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Adaptation of a DNA Replication Checkpoint Response Depends upon Inactivation of Claspin by the Polo-like Kinase

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Summary

The checkpoint mediator protein Claspin is essential for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing aphidicolin-induced DNA replication blocks. We show that, during this checkpoint response, Claspin becomes phosphorylated on threonine 906 (T906), which creates a docking site for Plx1, the *Xenopus* Polo-like kinase. This interaction promotes the phosphorylation of Claspin on a nearby serine (S934) by Plx1. After a prolonged interphase arrest, aphidicolin-treated egg extracts typically undergo adaptation and enter into mitosis despite the presence of incompletely replicated DNA. In this process, Claspin dissociates from chromatin, and Chk1 undergoes inactivation. By contrast, aphidicolin-treated extracts containing mutants of Claspin with alanine substitutions at positions 906 or 934 (T906A or S934A) are unable to undergo adaptation. Under such adaptation-defective conditions, Claspin accumulates on chromatin at high levels, and Chk1 does not decrease in activity. These results indicate that the Plx1-dependent inactivation of Claspin results in termination of a DNA replication checkpoint response.

Introduction

In eukaryotic cells, various checkpoint control mechanisms have evolved to maintain the integrity of the genome. For example, these regulatory pathways prevent the entry into mitosis if the cell contains incompletely replicated or damaged DNA (reviewed in Melo and Toczyski, 2002; Osborn et al., 2002). The checkpoint response to incompletely replicated DNA can be studied in cell-free extracts from *Xenopus* eggs (Dasso and Newport, 1990; Kumagai et al., 1998). In this system, treatment with the DNA replication inhibitor aphidicolin causes the formation of DNA replication blocks. These stalled replication forks elicit activation of Xchk1, the *Xenopus* homolog of the checkpoint effector kinase Chk1 (Kumagai et al., 1998). The activation of Xchk1 in egg extracts can also be triggered by addition of model DNA templates such as annealed oligomers of poly(dA)$_{70}$ and poly(dT)$_{70}$ (which we will refer to as pA-pT below) (Kumagai and Dunphy, 2000). These templates appear to mimic incompletely replicated DNA in the extracts.

In vertebrates, the activation of Chk1 depends upon ATR, which is homologous to budding yeast Mec1 and fission yeast Rad3 (Guo et al., 2000; Hekmat-Nejad et al., 2000; Liu et al., 2000). In egg extracts, *Xenopus* ATR (Xatr) carries out the activating phosphorylation of Xchk1 on four conserved Ser-Gln/Thr-Gln (SQ/TQ) motifs in its C-terminal regulatory domain (Guo et al., 2000). The Xatr-dependent phosphorylation of Xchk1 also depends on another protein called Claspin (Kumagai and Dunphy, 2000, 2003; Jeong et al., 2003; Lee et al., 2003). In this system, there is also a parallel replication checkpoint pathway that operates independently of Xatr, Claspin, and Xchk1 (Kumagai et al., 1998; Guo et al., 2000; Kumagai and Dunphy, 2000). A similar phenomenon has recently been described in mammalian cells (Brown and Baltimore, 2003).

Since Claspin also binds to Xchk1, a simple model is that Claspin promotes the phosphorylation of Xchk1 by Xatr by serving as a mediator or adaptor protein. In addition to this function, Claspin associates with chromatin in a highly regulated manner during S phase (Lee et al., 2003). The binding of Claspin to chromatin depends upon the prereplication complex (pre-RC), Cdc45, and Cdk2. These observations suggest that Claspin may also act as a sensor of DNA replication forks. Functional homologs of Claspin called Mrc1 were identified subsequently in budding and fission yeast (Alcasabas et al., 2001; Tanaka and Russell, 2001). Like Claspin, Mrc1 also associates with the replication apparatus in yeast cells (Katou et al., 2003; Osborn and Elledge, 2003).

Once Chk1 becomes activated, it prevents the entry into mitosis by blocking activation of the Cdc2-cyclin B complex. This process involves inhibition of Cdc25 and stimulation of Wee1, both of which are critical upstream regulators of Cdc2-cyclin B (Melo and Toczyski, 2002). The *Xenopus* Polo-like kinase (Plx1), which phosphorylates and activates Cdc25, is also involved in the entry into mitosis (Kumagai and Dunphy, 1996; Nigg, 2001). Plk1 is downregulated in human cells containing double-stranded DNA breaks (Smits et al., 2000). It has not previously been reported whether Plx1 is negatively regulated in *Xenopus* egg extracts containing DNA replication blocks.

One important aspect of checkpoint control involves the issue of how checkpoint-mediated arrests of the cell cycle come to an end. In the case of DNA damage responses, for example, the completion of DNA repair results in elimination of the checkpoint-activating signal, reversal of the checkpoint-signaling reactions, and recovery processes. A more intriguing manner for termination of a checkpoint arrest entails a process called adaptation. During adaptation, the checkpoint arrest becomes alleviated before the checkpoint-inducing DNA signal has been removed. This process has been most well characterized in the response of budding yeast to double-stranded DNA breaks (Sandell and Zakian, 1993; Toczyski et al., 1997; Pellicioli et al., 2001; Vaze et al.,

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2002; Leroy et al., 2003). In unicellular organisms such as yeast, adaptation may allow proliferation in cases where DNA damage that cannot be repaired in a timely manner is nonetheless survivable. The prevalence and biological role of checkpoint adaptation in multicellular organisms are unknown. In budding yeast, adaptation requires Cdc5 (the Polo-like kinase), Ckb1 and Ckb2 (subunits of casein kinase II), Ptc2 and Ptc3 (PP2C-like phosphatases), and the Srs2 helicase (Toczyski et al., 1997; Pellicioli et al., 2001; Vaze et al., 2002; Leroy et al., 2003). In fission yeast, phosphorylation of the Chk1-regulatory protein Crb2 by Cdc2 is necessary for exit from a DNA damage arrest (Esashi and Yanagida, 1999; Caspari et al., 2002). Despite these significant insights, no primary regulatory circuit for adaptation of a cell cycle checkpoint arrest has been delineated in any organism.

