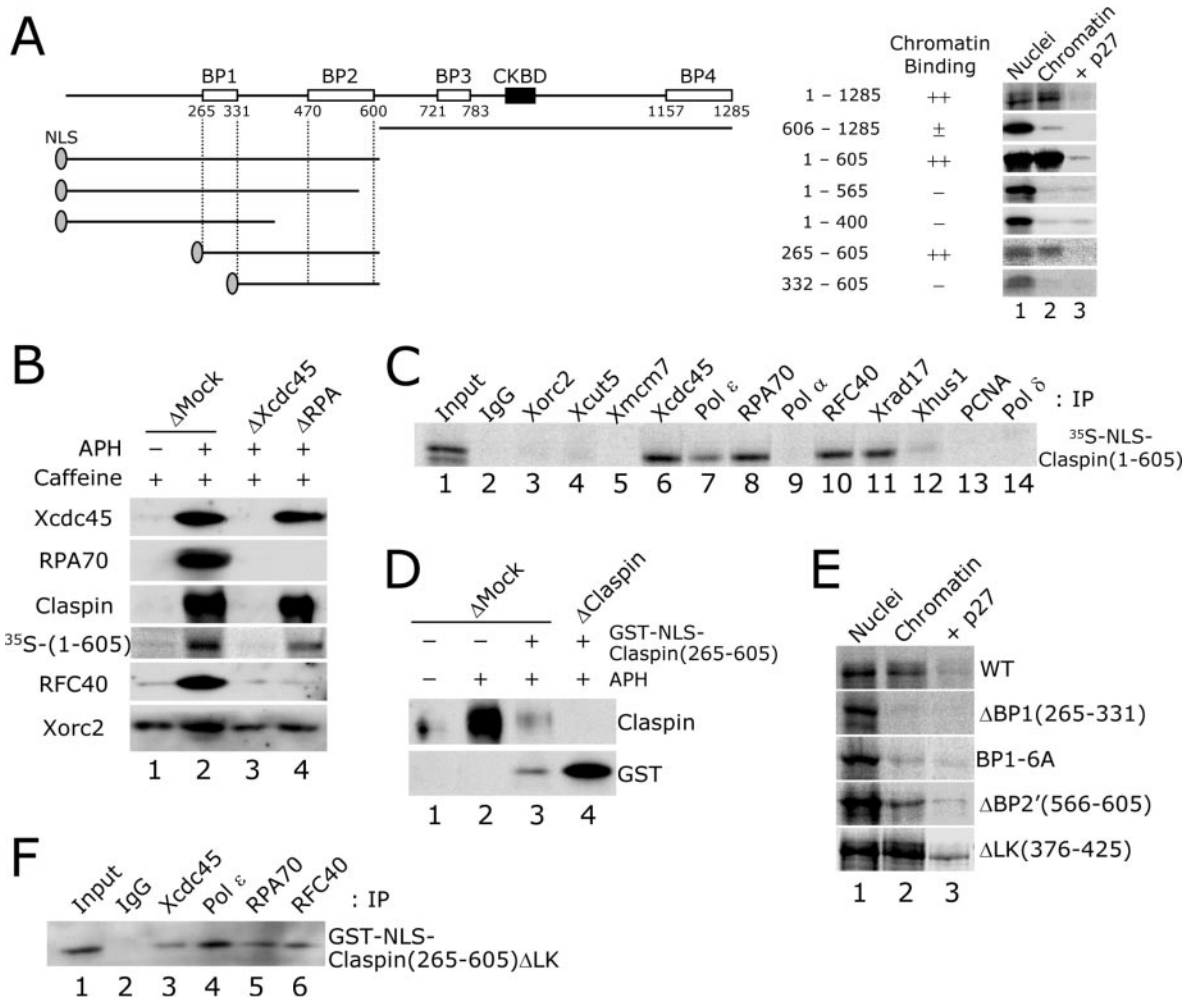

Figure 3. Identification and characterization of an RFID from Claspin. (A) Chromatin-binding fragments of Claspin. Various ^{35}S-labeled fragments from the N-terminal half of Claspin were incubated for 100 min in egg extracts containing sperm chromatin, aphidicolin, and caffeine. In this experiment, His6-Claspin(606-1285)-FLAG protein was used to represent the C-terminal half of the protein. Separate nuclear (lane 1) and chromatin fractions were prepared (lanes 2 and 3). To assess dependency of chromatin binding on S-phase cyclin-dependent kinase activity, extracts were treated with p27 (lane 3). Fragments were visualized by SDS-PAGE and phosphorimaging, except for the 606-1285 fragment, which was detected by immunoblotting with anti-FLAG antibodies. (B) The N-terminal domain of Claspin has very similar chromatin binding properties as full-length Claspin. Extracts were subjected to an immunodepletion procedure with control (lanes 1 and 2), anti-Xcdc45 (lane 3), or anti-RPA antibodies (lane 4). Extracts were incubated with ^{35}S-NLS-Claspin(1-605) and the indicated drugs. Binding to chromatin was assessed by phosphorimaging or immunoblotting for endogenous proteins as indicated. (C) Interaction of N-terminal domain of Claspin with various proteins on chromatin. ^{35}S-NLS-Claspin(1-605) was incubated in egg extracts as in A. Aliquots of chromatin eluates were immunoprecipitated with antibodies against the indicated proteins. The amount of bound ^{35}S-fragment was detected by SDS-PAGE and phosphorimaging. Lane 1 depicts 15% of the input chromatin eluate for each lane. (D) Interaction of bacterially expressed RFID with chromatin. Mock-depleted (lanes 1–3) and Claspin-depleted extracts (lane 4) were incubated in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of GST-NLS-Claspin(265-605). Chromatin fractions were prepared and immunoblotted with anti-Claspin (top) and anti-GST antibodies (bottom). (E) Contribution of the RFID to the binding of full-length Claspin to chromatin in undepleted extracts. ^{35}S-Labeled versions of full-length Claspin containing no deletion or deletions of residues 265–331 (ΔBP1), 566–605 (ΔBP2′), or 376–425 (ΔLK) were prepared. For the 6A mutant, the highly conserved residues Gln287, Arg288, Leu289, Pro298, Tyr299, and His300 were all changed to alanine. Nuclear accumulation (lane 1), chromatin binding (lane 2), and p27 sensitivity of chromatin binding (lane 3) were determined as described in A. (F) Interaction of the RFID with replication proteins on chromatin. GST-NLS-Claspin(265-605)ΔLK was incubated with egg extracts as in A. Aliquots of chromatin eluates were immunoprecipitated with the indicated antibodies and immunoblotted with anti-GST antibodies. Lane 1 depicts 15% of the input chromatin eluate for each sample.

