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Drf1-dependent Kinase Interacts with Claspin through a Conserved Protein Motif*[S]

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The Dbf4/Drf1-dependent kinase (DDK) is required for the initiation of DNA replication in eukaryotes. Another protein, Claspin, mediates the activation of a cellular checkpoint response to stalled replication forks and is also a regulator of replication. In this study, we found that DDK phosphorylates Claspin *in vitro* and forms a nuclear complex containing Cdc7, Drf1, and Claspin in *Xenopus* egg extracts. In addition, purified Claspin and DDK are capable of a direct *in vitro* interaction. We identified a conserved binding site on Claspin required for its interaction with DDK. This site corresponds to the first of two sequence repeats in the Chk1-binding domain of Claspin. Furthermore, we have established that two amino acids in this motif, Asp^{861} and Gln^{866}, are essential for the interaction between Claspin and DDK. We found that mutant forms of Claspin incapable of interacting with DDK are still able to associate with and activate Chk1 in response to DNA replication blockages. However, Claspin-depleted egg extracts that have been reconstituted with these mutants of Claspin undergo DNA replication more slowly. These findings suggest that the interaction of DDK with Claspin mediates a checkpoint-independent function of Claspin related to DNA replication.

The maintenance of genomic stability is of paramount importance to living organisms. For example, proliferating cells must ensure the fidelity of chromosomal DNA replication. To accomplish this task, eukaryotic organisms have evolved a highly ordered series of steps to carry out DNA replication as well as a system of checkpoints to monitor the integrity of this process.

The stepwise process of eukaryotic DNA replication begins with the formation of prereplication complexes at replication origins (1, 2). The conversion of the prereplication complexes into active replication forks is promoted by the action of two kinases, the S phase cyclin-dependent kinase and the Dbf4/Drf1-dependent kinase (DDK).[2] In higher eukaryotes, there are two versions of DDK that consist of the kinase subunit Cdc7 and either Dbf4 or Drf1 as regulatory subunits (3–5). Cdc7 is constitutively expressed throughout the cell cycle, but its kinase activity is dependent upon association with either Dbf4 or Drf1. Hence, the relationship between Cdc7 and Dbf4/Drf1 is functionally analogous to the cyclin-dependent kinase/cyclin relationship (5, 6). In early *Xenopus* embryos, Drf1 is the primary Cdc7-binding partner that drives replication (7, 8). The most well established target of DDK activity is the MCM2–7 complex, which is an important constituent of the putative replicative helicase. DDK phosphorylates multiple components of the MCM complex (9–11). Moreover, the action of DDK is required for the recruitment of Cdc45 to chromatin (12, 13). It is thought that Cdc45 stimulates replicative helicase activity, which allows binding of DNA polymerases and other DNA replication elongation factors to origins (14–17).

Cells frequently encounter barriers during the course of DNA replication. Cells respond to the resulting stalled replication forks mainly by employing the ATR-Chk1 checkpoint pathway (18, 19). ATR is a sensor kinase that becomes activated in response to stalled replication forks and thereby transduces signals through the effector kinase Chk1. These signaling steps lead to the prevention of the inappropriate entry into mitosis and the inhibition of late replication origin firing (18, 19). ATR directly phosphorylates Chk1 by a mechanism that depends on the checkpoint mediator protein Claspin (20–22). Besides its well established function in mediating the activation of Chk1 in response to replication stress, Claspin also has roles in the regulation of unperturbed DNA replication and in the stabilization of disrupted replication forks (23–25). Claspin is a component of both normal and stalled replication forks. It interacts with a number of replication factors, including Cdc45, replication factor C, replication protein A, and DNA polymerase ϵ, and is necessary for a normal rate of fork progression (23–27). The association of Claspin with chromatin depends on Cdc45 and occurs at approximately the same time as polymerase ϵ (23). Likewise, the yeast ortholog of Claspin, Mrc1, moves with the replication fork and is necessary for a normal rate of DNA replication (28–33).

Despite the inhibition of late origin firing under conditions of checkpoint-inducing replication stress, the Cdc7/Drf1 kinase remains active (34–36). Several lines of evidence indicate that DDK may play a role in the regulation of checkpoint responses in metazoan systems. Drf1 accumulates on replication-arrested chromatin in an ATR- and Claspin-dependent manner in *Xenopus* egg extracts (34). In addition, human Cdc7 has been shown to phosphorylate and interact with human Claspin in HeLa cells (37). Moreover, when Cdc7 is knocked down by small interfering RNA, mammalian cells are more sensitive to agents that

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[2] The abbreviations used are: DDK, Dbf4/Drf1-dependent kinase; MCM, minichromosome maintenance complex; ATR, ATM and Rad3-related; CKBD, Chk1-binding domain; IP, immunoprecipitation; GST, glutathione *S*-transferase; CBD, cellulose-binding domain; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

elicit replication stress, such as hydroxyurea. These Cdc7-ablated cells are defective in the checkpoint-associated phosphorylation of Chk1 and Claspin (37). Finally, the ectopic addition of excess Dbf4-Cdc7 to *Xenopus* egg extracts or overexpression of Dbf4 in HeLa cells is able to down-regulate the ATR-Chk1 pathway signaling elicited by agents that induce replication stress (36).

For these reasons, we sought to examine the potential role of DDK in response to replication stress by further examining the relationship between DDK and the replication-monitoring protein Claspin. In this study, we established that DDK phosphorylates and interacts with Claspin in *Xenopus* egg extracts. Furthermore, we precisely mapped the site of this interaction to an evolutionarily conserved protein motif on Claspin. We found that two amino acids located within the region of Claspin shown to be essential for binding and activating Chk1 were required for the interaction with DDK. Interestingly, however, we demonstrate that this interaction is not required for Claspin to mediate activation of Chk1. These findings suggest that interaction with DDK reflects a checkpoint-independent function of Claspin.

EXPERIMENTAL PROCEDURES

Xenopus Egg Extracts—Interphase egg extracts were treated with 50 μg/ml annealed $(dA)_{70}$-$(dT)_{70}$ (pA-pT) to activate checkpoint responses as described previously (20). To produce chromatin containing DNA replication blocks, the extracts were incubated with demembranated sperm nuclei (3000–4000/μl) and 50 μg/ml aphidicolin.

Antibodies—Immunodepletion and immunoblotting of Claspin were performed with antibodies raised against residues 232–606 of Claspin (38). The anti-Claspin immunoprecipitations (IPs) in this paper were carried out with antibodies generated against residues 1–464 of Claspin (20). Anti-Drf1, anti-Cdc7, anti-Plx1, anti-replication protein A 70, and anti-Chk1 antibodies were described previously (23, 34, 39, 40). Anti-GST (Santa Cruz Biotechnology), anti-FLAG M2 (Sigma), and antibodies that detect phosphorylated Ser^{344} of *Xenopus* Chk1 (Cell Signaling Technology) were purchased from commercial sources. Anti-Scc2 serum (41) was a generous gift of T. Takahashi (Osaka University, Osaka, Japan).