Together, these results indicate that neither BP1 nor the whole DBD region is required for activation of Xchk1. Deletion of BP1 or the DBD region does weaken the interaction of Claspin with chromatin. However, without competition from endogenous full-length Claspin, mutants lacking these sequences bind very well to chromatin, which implies that the remaining sequences of Claspin are sufficient for chromatin binding.

We have previously shown that Claspin does not bind detectably to either single-stranded or double-stranded DNA in egg extracts (Lee *et al*., 2003; Kumagai *et al*., 2004). More recently, it was reported that recombinant human Claspin seems to have a stronger in vitro binding activity toward branched DNA than single-stranded or double-stranded DNA (Sar *et al*., 2004). Therefore, we tested directly whether endogenous Claspin in egg extracts can associate

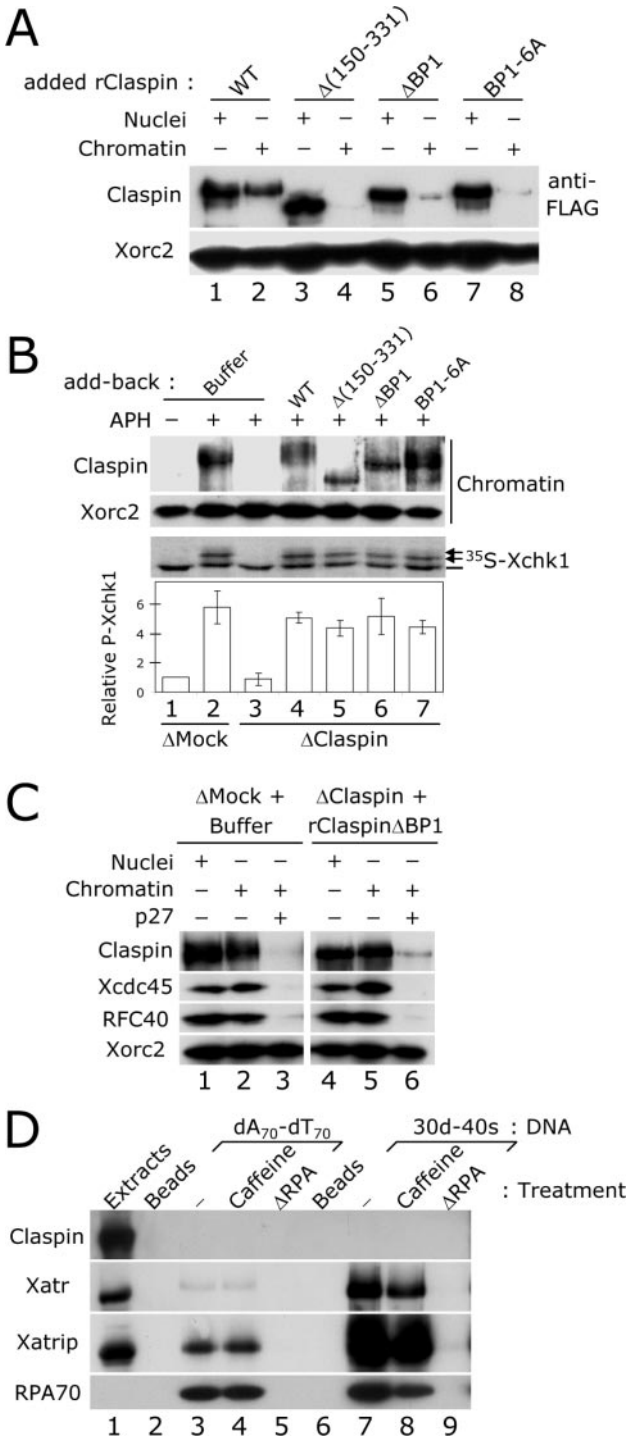

Figure 4. Role of the BP1 region from the RFID in binding of Claspin to chromatin and activation of Xchk1 in extracts lacking endogenous Claspin. (A) Various His6-Claspin-FLAG proteins were prepared from baculovirus-infected insect cells and added directly into undepleted egg extracts containing sperm chromatin, aphidicolin, and caffeine. The proteins included full-length Claspin (WT) (lanes 1 and 2), a deletion of residues 150–331 (lanes 3 and 4), and a deletion of residues 265–331 (ΔBP1, lanes 5 and 6), and the 6A mutant (lanes 7 and 8). Nuclear (lanes 1, 3, 5, and 7) and chromatin fractions (lanes 2, 4, 6, and 8) were immunoblotted with anti-FLAG antibodies (top). Samples were also immunoblotted for Xorc2 as a loading control (bottom). (B) Mock-depleted (lanes 1 and 2) and Claspin-depleted extracts (lanes 3–7) containing control buffer (lanes 1–3) or equivalent amounts of the indicated forms of His6-Claspin-FLAG (lanes 4–7) were incubated in the absence (lane 1) or presence (lanes 2–7) of aphidicolin. Chromatin fractions were immunoblotted for Claspin and Xorc2 (top two panels). Phosphorylation of ^{35}S-Xchk1 in nuclear fractions was assessed by phosphorimaging (third panel from top). For quantitation (bottom), the amount of shifted ^{35}S-Xchk1 was divided by the total ^{35}S-Xchk1 in each lane and normalized to the signal in the absence of aphidicolin (lane 1). Results are the mean ± SD for two experiments. (C) Binding of the ΔBP1 mutant to chromatin is sensitive to p27. Mock-depleted (lanes 1–3) and Claspin-depleted extracts (lanes 4–6) containing control buffer (lanes 1–3) or the ΔBP1 mutant of His6-Claspin-FLAG were incubated with aphidicolin and caffeine. p27 was also added in lanes 3 and 6. Nuclear (lanes 1 and 4) and chromatin fractions (lanes 2, 3, 5, and 6) were immunoblotted for the indicated proteins. (D) Claspin does not bind detectably to branched DNA in egg extracts. Magnetic beads