Recombinant Proteins—GST-fused Claspin fragments 847–903 and 847–903(2AG) were described previously (42). DNA sequences for additional GST-fused Claspin fragments (amino acids 1–258, 258–518, 518–775, 776–851, 847–962, 961–1078, 1076–1285, 776–877, 776–867, 776–856, 847–877, 857–903, 868–903, 878–903, 902–961, 776–1078, and 878–961) were cloned into the pGEX-2T expression vector using PCR-based methods. Sequences encoding the minimal DDK binding site (residues 856–867) and point mutants thereof (D861E, Q866K, and D861E/Q866K) were cloned into pGEX-2T by producing oligonucleotide linkers that create 5′ BamHI and 3′ EcoRI cohesive ends upon annealing. These oligonucleotides were subsequently ligated into the linearized vector prepared with the appropriate restriction sites. Constructs for fragments of human Claspin (HuCKBD1, residues 908–919; HuCKBD2, residues 937–948; and HuCKBD3, residues 974–985) were prepared in a similar manner. The above GST-fused peptides were expressed in *Escherichia coli* BL21 CodonPlus (RIL) cells and purified with glutathione-agarose beads. Site-directed mutagenesis using the QuikChange kit (Stratagene) was performed to create the D861E, Q866K, and D861E/Q866K mutants with pFastBac-His_6-Claspin-FLAG (full-length) and pGEX-2T(776–1078) as templates. For baculovirus expression in Sf9 insect cells, the sequence encoding full-length *Xenopus* Drf1 was cloned into pFastBacHTa by standard methods. Recombinant baculoviruses were created using the Bac-to-Bac system (Invitrogen). Wild-type Chk1-GST-His_6 protein was expressed and purified in Sf9 insect cells as described previously (20). Full-length Claspin proteins were expressed in Sf9 insect cells and purified with M2 anti-FLAG-agarose beads (Sigma) and eluted with 3× FLAG peptide (Sigma) in HEPES-buffered saline. The recombinant *Xenopus* DDK complex was expressed in Sf9 cells that were co-infected with baculoviruses encoding His_6-Drf1 and a cellulose-binding domain (CBD)-tagged version of *Xenopus* Cdc7 (a gift of Dr. James Maller, U. of Colorado, Denver) (43). The complex was purified with nickel-agarose beads according to methods described previously (20).

Direct Binding Assay of the DDK-Claspin Interaction—Cellulose resin (Sigmacell 20) was hydrated in binding buffer (10 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, and 5 mM EGTA) and washed several times to remove smaller cellulose fibers. The cellulose was preblocked with binding buffer supplemented with 20 mg/ml bovine serum albumin for 30 min at 4 °C. Recombinant His_6-Claspin-FLAG and/or His_6-Drf1/CBD-Cdc7 was incubated for 30 min at 4 °C in binding buffer supplemented with 1 mM dithiothreitol; 1 mM phenylmethylsulfonyl fluoride; 10 μg/μl each of pepstatin, chymostatin, and leupeptin; and 2 mg/ml bovine serum albumin. Next, blocked cellulose (15 μl) was added to each reaction, and the incubation was continued for 25 min at 4 °C with agitation. Finally, the cellulose was washed five times with binding buffer and subsequently resuspended and boiled in 20 μl of SDS-PAGE sample buffer.

Immunoprecipitations, Immunodepletions, and GST Pulldowns—Interphase immunoprecipitations were carried out according to previously defined methods (34). Immunodepletions of Claspin from extracts as well as GST pulldowns from extracts were performed as in an earlier work (20).

Immunoprecipitation from Nuclear Lysates, Isolation of Whole Nuclei from Extracts, and Replication Assays—Nuclear IPs were performed by incubating 100 μl (per IP reaction) of extracts lacking or containing aphidicolin (4,000 nuclei/μl) for 65 min at room temperature and subsequently centrifuging the extracts through 1 ml of ice-cold sucrose cushion (20 mM HEPES-KOH, pH 7.5, 1 M sucrose, 80 mM KCl, 2.5 mM potassium gluconate, and 10 mM magnesium gluconate) at 6,100 × *g* for 5 min at 4 °C. The supernatant was removed, leaving a loose pellet, to which another 500 μl of sucrose cushion was added. The sample was centrifuged again as above. The supernatant was removed, and 25 μl of high salt lysis buffer (10 mM HEPES-KOH, pH 7.5, 300 mM NaCl, 2.5 mM EGTA, 0.1% CHAPS, and 0.5% Nonidet P-40) was added to resuspend the pellets. The samples were incubated for 10 min at 4 °C, diluted 4-fold with 75 μl of 20 mM HEPES-KOH (pH 7.5) to reduce the salt con-

centration, and subsequently centrifuged at 10,000 rpm for 10 min at 4 °C. For immunoprecipitations, the supernatant was incubated with agitation at 4 °C for 1 h with Affiprep protein A beads (Bio-Rad) that had been coated with the appropriate antibodies. The beads were then washed four times with a buffer containing 10 mM HEPES-KOH (pH 7.5), 80 mM NaCl, 2.5 mM EGTA, 20 mM β-glycerolphosphate, and 0.1% Nonidet P-40 and finally boiled in SDS-PAGE sample buffer. Whole nuclei were obtained by centrifuging 25 μl of extracts (mock depleted or Claspin-depleted) twice through a 500-μl sucrose cushion as described above and then resuspending and boiling the pellets in 20 μl of SDS-PAGE sample buffer. Assays of chromosomal DNA replication in egg extracts were performed with [α-^{32}P]dATP as described previously (23).

RESULTS

DDK Forms a Stable Complex with Claspin in S Phase Egg Extracts—An active form of the kinase Cdc7 can be isolated from interphase *Xenopus* egg extracts by immunoprecipitating the Drf1 protein (34). In the course of testing substrates for this kinase activity, we observed that Cdc7 was able to phosphorylate recombinant full-length His$_6$-Claspin-FLAG protein *in vitro* (supplemental Fig. S1, *lane 3*). This result led us to ask whether Drf1 and Claspin can interact with one another in egg extracts. To this end, we subjected interphase egg extracts to immunoprecipitation with either anti-Drf1 or anti-Claspin antibodies and then immunoblotted the immunoprecipitates for Claspin and Drf1, respectively. These extracts were also incubated in the absence or presence of the checkpoint-inducing DNA oligonucleotides (dA)$_{70}$-(dT)$_{70}$. We could easily detect Claspin in anti-Drf1 immunoprecipitates and vice versa. These interactions were not affected by the presence of (dA)$_{70}$-(dT)$_{70}$ (Fig. 1*A*). These results are consistent with studies on human Claspin (37).