coated with no DNA (lanes 2 and 6), $(dA)_{70}$-$(dT)_{70}$ (lanes 3–5), or 30d-40s DNA (a branched structure that contains a 30-mer double-stranded region and two 40-mer single-stranded regions) (lanes 7–9) were incubated in mock-depleted (lanes 3, 4, 7, and 8) or RPA-depleted egg extracts (lanes 5 and 9). Caffeine was included in some incubations (lanes 4 and 8). The beads were recovered, washed, and immunoblotted for the indicated proteins. Lane 1 shows 1 μl of egg extract.

stably with branched DNA. As the template (30d-40s DNA), we annealed two 70-mers that are complementary for 30 nucleotides at one end. As shown in Figure 4D, we could not detect any binding of Claspin to either the branched 30d-40s DNA or double-stranded $(dA)_{70}$-$(dT)_{70}$. On the other hand, we could readily observe binding of RPA, Xatr, and Xatrip to both templates. Furthermore, addition of caffeine or immunodepletion of RPA, both of which greatly increase the binding of Claspin to chromatin in aphidicolin-containing egg extracts (Lee *et al*., 2003), did not result in any binding of Claspin to either template. The concentration of Claspin in egg extracts (240 nM) (Kumagai and Dunphy, 2000) is well over the observed K_d for in vitro binding of recombinant human Claspin to branched DNA and thus should not be a limiting factor. Together, these results suggest that endogenous Claspin in egg extracts cannot bind stably to DNA alone in egg extracts and that interaction with proteins at the replication fork is necessary for stable association with chromatin.

The BP2 Region of Claspin Potentiates Its Chk1-activating Function

To study the role of the RFID more systematically, we prepared various mutant versions of Claspin containing serial N-terminal deletions and compared their chromatin binding properties in the absence of endogenous Claspin. We performed these experiments in the presence of aphidicolin and caffeine to maximize binding, but we obtained qualitatively similar binding in the presence of aphidicolin alone (our unpublished data). As shown in Figure 5A, both Claspin(265-1285) and Claspin(332-1285), which lack part of or the entire DBD region, bind as well as full-length Claspin to chromatin. On the other hand, further deletions from the N-terminal end resulted in a dramatic drop in association with chromatin. In particular, binding of the Claspin(470-1285), Claspin(606-1285), and Claspin(774-1285) fragments was markedly reduced, although the 470-1285 fragment did reproducibly bind somewhat better than the 606-1285 and 774-1285 fragments.

We proceeded to compare the ability of the various truncation mutants to mediate the activation of Xchk1. For these experiments, we immunodepleted endogenous Claspin from egg extracts and then added back the various recombinant Claspin proteins at two different concentrations to

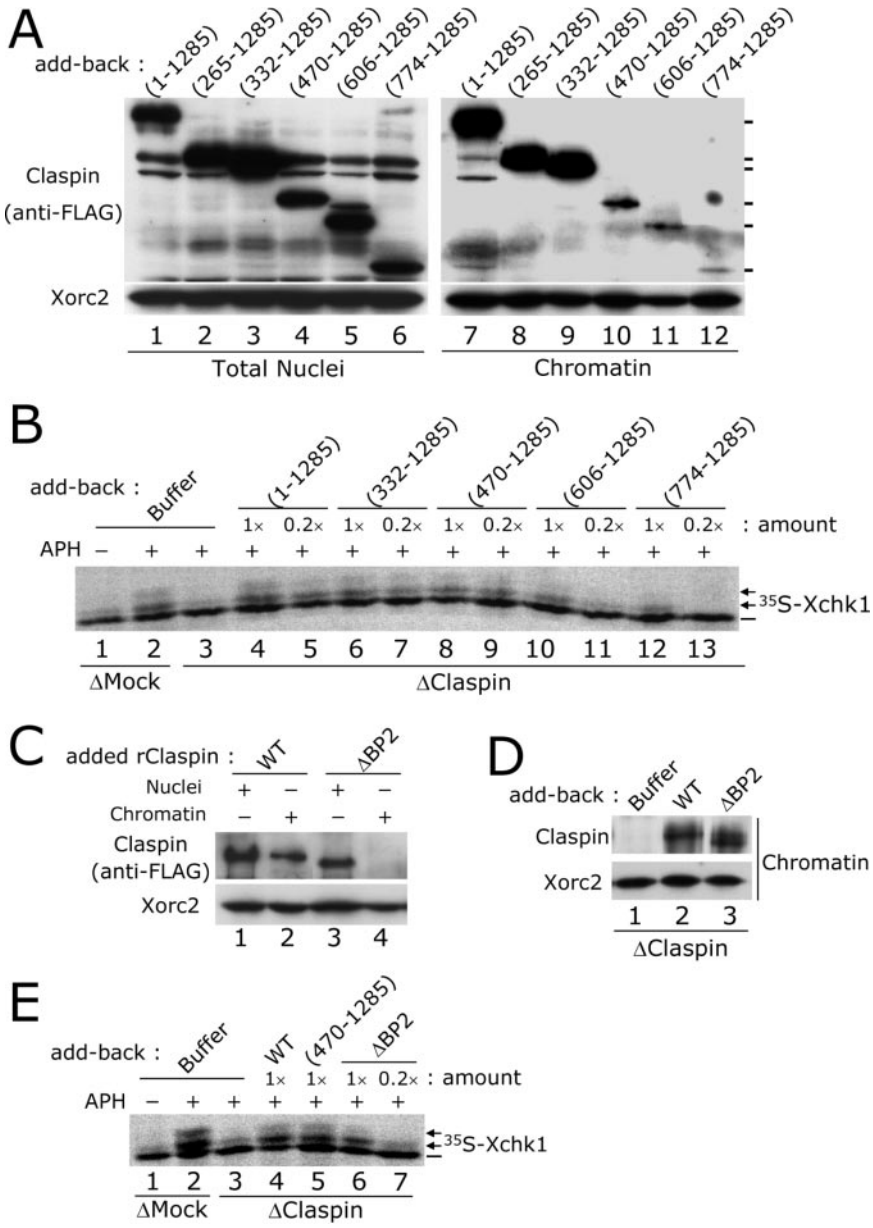

Figure 5. C-Terminal fragments of Claspin display reduced potency in mediating activation of Xchk1. (A) Full-length (residues 1–1285) and N-terminally truncated forms of Claspin (residues 265-1285, 332-1285, 470-1285, 606-1285, and 774-1285) were prepared as His6-Claspin-FLAG proteins in insect cells and added into Claspin-depleted extracts containing sperm chromatin, aphidicolin, and caffeine. Nuclear (lanes 1–6) and chromatin fractions (lanes 7–12) were prepared and immunoblotted with anti-FLAG (top) and anti-Xorc2 antibodies (bottom). The bars on right denote positions of the different recombinant Claspin proteins. (B) Mock-depleted (lanes 1 and 2) and Claspin-depleted extracts (lanes 3–13) containing control buffer (lanes 1–3) or the indicated forms of His6-Claspin-FLAG (lanes 4–13) were incubated in the absence (lane 1) or presence (lanes 2–13) of aphidicolin. Recombinant Claspin proteins were added at a molar concentration equal to (1×) or one-fifth (0.2×) that of endogenous Claspin. Phosphorylation of ^{35}S-Xchk1 in nuclear fractions from the extracts was assessed by phosphorimaging. (C) Wild-type His6-Claspin-FLAG (lanes 1 and 2) and a deletion mutant lacking residues 470–605 (ΔBP2, lanes 3 and 4) were added into undepleted egg extracts containing sperm chromatin, aphidicolin, and caffeine. Nuclear (lanes 1 and 3) and chromatin fractions (lanes 2 and 4) were immunoblotted with anti-FLAG (top) and anti-Xorc2 antibodies (bottom). (D) Claspin-depleted extracts were prepared and incubated in the presence of aphidicolin with buffer alone (lane 1), wild-type His6-Claspin-FLAG (lane 2), or the ΔBP2 mutant (lane 3). Chromatin fractions were immunoblotted for Claspin (top) and Xorc2 (bottom). (E) Phosphorylation of ^{35}S-Xchk1 was compared in extracts containing the full-length, 470-1285, and ΔBP2 versions of Claspin at the indicated concentrations as described in B.

compare their relative potencies. In particular, we used molar concentrations corresponding to the amount of endogenous Claspin (1×) and a fivefold dilution of this amount (0.2×), respectively. Finally, we added sperm chromatin and aphidicolin to the extracts and then monitored phosphorylation of Xchk1. We observed that the full-length, 332-1285, and 470-1285 polypeptides all could restore phosphorylation of Xchk1 in Claspin-depleted extracts at the both 1× and 0.2× concentrations (Figure 5B). However, the shorter fragments of Claspin, namely, 606-1285 and 774-1285 were significantly less potent at promoting the phosphorylation of Xchk1. This effect was most evident at the diluted concentration of these fragments. For example, an extract containing the 774-1285 mutant at the 0.2× concentration displayed little or no phosphorylation of Xchk1. These observations indicate that the C-terminal region of Claspin, which does not bind strongly to chromatin, shows reduced capacity to mediate the activation of Xchk1. However, the 470-1285 fragment seemed similar to full-length Claspin in the ability to mediate phosphorylation of Xchk1, even though it did not display the stable chromatin binding capacity of full-length Claspin.