To examine this finding in greater detail, we also prepared from interphase egg extracts lysates of replicating nuclei in which we had eluted proteins from chromatin by salt treatment. We immunoprecipitated these lysates with anti-Claspin, anti-Drf1, and anti-Cdc7 antibodies and then immunoblotted for all three proteins. In these experiments, we also found that Claspin, Cdc7, and Drf1 formed a specific complex (Fig. 1*B*). In addition, we observed that the formation of this complex was not affected by aphidicolin, a drug that causes the formation of stalled DNA replication forks in egg extracts. Recently, Cdc7 and Drf1 have been shown to form a stable complex with the cohesin loading protein Scc2 in *Xenopus* egg extracts. Moreover, this interaction is required for the loading of the cohesin complex onto chromatin during replication (41). In our experiments, we observed that Scc2 and Claspin also associate with each other in egg extracts in both the absence and the presence of aphidicolin (Fig. 1*B*).

Next, we sought to determine whether the interaction between DDK and Claspin was direct. For this purpose, we performed *in vitro* binding assays with recombinant DDK and Claspin. To prepare recombinant *Xenopus* DDK, we co-expressed His$_6$-Drf1 and CBD-tagged Cdc7 in Sf9 insect cells and then purified the complex with nickel-agarose beads. We then incubated the purified DDK complex with recombinant full-length His$_6$-Claspin-FLAG or buffer alone. We also incubated His$_6$-Claspin-FLAG without DDK as a control. Finally, we added cellulose resin to the incubations to isolate CBD-Cdc7

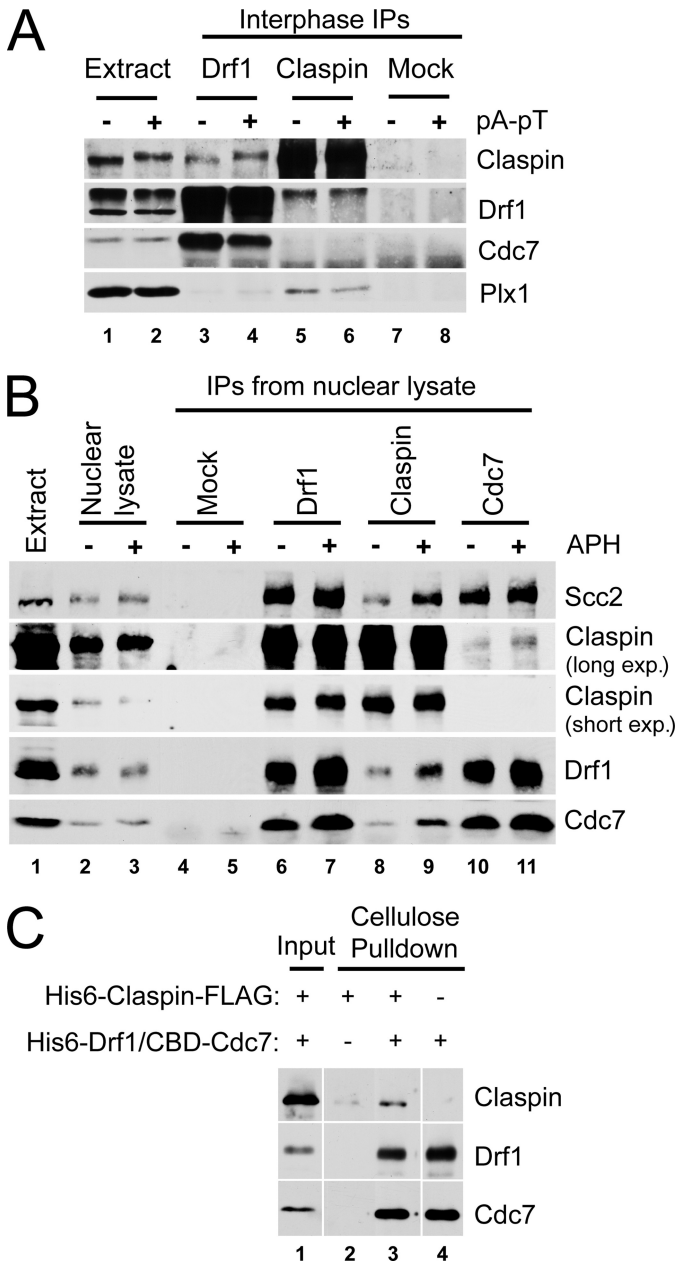

FIGURE 1. **Claspin forms a complex with Cdc7 and Drf1 (DDK) in *Xenopus* egg extracts.** *A*, IPs with control (Mock), anti-Drf1, and anti-Claspin antibodies were prepared from nuclei-free interphase egg extracts lacking or containing (dA)$_{70}$-(dT)$_{70}$ (pA-pT) and immunoblotted for Claspin, Drf1, Cdc7, and Plx1. *Lanes 1* and *2* depict 2% of pre-IP extracts. *B*, interphase extracts containing sperm nuclei (4,000/μl) were incubated in the absence or presence of aphidicolin (*APH*). Nuclear lysates were prepared and immunoprecipitated with control (*Mock*), anti-Drf1, anti-Claspin, and anti-Cdc7 antibodies. These immunoprecipitates were immunoblotted for Scc2, Claspin, Drf1, and Cdc7. The Claspin immunoblots are presented as both long and short film exposures. *Lane 1* is an aliquot of interphase egg extract, and *lanes 2* and *3* show 2% of the pre-IP nuclear lysate lacking or containing aphidicolin. *C*, *in vitro* binding assays for the interaction of His$_6$-Drf1/CBD-Cdc7 (DDK) and His$_6$-Claspin-FLAG. Full-length His$_6$-Claspin-FLAG (*lanes 2* and *3*) or buffer alone (*lane 4*) was incubated in the absence (*lane 2*) or presence of His$_6$-Drf1/CBD-Cdc7 (*lanes 3* and *4*). Cellulose pulldowns were immunoblotted for Claspin, Drf1, and Cdc7. *Lane 1* shows 10% of the reaction in *lane 3* before cellulose was added.