These observations suggested that all or part of the BP2 region in Claspin (residues 470–600) might be important for checkpoint signaling. To pursue this possibility, we prepared a mutant of full-length His6-Claspin-FLAG with a deletion of residues 470–605 (ΔBP2). Consistent with the results observed for the smaller deletion of residues 566–605, the ΔBP2 mutant could bind to chromatin in Claspin-depleted extracts but not in undepleted extracts containing endogenous Claspin (Figure 5, C and D). When we examined checkpoint regulation, we observed that aphidicolin-treated extracts containing a fivefold dilution of the ΔBP2 mutant showed significantly reduced phosphorylation of Xchk1 (Figure 5E). Therefore, deletion of BP2 seems to compromise the checkpoint-signaling potency of Claspin.

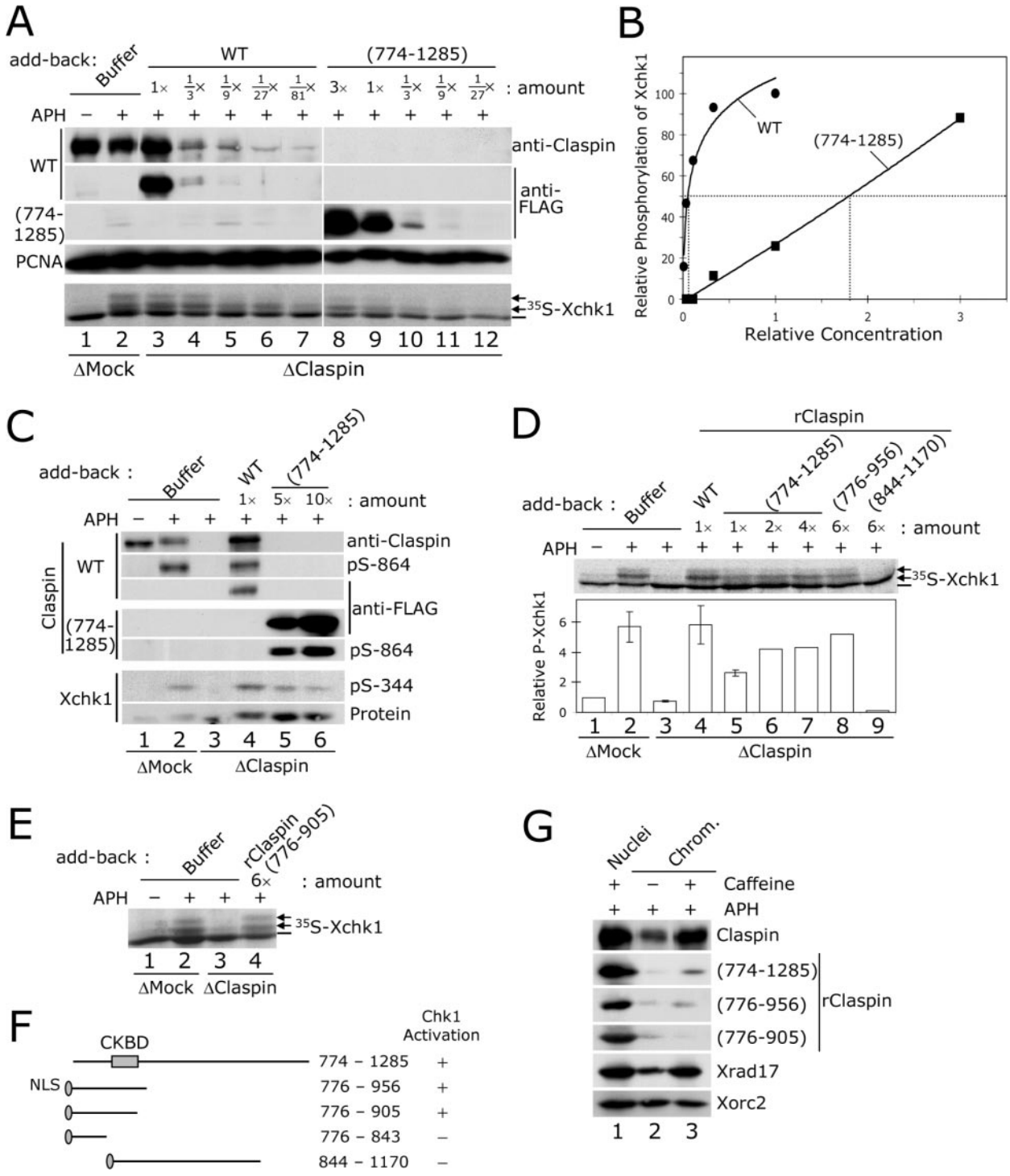

Figure 6. A small fragment from the C-terminal end of Claspin is sufficient at high concentrations for mediating phosphorylation of Xchk1. (A) Mock-depleted (lanes 1 and 2) and Claspin-depleted extracts (lanes 3–12) were incubated with control buffer (lanes 1 and 2) and the indicated dilutions of full-length His6-Claspin-FLAG (lanes 3–7) or the His6-Claspin(774-1285)-FLAG fragment (lanes 8–12) in the absence (lane 1) or presence (lanes 2–12) of aphidicolin. Nuclear fractions were prepared and immunoblotted with anti-Claspin antibodies (top), anti-FLAG antibodies to detect both full-length Claspin (second panel from top) and the 774-1285 fragment (third panel from the top), and anti-PCNA antibodies (fourth panel from top). Note that the anti-Claspin antibodies do not detect the 774-1285 fragment. Phosphorylation of ^{35}S-Xchk1 was assessed by phosphorimaging (bottom). (B) Quantitation of results from A for full-length Claspin (closed circles) and the 774-1285 fragment (closed squares). (C) Phosphorylation of the 774-1285 fragment on Ser864. Mock-depleted (lanes 1 and 2) and Claspin-depleted extracts (lanes 3–6) containing control buffer (lanes 1–3), full-length His6-Claspin-FLAG (lane 4), or His6-Claspin(774-1285)-FLAG (lanes 5 and 6) at the indicated amounts relative to endogenous Claspin were incubated in the absence (lane 1) or presence (lanes 2–6) of aphidicolin. Nuclear fractions were immunoblotted with anti-Claspin, anti-P-Ser864 of Claspin, and anti-FLAG antibodies as indicated. To examine phosphorylation of Xchk1, samples were immunoblotted with anti-P-Ser344 of Xchk1 and anti-Xchk1 antibodies (bottom two panels). (D) C-terminal fragments of Claspin fully rescue phosphorylation of Xchk1 at high concentrations. Mock-depleted (lanes 1 and 2) and

The C-Terminal Region of Claspin Contains a Small Chk1-activating Domain

These observations suggested that the C-terminal domain of Claspin has a significantly reduced potency for activation of Xchk1. To pursue this issue, we systematically compared the potencies of full-length Claspin and the C-terminal 774-1285 fragment for mediating the activation of Xchk1 (Figure 6, A and B). For this purpose, we immunodepleted endogenous Claspin from egg extracts and added back different dilutions of full-length Claspin and the 774-1285 fragment. This procedure enabled us to compare the relative potencies of the two proteins. Significantly, full-length Claspin was highly resistant to dilution, indicating that there is a large functional surplus of Claspin in egg extracts. For example, we observed half-maximal phosphorylation of Xchk1 in extracts containing a concentration of recombinant full-length Claspin ~0.055× the level of endogenous Claspin (Figure 6B). By contrast, a molar concentration of the 774-1285 fragment ~1.8-fold greater than that of endogenous full-length Claspin was required to elicit a similar extent of phosphorylation. Therefore, the 774-1285 fragment seems to be ~33-fold less potent than full-length Claspin. To evaluate whether the 774-1285 fragment acts by a similar mechanism as full-length Claspin, we examined phosphorylation of Claspin on Ser864, which is required for phosphorylation of Xchk1 (Kumagai and Dunphy, 2003). As shown in Figure 6C, the 774-1285 fragment became very efficiently phosphorylated on Ser864 in aphidicolin-treated extracts.

These results imply that the C-terminal region of Claspin should contain the minimal sequences necessary to carry out the biochemical process required for the ATR-dependent phosphorylation of Xchk1. To identify these sequences, we prepared various C-terminal fragments of Claspin as fusion proteins containing GST and an ectopic NLS. The fusion constructs were designed to contain the previously identified Chk1-binding domain of Claspin (CKBD, residues 847–903), in which serines 864 and 895 must be phosphorylated for binding to Xchk1 (Kumagai and Dunphy, 2003). We could observe phosphorylation of Xchk1 in the presence of a fragment from Claspin containing residues 776–956 but not an overlapping fragment containing residues 844-1170 (Figure 6D). Further analysis indicated that a 130-amino acid fragment containing amino acids 776–905 is sufficient for restoring phosphorylation of Xchk1 to Claspin-depleted extracts (Figure 6, E and F). We named this fragment the Chk1-activating domain (CKAD). The CKAD, as well as the longer 776–956 and 774-1285 fragments, displayed little or no binding to chromatin (Figure 6G). Importantly, however, the CKAD is considerably less potent than full-length Claspin. For example, in Figure 6E, we needed to add the CKAD fragment at a sixfold molar excess over the amount of endogenous Claspin, which is already in surplus, to observe a similar extent of Xchk1 phosphorylation.

Overall, these findings indicate that the capacity of Claspin to mediate the activation of Xchk1 resides in a small C-terminal region containing no more than 130 amino acids. However, sequences from the BP2 region greatly increase the overall potency of Claspin for activation of Xchk1. Deletion of BP2 weakens the interaction of Claspin with chromatin. On the other hand, a truncated form of Claspin (residues 470-1285) that contains BP2 does not bind stably to chromatin and nonetheless induces phosphorylation of Xchk1 as efficiently as full-length Claspin. These observations indicate that stable retention of Claspin on chromatin is not necessary for activation of Xchk1.