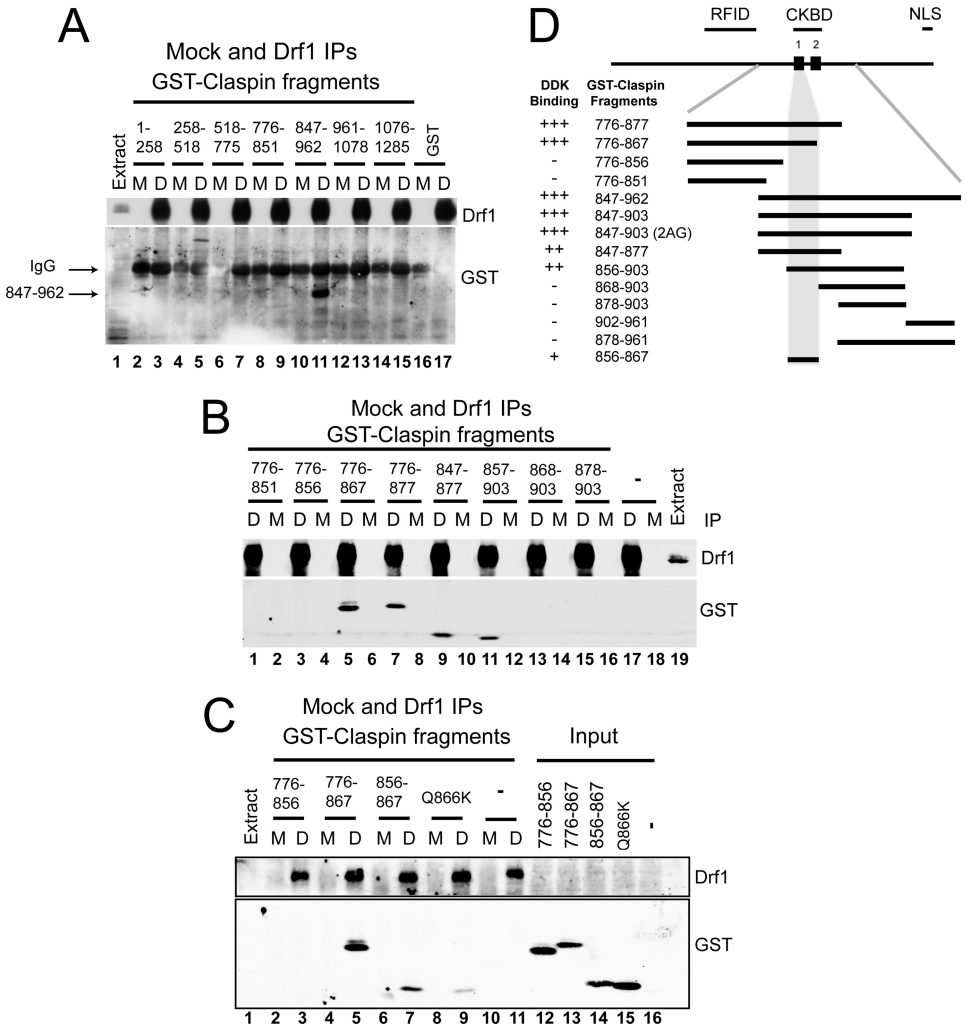

FIGURE 2. **The region of Claspin required for interaction with DDK maps to the first repeat sequence of the CKBD.** *A*, interphase egg extracts containing the indicated GST-Claspin fragments or GST alone were immunoprecipitated with anti-Drf1 (*D*) or control (mock, *M*) antibodies and immunoblotted for Drf1 and GST. The *top arrow* denotes a cross-reacting IgG band. *Lane 1* depicts 2% of the pre-IP extract. Input levels for the recombinant peptides are shown in supplemental Fig. 2*A*. *B*, interphase, nuclei-free egg extracts containing the indicated GST-Claspin fragments or no recombinant protein were immunoprecipitated with anti-Drf1 (*D*) or control (*M*) antibodies and immunoblotted for Drf1 and GST. *Lane 19* depicts an aliquot of pre-IP extract. Input levels (2% of the pre-IP extracts) for the recombinant peptides are shown in supplemental Fig. 2*B*. *C*, interphase, nuclei-free egg extracts containing GST-Claspin(776–856), GST-Claspin(776–867), GST-Claspin(856–867), GST-Claspin(856–867)-Q866K, or no recombinant protein were immunoprecipitated with anti-Drf1 (*D*) or control (*M*) antibodies and immunoblotted for Drf1 and GST (*lanes 2–11*). *Lane 1* depicts an aliquot of the initial extract. *Lanes 12–16* represent 2% of the pre-IP extracts containing the recombinant peptides listed above. *D*, *top panel*, a schematic depiction of several known domains of Claspin. The domains are the replication fork-interacting domain (*RFID*), the first and second repeats of the Chk1-binding domain (*CKBD*), and the nuclear localization sequence (*NLS*). *Bottom panel*, a summary of the abilities of Claspin peptide fragments to bind DDK.

and its associated His_6-Drf1 partner. We found that, in the presence of DDK, Claspin associated with the cellulose at a level significantly above that in the control incubation containing only His_6-Claspin-FLAG (Fig. 1*C*, *lanes 2* and *3*). Therefore, we conclude that the interaction between Claspin and DDK appears to be direct.

Mapping a DDK-binding Site on Claspin—To characterize further the interaction between DDK and Claspin, we sought to identify the minimal region of Claspin that is required for this binding. For this purpose, we first constructed a series of seven GST-fused Claspin fragments that spanned the entirety of the protein. Immunoprecipitations of Drf1 were carried out with interphase extracts that had been supplemented with these GST-Claspin protein fragments. We found that Drf1 strongly interacted with the Claspin fragment containing amino acids 847–962 (Fig. 2*A*, *lane 11*, and supplemental Fig. S2*A*). There was also a weak interaction of Drf1 with the fragment containing amino acids 258–518 (supplemental Fig. S2*A*, *lane 5*).

Subsequently, we focused on the 847–962 fragment, both because this interaction was very strong and because this fragment contains the Chk1-binding domain (CKBD), a region required for the binding and activation of Chk1 (42). The CKBD of Claspin is composed of two repeat sequences, each containing serines that are phosphorylated during a replication checkpoint response. To more finely map the sequence that is required for Claspin to associate with Drf1, we prepared a series of overlapping GST-fused Claspin peptide fragments ranging from amino acids 776 to 903. We found that peptide fragments starting at position 776 and ending at or before amino acid 856 could not interact with Drf1 (Fig. 2, *B* and *D*, and supplemental Fig. S2*B*). Conversely, Claspin fragments starting at or before residue 856 could associate with Drf1, whereas there was no binding of the 868–903 and 878–903 fragments (Fig. 2, *B* and *D*, and supplemental Fig. S3). Thus, the region comprising amino acids 856–867 of Claspin is required for this interaction (Fig. 2*D*). Interestingly, this region corresponds to the first repeat of the CKBD (856–867). Notably, fragments containing the second CKBD repeat (amino acids 887–898) failed to bind to Drf1. To ascertain whether this minimal region was sufficient for the interaction with DDK, we constructed a GST fusion protein containing only residues 856–867 from Claspin. When we tested this fragment in the binding assay, we could readily observe binding to Drf1 (Fig. 2*C*, *lane 7*). Thus, residues 856–867 of Claspin are both necessary and sufficient for recognition of Drf1.

It is striking that only the first CKBD repeat and not the second one could interact with Drf1, given that only two amino acids (namely, aspartate 861 and glutamine 866) are different between the repeats (Fig. 3*C*). Some Claspin homologs, includ-

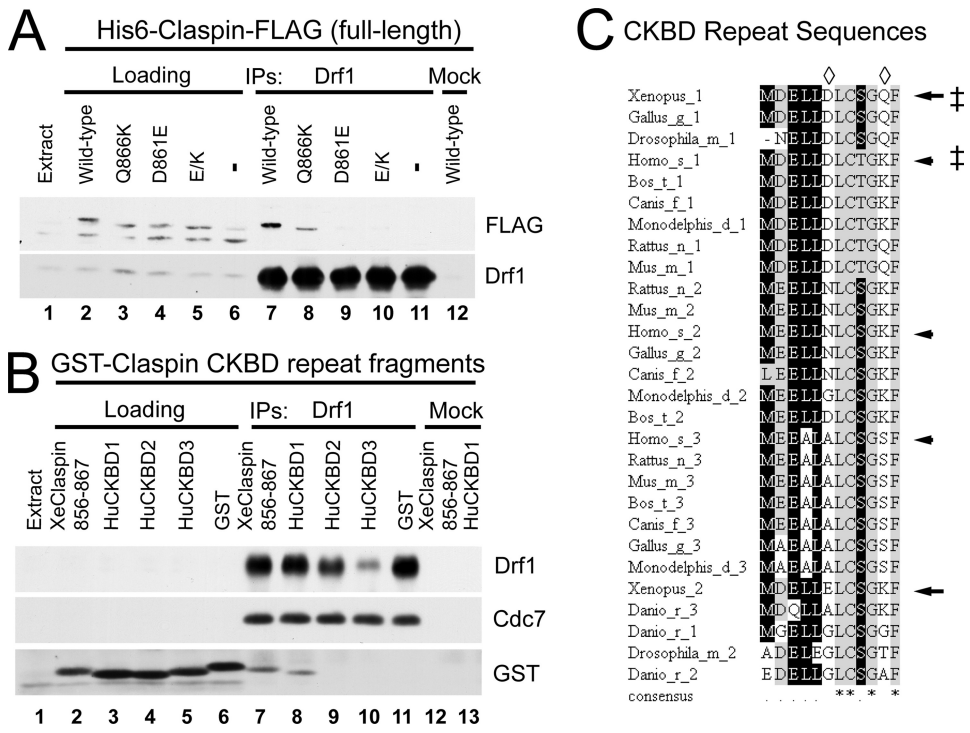

FIGURE 3. **Identification of conserved sites in Claspin required for interaction with DDK.** *A*, interphase, nuclei-free egg extracts containing full-length versions of wild-type His_6-Claspin-FLAG, His_6-Claspin-FLAG-Q866K, His_6-Claspin-FLAG-D861E, His_6-Claspin-FLAG-E/K, or no recombinant protein were immunoprecipitated with anti-Drf1 antibodies (*lanes 7–11*). Extracts containing wild-type His_6-Claspin-FLAG were also mock immunoprecipitated with control antibodies (*lane 12*). *Lanes 2–6* depict 2% of the pre-IP extracts supplemented with the recombinant proteins listed above. *Lane 1* shows an aliquot of interphase extract alone. The immunoprecipitates were immunoblotted with anti-FLAG and anti-Drf1 antibodies. *B*, interphase, nuclei-free egg extracts containing GST-XeClaspin(856–67), GST-HuClaspin-CKBD1, GST-HuClaspin-CKBD2, GST-HuClaspin-CKBD3, and GST were immunoprecipitated with anti-Drf1 antibodies (*lanes 7–11*). Extracts containing GST-Claspin(856–867) and GST-HuCKBD1 were also mock immunoprecipitated with control antibodies (*lanes 12* and *13*). *Lane 1* contains an aliquot of pre-IP interphase extract, and *lanes 2–6* depict 2% of the pre-IP extracts supplemented with the recombinant proteins listed above. The immunoprecipitates were immunoblotted for Drf1, Cdc7, and GST. *C*, a sequence alignment of the CKBD repeat sequences across a selection of metazoan Claspin homologs. The organisms represented here are *X. laevis* (South African claw-toed frog), *Gallus gallus* (domestic chicken), *Drosophila melanogaster* (fruit fly), *Homo sapiens* (human), *Bos taurus* (domestic cow), *Canis familiaris* (domestic dog), *Monodelphis domestica* (gray short-tailed opossum), *Rattus norvegicus* (Norway rat), *Mus musculus* (house mouse), and *Danio rerio* (zebrafish). The *arrows* point to the *X. laevis* Claspin CKBD repeats, and the *arrowheads* point to the *H. sapiens* CKBD repeats. *Asterisks* denote positions that are conserved in all CKBD repeats. *Diamonds* indicate crucial residues for binding of DDK, and *crosses* denote the CKBD repeats in *X. laevis* and *H. sapiens* that associate with DDK.

ing human Claspin, have three repeats instead of the two found in *Xenopus laevis* (44, 45). When we compared CKBD repeat sequences across a selection of metazoan Claspin homologs, we noticed that these two positions displayed the most variability (Fig. 3*C*). We initially focused on the position containing glutamine 866, which changes from a glutamine to a lysine in the second *Xenopus* repeat sequence. This variation represents a difference in charge. By contrast, the position corresponding to aspartate 861 contains a similar acidic residue (glutamate) in the second CKBD repeat.

We constructed a GST-fused version of the 856–867 fragment of Claspin in which glutamine 866 was changed to lysine (hereafter referred to as the Q866K mutant). Upon testing this mutated fragment, we observed that binding to Drf1 was significantly reduced but not completely abolished relative to the original fragment (Fig. 2*C*, *lane 9*, and supplemental Fig. S4*A*). Next, we proceeded to change aspartate 861 to glutamate (to generate the D861E mutant). We also prepared the D861E/Q866K double mutant (referred to as E/K). In the context of the 856–867 fragment, the D861E mutant displayed a more marked reduction of binding to Drf1 in comparison with the Q866K mutant. Moreover, the double E/K mutation essentially abolished the interaction with Drf1 (supplemental Fig. S4*A*). We observed similar results in a larger Claspin GST-fused fragment (776–1078) that contains the entire CKBD and is by itself sufficient to rescue activation of Chk1 in Claspin-depleted extracts (supplemental Fig. S4*B*) (24). Finally, we decided to test the capacity of these mutants to interact with DDK in the context of the full-length Claspin protein. We expressed and purified FLAG-tagged recombinant versions of wild-type, D861E, Q866K, and E/K full-length Claspin proteins from insect cells using a baculovirus protein expression system. The full-length proteins followed the same pattern as the smaller Claspin fragments in that binding of the Q866K mutant to Drf1 was reduced, whereas binding of the D861E single mutant and the double E/K binding was nearly eliminated (Fig. 3*A*).

Because the Claspin CKBD sequences are well conserved across metazoans, we decided to test whether these amino acid sites were important in the human ortholog of Claspin. At the amino acid position equivalent to aspartate 861 in *Xenopus* Claspin, the human Claspin CKBD repeats contain the residues aspartate, asparagine, and alanine in the first, second, and third repeat, respectively. Accordingly, we expressed and purified the three human Claspin CKBD repeats as GST-fused peptides, added them to *Xenopus* egg extracts, and assayed binding to Drf1 by immunoprecipitating with anti-Drf1 antibodies as above. We observed that the first human Claspin CKBD repeat (HuCKBD1) bound efficiently to Drf1, whereas the second and third repeats (HuCKBD2 and huCKBD3) did not bind at all (Fig. 3*B*, *lanes 8–10*). These findings suggest that the DDK-binding domain of Claspin may be conserved across metazoan species.