Figure 6 (cont). Claspin-depleted extracts (lanes 3–9) containing control buffer (lanes 1–3), full-length His6-Claspin-FLAG (lane 4), His6-Claspin(774-1285)-FLAG (lanes 5–7), GST-NLS-Claspin(776-956) (lane 8), and GST-NLS-Claspin(844-1170) (lane 9) at the indicated concentrations relative to endogenous Claspin were incubated in the absence (lane 1) or presence (lanes 2–9) of aphidicolin. Phosphorylation of ^{35}S-Xchk1 was detected with a phosphorimager (top) and quantitated (bottom). Results are mean ± SD for two experiments. (E) The 776–905 fragment of Claspin is sufficient at a high concentration for full phosphorylation of Xchk1. Mock-depleted (lanes 1 and 2) and Claspin-depleted extracts (lanes 3 and 4) were incubated with control buffer (lanes 1–3) or GST-NLS-Claspin(776-905) (lane 4) at a molar concentration sixfold higher than that of endogenous Claspin and examined for phosphorylation of ^{35}S-Xchk1. (F) Summary for identification of the CKAD from Claspin. The CKBD is denoted with the shaded box. (G) Fragments from C-terminal half of Claspin display little or no stable binding to chromatin. Extracts were incubated with His6-Claspin(774-1285)-FLAG, GST-NLS-Claspin(776-956), or GST-NLS-Claspin(776-905) in the presence of aphidicolin (lane 2) or aphidicolin plus caffeine (lanes 1 and 3). Polypeptides were added at 4–6× the molar concentration of endogenous Claspin. Nuclear (lane 1) and chromatin fractions (lanes 2 and 3) were immunoblotted with anti-FLAG or anti-GST antibodies to detect recombinant Claspin proteins as appropriate. Samples were also immunoblotted for endogenous Claspin, Xrad17, and Xorc2.

DISCUSSION

One characteristic of the checkpoint mediator protein Claspin is that it interacts specifically with both normal and stalled DNA replication forks during S phase. In this report, we have investigated the mechanism and functional significance of this interaction. To this end, we have mapped the regions of Claspin that enable binding to chromatin and searched for chromatin-bound proteins that mediate this interaction. Our studies have indicated that Claspin contains a relatively large RFID that is responsible for interaction with chromatin. This domain associates with Xcdc45, RPA, and both the replicative and Rad17-containing RFC complexes (Figure 7). We have also established that there is specific binding of Claspin to one of the major replicative DNA polymerases, namely, Pol ε. Therefore, Claspin interacts with both essential replication proteins and a key checkpoint regulator on chromatin. Consistent with these observations, it has been reported that budding yeast Mrc1 and Cdc45 can be coimmunoprecipitated from nuclease digests of chromatin (Katou *et al*., 2003). However, these studies did not resolve whether Mrc1 and Cdc45 associate by protein–protein interactions or simply bind near one another on the same digested DNA fragments.

Claspin Interacts Successively with Replication Fork Proteins

We have found that Xcdc45 forms a complex with Claspin without loading of RPA or RFC onto chromatin. By contrast, RFC depends upon the loading of RPA to interact with Claspin. These dependencies reflect the hierarchy in which these proteins associate with chromatin. Therefore, our results indicate that upon binding to chromatin Claspin interacts first with Xcdc45. After unwinding of the DNA, loading of RPA, and synthesis of initiating primers, the replicative RFC complex would recognize the 3′ recessed ends of nascent DNA strands. At this point, RFC would also become incorporated into a complex with Claspin. We also find that Claspin interacts with the Rad17-containing RFC complex. Recent evidence has indicated that the Rad17-containing complex interacts with the 5′ recessed ends of DNA strands (Ellison and Stillman, 2003; Zou *et al*., 2003). Thus, Claspin

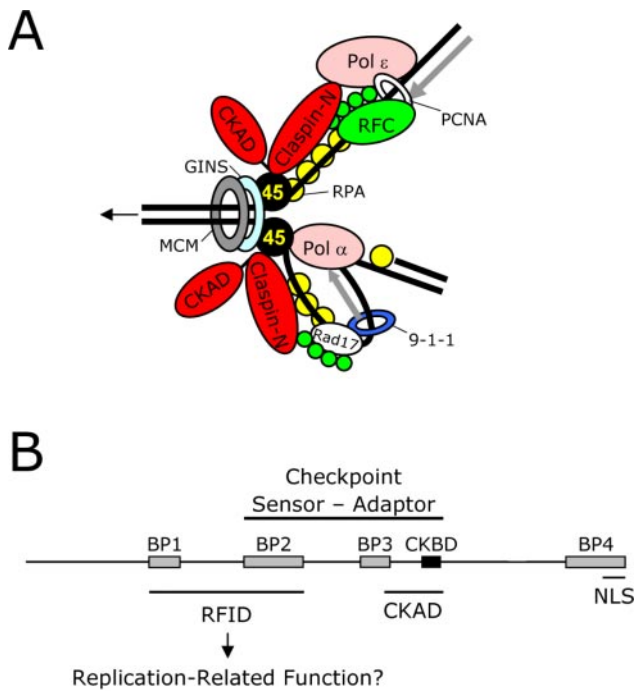

Figure 7. Model for the interaction of Claspin with stalled replication forks. (A) Diagram summarizes the protein–protein interactions of Claspin at replication forks. See text for discussion. (B) Summary of the different functional domains within Claspin.

would be in a position to participate in the detection of an array of different DNA replication intermediates (Figure 7A).

Role of Claspin as a Checkpoint Sensor Protein

One important question in the cell cycle checkpoint field involves the issue of how cells detect the presence of incompletely replicated or damaged DNA. Numerous studies in recent years have suggested that cells use a combinatorial mechanism to detect and discriminate between arrays of checkpoint-triggering signals (Sancar *et al*., 2004). Mediator proteins such as Claspin and the BRCT-containing proteins can provide additional modes of discrimination to checkpoint-sensing mechanisms, and indeed they may be crucial for cells to distinguish between different checkpoint-inducing signals. The fact that Claspin associates strongly with replication forks raised the possibility that this binding would be closely related with the ability of Claspin to mediate the activation of Xchk1. Interestingly, however, we find that certain fragments of Claspin retain the ability to mediate the activation of Xchk1 without being able to associate stably with chromatin. These fragments fall into two classes. Fragments from the C-terminal half of Claspin retain full efficacy for activation of Xchk1 but are significantly less potent. For example, the 774-1285 fragment is ~33-fold less potent than full-length Claspin for half-maximal activation of Xchk1. By contrast, a fragment containing residues 470-1285 does not bind to chromatin stably but is nonetheless comparable to full-length Claspin in its potency for activation of Xchk1. Significantly, this fragment retains a substantial portion of the RFID, namely, BP2 (residues 470–600) (Figure 7B). The presence of the BP2 region may allow the 470-1285 fragment to interact transiently with replication forks and thereby enhance the ability of Claspin to mediate the activation of Xchk1. Alternatively, it is possible that the BP2 region increases the activity of Claspin as a mediator by a mechanism that does not involve any interaction with chromatin. However, our mapping studies have clearly indicated that this region is important for optimal interaction with chromatin. For example, deletion of BP2 (residues 470–600) from full-length Claspin impaired chromatin binding in egg extracts containing all of its endogenous Claspin, indicating that this mutant cannot compete effectively with normal Claspin. Furthermore, in Claspin-depleted extracts, the ΔBP2 mutant can bind to chromatin, but it displays significantly reduced ability to mediate the activation of Xchk1. Therefore, this mutant seems to associate with chromatin in an aberrant manner that is not compatible with normal activation of Xchk1.

Recently, it has been shown that human Claspin and its fission yeast relative Mrc1 possess a DBD (Sar *et al*., 2004; Zhao and Russell, 2004). The DBD in human Claspin (residues 149–340) is highly homologous to residues 150–331 in *Xenopus* Claspin and contains the BP1 region of the RFID that we have identified in this study. Our results indicate that the DBD region is not essential either for interaction with chromatin or for activation of Xchk1. We found that versions of Claspin that lack this region, such as the Δ(150-331) and 332-1285 constructs, bind very well to chromatin in Claspin-depleted extracts and display a comparable potency with full-length Claspin for mediating the activation of Xchk1. Nonetheless, these mutants do seem to have a lower affinity for replication forks in that they are unable to bind to chromatin in undepleted extracts containing the full complement of endogenous Claspin. The physiological role of the DBD in human Claspin is not known. Fission yeast harboring a version of Mrc1 with a mutated DBD displayed a modest increase in sensitivity to hydroxyurea and a partial defect in the DNA replication checkpoint. However, the abilities of this mutant to associate with chromatin in fission yeast cells and to mediate the activation of Cds1, the checkpoint effector kinase downstream of Mrc1, have not yet been described. We suspect that the DBD/BP1 region may have a role in some other function of Claspin besides mediating the activation of Xchk1.

Identification of a Minimal Chk1-activating Domain

Another finding of this work is that a very small fragment of Claspin (the CKAD), only 130 amino acids long, is fully sufficient at high concentrations to sustain the Xatr-dependent activation of Xchk1 in aphidicolin-treated extracts. We can detect little or no association of the CKAD with chromatin, which reinforces the concept that stable binding of Claspin to chromatin is not obligatory for checkpoint activation. Nonetheless, even this small fragment presumably interacts transiently with sites of replication to collaborate with replication fork-associated Xatr in the phosphorylation of Xchk1. Similarly, we can never detect binding of Xchk1 to chromatin (our unpublished data), which implies that Xchk1 would also interact only transiently with Xatr-Xatrip and Claspin at replication forks. Consistent with this idea, Claspin has a low affinity for the fully activated form of Xchk1, which presumably dissociates from Claspin immediately upon kinase activation (Jeong *et al*., 2003). Together, these observations indicate that Xatr-Xatrip, Claspin, and Xchk1 associate evanescently with one another during the process that results in activation of Xchk1.

Stable Chromatin Binding of Claspin May Reflect an Additional Function

Our observations support the possibility that the RFID has another function in addition to initial activation of Xchk1. In the absence of Claspin, DNA replication in egg extracts occurs somewhat more slowly than normal (Lee *et al*., 2003). Interestingly, overexpression of Claspin in human cells enhances the rate of cell proliferation (Lin *et al*., 2004). Furthermore, it is well established that the budding yeast Mrc1 protein, the apparent functional counterpart of Claspin, has a role in DNA replication. Yeast mutants lacking Mrc1 replicate their DNA more slowly, accumulate DNA damage in S phase, and exhibit defects in sister chromatid cohesion (Alcasabas *et al*., 2001; Osborn and Elledge, 2003; Xu *et al*., 2004). Although the exact role that Claspin/Mrc1 plays in S-phase regulation remains to be established, our data raise the possibility that the stable retention of Claspin on chromatin may be related to this function.

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