The Interaction of Claspin with DDK Is Separable from Its Role in Mediating the Activation of Chk1—Because we have determined that the region of Claspin that interacts with DDK also encompasses a key portion of its binding domain for Chk1, we decided to compare the ability of Claspin to associate with DDK *versus* Chk1. We first tested whether or not Chk1 could bind to mutants that were defective for the interaction with DDK. To this end, we performed GST pulldowns with our array

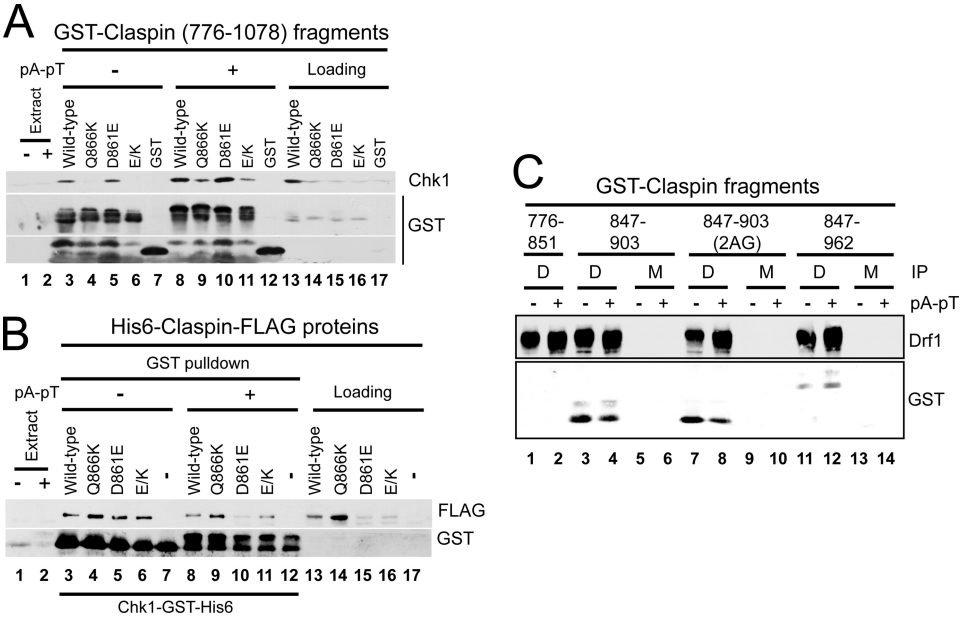

FIGURE 4. **Interactions of Claspin with DDK and Chk1 have distinct requirements.** *A*, interphase extracts, lacking or containing $(dA)_{70}$-$(dT)_{70}$ (pA-pT), were supplemented with wild-type GST-Claspin(776–1078), GST-Claspin(776–1078)-Q866K, GST-Claspin(776–1078)-D861E, GST-Claspin(776–1078)-E/K, and GST. The extracts were subjected to pulldowns with glutathione-agarose beads (*lanes 3–12*). *Lanes 13–17* depict 2% of the prepulldown extracts supplemented with the above recombinant peptides. *Lanes 1* and *2* depict extracts without and with oligonucleotides but with no added recombinant proteins. The pulldown fractions were immunoblotted for Chk1 and GST. *B*, interphase extracts, lacking or containing $(dA)_{70}$-$(dT)_{70}$ (pA-pT), were supplemented with recombinant Chk1-GST-His_6. The extracts were also supplemented with the indicated versions of recombinant His_6-Claspin-FLAG or no recombinant Claspin. The extracts were subjected to pulldowns with glutathione-agarose beads (*lanes 3–12*). *Lanes 13–17* depict 2% of the extracts prior to the pulldowns. *Lanes 1* and *2* depict extracts without and with oligonucleotides but with no added recombinant proteins. The samples were immunoblotted for the FLAG epitope and GST. *C*, interphase extracts containing GST-Claspin(776–851), wild-type GST-Claspin(847–903), GST-Claspin(847–903)-2AG, and GST-Claspin(847–962) were incubated in the absence or presence of checkpoint-inducing oligonucleotides and immunoprecipitated with anti-Drf1 antibodies (*D*) or mock (*M*) control antibodies. The immunoprecipitates were immunoblotted for Drf1 and GST.

of DDK interaction mutants in the context of the GST-fused 776–1078 Claspin peptide fragment mentioned previously. This peptide fragment of Claspin contains both the CKBD and additional sequences necessary for rescuing the activation of Chk1 in Claspin-depleted extracts (24).

When we used glutathione beads to isolate the wild-type, D861E, Q866K, and E/K mutant Claspin (776–1078) fragments from nuclei-free interphase extracts, we found that all of these proteins could bind Chk1 in extracts that had been treated with the checkpoint-inducing $(dA)_{70}$-$(dT)_{70}$ oligonucleotides (Fig. 4*A*). Notably, the mutant Claspin fragments displayed a reduction in electrophoretic mobility equivalent to that of the wild-type fragment in extracts treated with $(dA)_{70}$-$(dT)_{70}$ (Fig. 4*A*). This electrophoretic shift is characteristic of Claspin peptides containing the CKBD and is mediated by phosphorylation in response to checkpoint activation (24, 42).

Next, we tested the Claspin-Chk1 interaction in a reciprocal manner by incubating glutathione beads in extracts supplemented with both a GST-tagged version of Chk1 (Chk1-GST-His_6) and the different versions of the full-length His_6-Claspin-FLAG protein. By this methodology, we also observed that D861E, Q866K, and E/K mutants could associate normally with Chk1 (Fig. 4*B*). Taken together, these results indicate that elimination of the ability of Claspin to associate with DDK has virtually no effect on binding to Chk1.

Subsequently, we endeavored to test whether Claspin fragments that are unable to bind to Chk1 were still competent to interact with DDK. Chk1 interacts with Claspin through two phosphoserines ($Ser(P)^{864}$ and $Ser(P)^{895}$) that become phosphorylated in response to activation of the DNA replication checkpoint (42). We performed anti-Drf1 immunoprecipitations with interphase extracts supplemented with CKBD-containing GST-fused Claspin fragments (847–903) that were either wild type or had Ser^{864} and Ser^{895} mutated to alanines (designated as the 2AG mutant). The wild-type and 2AG fragments were found at equivalent levels in the anti-Drf1 immunoprecipitates, regardless of whether the extracts contained the checkpoint-inducing $(dA)_{70}$-$(dT)_{70}$ oligonucleotides (Fig. 4*C* and supplemental Fig. S5). Thus, the binding of Chk1 to Claspin is distinct from the interaction of DDK with Claspin.

Next, we tested whether a mutant version of Claspin that was compromised for interacting with DDK was competent to activate Chk1 in response to replication arrest. To address this question, we immunodepleted Claspin from extracts and then added back wild-type or mutant full-length His_6-Claspin-FLAG proteins to approximately endogenous levels. The extracts were incubated with demembranated sperm chromatin in the absence or presence of aphidicolin. The activation of Chk1 was monitored with anti-phosphopeptide antibodies that detect phosphorylation of Chk1 on Ser^{344}, a well established marker for activation of this kinase. As expected, activation of Chk1 in response to aphidicolin was ablated in the absence of Claspin, and recombinant wild-type Claspin could reverse this defect (Fig. 5*A*, *lanes 8* and *9*). Significantly, the E/K mutant Claspin protein was also fully able to rescue the activation of Chk1 (Fig. 5*A*, *lane 10*). The D861E mutant Claspin protein had similar efficacy as the E/K protein for activating Chk1 (data not shown). These results are entirely consistent with the fact that these mutants can still bind Chk1 normally.

Claspin associates with chromatin during the S phase in a manner that depends on both the prereplication complex and Cdc45 (23). The most important known kinase target of DDK is the MCM2–7 complex, and this phosphorylation leads to the recruitment of Cdc45 (9–11). Accordingly, we examined the ability of the DDK interaction mutants of Claspin to associate with chromatin that is undergoing replication in egg extracts. For this purpose, we isolated chromatin from extracts in which we had immunodepleted the endogenous Claspin and replaced

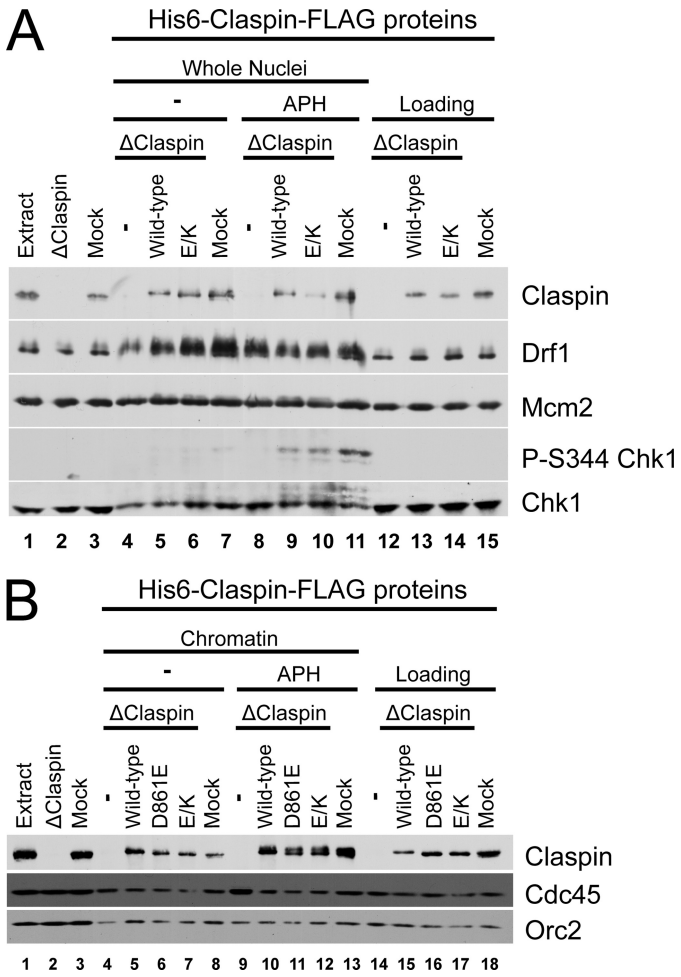

FIGURE 5. **DDK interaction mutants of Claspin can still mediate activation of Chk1 and associate with replicating chromatin.** *A*, nuclei were isolated from interphase extracts supplemented with 3,000 sperm nuclei/μl that were either immunodepleted with anti-Claspin antibodies (*lanes 4–6* and *8–10*) or mock depleted with control antibodies (*lanes 7* and *11*). The extracts were also incubated in the absence (*lanes 4–7*) or presence of aphidicolin (*lanes 8–11*). The extracts were supplemented with full-length versions of wild-type His_6-Claspin-FLAG (*lanes 5* and *9*), His_6-Claspin-FLAG-E/K (*lanes 6* and *10*), or no recombinant protein (*lanes 4* and *8*). *Lanes 12–15* depict 4% of the extracts taken before isolation of nuclei. Aliquots of extracts were taken before (*lane 1*) and after immunodepletion (*lanes 2* and *3*). The extracts and nuclear fractions were immunoblotted for Claspin, Drf1, Mcm2, phosphorylated Ser^{344} of Chk1, and Chk1. *B*, chromatin was isolated from interphase extracts supplemented with 3,000 sperm nuclei/μl and incubated in the absence (*lanes 4–8*) or presence of aphidicolin (*lanes 9–13*). The extracts were immunodepleted with either anti-Claspin (*lanes 4–7* and *9–12*) or control antibodies (*lanes 8* and *13*). The extracts were also supplemented with the indicated versions of the His_6-Claspin-FLAG protein (*lanes 5–7* and *10–12*) or buffer alone (*lanes 4*, *8*, *9*, and *13*). *Lanes 14–18* depict 4% of the extract taken before isolation of chromatin. Aliquots of extract were taken before (*lane 1*) and after immunodepletion (*lanes 2* and *3*). The samples were immunoblotted for Claspin, Cdc45, and Orc2.

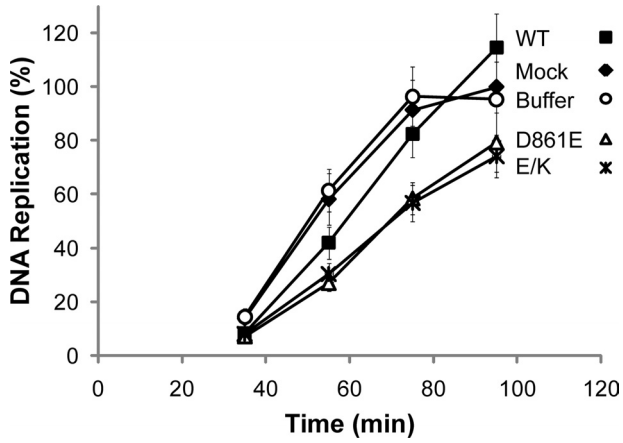

FIGURE 6. **Egg extracts containing DDK interaction mutants of Claspin replicate chromosomal DNA more slowly.** Claspin-depleted extracts were prepared and supplemented with recombinant wild-type (*WT*, *closed squares*), D861E (*open triangles*), or E/K (*asterisks*) His_6-Claspin-FLAG proteins or buffer alone (*open circles*). Recombinant Claspin proteins were added back at approximately the concentration of endogenous Claspin in egg extracts (see supplemental Fig. S6). Mock depleted extracts (*closed diamonds*) were prepared in parallel. Aliquots of extracts were taken at 35, 55, 75, and 95 min and assayed for chromosomal DNA replication. Incorporation of ^{32}P into chromosomal DNA was determined by agarose gel electrophoresis and phosphorimaging. Incorporation was normalized to the level observed in the mock depleted extracts at 95 min. The data points represent the means ± S.E. for seven independent experiments.

it with wild-type recombinant Claspin or various DDK interaction mutants. For these experiments, we also incubated the various extracts in the absence or presence of aphidicolin. We observed that, in the absence of aphidicolin, both the D861E and E/K mutants bound to chromatin as efficiently as wild-type Claspin (Fig. 5*B*). In the presence of aphidicolin, Claspin binds in elevated amounts to chromatin and undergoes an electrophoretic shift caused by checkpoint-dependent phosphorylation. We found that both the D861E and E/K mutants, like wild-type Claspin, associated in higher amounts with aphidicolin-treated chromatin and displayed a checkpoint-dependent shift in electrophoretic mobility (Fig. 5*B*). Finally, our laboratory previously observed that Cdc45 accumulates in higher amounts on chromatin in the absence of Claspin (23, 34). The addition of recombinant wild-type Claspin reduced the binding of Cdc45 to normal levels (Fig. 5*B*). Likewise, both the D861E and E/K mutant Claspin proteins could elicit the normal binding pattern of Cdc45. Taken together, these results indicate that Claspin can associate normally with chromatin under a variety of different conditions, even if it has lost the ability to interact with DDK.

The Interaction of Claspin with DDK Is Required for Maintaining a Normal Rate of DNA Replication—Claspin is a regulator of replication in mammalian cells and is required for a normal rate of DNA replication, even under unperturbed conditions (23, 24, 26, 27). However, in *Xenopus* egg extracts, immunodepletion of Claspin results in only a very modest decrease in DNA replication (23). Because the primary role established for DDK is as an S phase regulator, we examined the status of DNA replication in egg extracts containing the D861E and E/K Claspin mutant proteins. To this end, we immunodepleted Claspin from interphase extracts and added back either buffer alone or various recombinant His_6-Claspin-FLAG proteins. We measured DNA replication by adding [α-^{32}P]dATP before the onset of replication and taking samples at selected time points until replication typically reaches completion. Replication usually starts at ~35–45 min after entry into interphase in *Xenopus* egg extracts. We found that under these conditions Claspin-depleted extracts with no recombinant protein added (buffer alone) did not display a consistent decrease in replication in comparison with mock depleted extracts or Claspin-depleted extracts reconstituted with wild-type Claspin (Fig. 6). However, when the D861E and E/K mutants were

added back to Claspin-depleted extracts, there was an approximately 30% decrease in replication (Fig. 6 and supplemental Fig. S6). The different rates of replication are apparent even by the second time point (*i.e.* at 60 min). Thus, these mutants appear to be having a negative effect on replication. These results are consistent with the involvement of Claspin in maintaining a normal basal replication rate in other systems (24, 25, 27) and suggest that DDK may play a part in this regulation.

DISCUSSION

The role of Claspin as a mediator in the activation of Chk1 in response to replication stress has been well studied. On the other hand, the role of Claspin as a regulator of replication is still being elucidated. In this study, we have confirmed that Claspin associates specifically with the replication-initiating kinase DDK in *Xenopus* egg extracts. A similar finding has been made in human cells (37). Moreover, this complex includes Claspin, Cdc7, Drf1, and the cohesin-loading protein Scc2. The discovery that Claspin can be co-immunoprecipitated with Scc2 coincides with the finding that the Drf1-Cdc7 complex interacts with Scc2 and is necessary for the loading of Scc2 onto chromatin during the S phase (41). Even though we did not observe a defect in the loading of Scc2 onto chromatin in Claspin-depleted extracts with no perturbation of replication (data not shown), it remains possible that Claspin may play a role in regulating the increased loading of the cohesin complex onto chromatin in the presence of stalled replication forks (46).

We found that the minimal sequence of Claspin required for binding to DDK was the first repeat of the CKBD. By comparing CKBD repeat sequences both within *Xenopus* Claspin and between other metazoan Claspin homologs, we identified two amino acid positions of maximum variability within the repeats. By changing aspartate 861 to glutamate and glutamine 866 to lysine (thereby converting the first *Xenopus* CKBD repeat into the second), we were able to abolish almost completely the ability of Claspin to interact with Drf1. We examined the evolutionary conservation of this binding by testing the ability of GST-fused human CKBD minimal repeat peptides to interact with *Xenopus* Drf1. There are three human CKBD repeats, but only the first two are required for activation of Chk1 (44, 45). The first human CKBD repeat, which has an aspartate at the position equivalent to aspartate 861 of *Xenopus* Claspin, was the only fragment that could interact with *Xenopus* Drf1 in egg extracts. Although this is an admittedly heterologous interaction, there is a high degree of sequence identity between CKBD repeats across metazoan species. The first repeat seems to contain aspartic acid at this position in nearly every case (except zebrafish), whereas the other repeats have a higher variability of residues, and none contain aspartic acid. Therefore, it seems reasonable to speculate that there is a high degree of conservation across metazoan Claspin molecules for this site to interact with DDK.

The mechanism by which Claspin activates Chk1 has been well described in *Xenopus* egg extracts. In response to replication stress, serines 864 and 895 are phosphorylated in an ATR-dependent manner by an unidentified kinase. These phosphoserines associate with the kinase domain of Chk1 during a process that ultimately leads to the activation of Chk1 (22, 42, 47). The proximity of aspartate 861 and glutamine 866 to serine 864 was striking and led us to test whether the D861E or E/K mutants would affect the ability of Claspin to activate Chk1. Subsequently, we found that these mutants were fully competent to associate with Chk1 and mediate its activation in response to replication stress. Additionally, a 2AG mutant version of Claspin fragment that is incapable of binding to Chk1 (because of mutations in serines 864 and 895) was still able to interact with Drf1. These findings suggest that the role of Claspin in interacting with DDK is distinct from that of activating Chk1. It has been reported that the ablation of Cdc7 by small interfering RNA leads to reduced activation of the ATR-Chk1 pathway in mammalian cells (37). However, this effect may be the result of a reduced number of replication forks as a result of the absence of Cdc7 rather than a direct modulation of Claspin. Other work has shown that overexpression or ectopic addition of excess Dbf4-Cdc7 can lead to an attenuation of Chk1 signaling upon the addition of etoposide in mammalian cells and *Xenopus* egg extracts (36). Although we observed no defect in the ability of the DDK interaction mutants to activate Chk1 in response to aphidicolin, it remains possible that there could be a defect in recovery from damage because of the loss of interaction with DDK. Additionally, there could be differences between the Dbf4-Cdc7 and Drf1-Cdc7 complexes or in response to different types of damage.

On the other hand, our experiments indicate that addition of the D861E and E/K mutants of Claspin to Claspin-depleted extracts results in a slower rate of DNA replication in comparison with Claspin-depleted extracts (23). There is also a modest delay in mitosis in the presence of these mutants (data not shown), which would be consistent with delayed replication. Although Claspin and its yeast homolog Mrc1 are not absolutely required for replication, these proteins are involved in the control of replication-related processes. Our results suggest that the inability of Claspin to associate with DDK normally results in the perturbation of DNA replication.

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