In this report, we show that the *Xenopus* Polo-like kinase Plx1 promotes inactivation of Claspin in aphidicolin-treated *Xenopus* egg extracts after a prolonged interphase arrest. As a consequence, the extracts enter mitosis despite the ongoing presence of DNA replication blocks. These studies indicate that inactivation of a checkpoint mediator protein such as Claspin by a mitotic regulatory kinase results in checkpoint adaptation. In principle, such adaptive processes in multicellular organisms could promote cell death or lead to the perpetuation of genetic errors, depending on the circumstances.

Results

Plx1 Associates with Claspin in a Checkpoint-Regulated Manner

Our laboratory has been interested in the function and regulation of Plx1 in *Xenopus* egg extracts, especially during checkpoint responses. For these studies, we searched for Plx1-interacting proteins by immunoprecipitating Plx1 from egg extracts (Figure 1A). Two closely spaced bands at approximately 180 kDa attracted our attention, because the pattern of these proteins changed subtly after treatment with pA-pT (not shown), suggesting that there is some change in state of modification or abundance among these proteins in the immunoprecipitates upon checkpoint activation. Mass spectrometric analysis indicated that these bands contain multiple polypeptides, including Claspin (see Experimental Procedures).

To assess the specificity of the interaction between Plx1 and Claspin, we performed reciprocal immunoprecipitation experiments. First, we immunoprecipitated Plx1 from interphase extracts, M phase extracts, and interphase extracts containing pA-pT and then performed immunoblotting with anti-Claspin antibodies (Figure 1B). We observed similar amounts of Claspin in anti-Plx1 immunoprecipitates from interphase and M phase extracts, but there was highly elevated binding of Claspin to Plx1 in extracts containing pA-pT. Conversely, we also detected Plx1 in anti-Claspin immunoprecipitates by immunoblotting with anti-Plx1 antibodies (Figure 1C). We likewise observed enhanced binding of Plx1 to Claspin in the presence of pA-pT by this method. Finally, we performed pull-down experiments with a recombinant, kinase-inactive version of Plx1 (Plx1-N172A-GH). As shown in Figure 1D, Claspin associated specifically with nickel beads containing Plx1-N172A-GH. There was no binding to control nickel beads (data not shown). The binding of Claspin to Plx1-N172A-GH was also greatly enhanced in the presence of pA-pT. Addition of caffeine, an inhibitor of ATR and ATM, abolished the increased association of Plx1 and Claspin. Thus, according to three different methods, Plx1 and Claspin form a specific, checkpoint-regulated complex.

Phosphorylation of Claspin on T906 Recruits Plx1

To characterize the interaction between Claspin and Plx1, we attempted to identify the Plx1 binding site on Claspin. For the binding assay, we incubated various ^{35}S-labeled fragments of Claspin in egg extracts containing recombinant Plx1-N172A-GH in the absence and presence of pA-pT. Subsequently, we reisolated tagged Plx1 and examined binding of the ^{35}S-labeled Claspin fragments by gel electrophoresis and phosphorimaging. Initial experiments indicated that Plx1 binds to fragments from the C-terminal end of Claspin (e.g., residues 776–1285 and 776–1174) but not to an N-terminal fragment (residues 1–744) (Figure 1E). Further detailed mapping indicated that residues 878–920 of Claspin are essential for binding to Plx1 (see Figure 1F).

The Polo-like kinase (Plk) docks with prospective substrates by a phosphorylation-mediated mechanism (Elia et al., 2003). Plk contains a Polo-box domain (PBD) that interacts with phosphorylated sequences in substrates such as Cdc25C. We investigated whether phosphorylation of Claspin is necessary for binding to Plx1 by mutating candidate serines and threonines in the region of Claspin that is required for binding to Plx1 (residues 878–920) (Figure 2A). Since this region partially overlaps the previously identified Chk1 binding domain (CKBD) of Claspin at residues 847–903, in which we had already prepared a number of Ser/Thr to Ala mutations (Kumagai and Dunphy, 2003), we also examined these sites. Initially, we examined mutations that were introduced into the Claspin(776–1174) fragment, which binds well to Plx1. None of the mutations in the CKBD affected binding to Plx1 (Figure 2B). For example, the 2AG mutant, which lacks S864 and S895 and thus cannot bind to Xchk1, interacts normally with Plx1. By contrast, a quadruple mutant of Claspin(776–1174) lacking S905, T906, S909, and S910 was unable to associate with Plx1 (Figure 2C). Upon further analysis, we observed that a single point mutant of Claspin(776–1174) in which T906 was changed to alanine (T906A) also could not interact with Plx1. By comparison, a single S910A mutant could still bind to Plx1.

We proceeded to introduce the T906A mutation into the full-length Claspin protein. As shown in Figure 3A, ^{35}S-labeled Claspin-T906A displayed only background levels of binding to Plx1. We also prepared a full-length version of His6-Claspin-T906A in baculovirus-infected insect cells. Next, we removed the endogenous Claspin from egg extracts by immunodepletion with anti-Claspin antibodies and subsequently replaced this protein with either wild-type His6-Claspin or the His6-Claspin-T906A mutant (Figure 3B). Wild-type His6-Claspin bound well to exogenously added Plx1-N172A-GH, but there was no binding of His6-Claspin-T906A (Figure 3C).

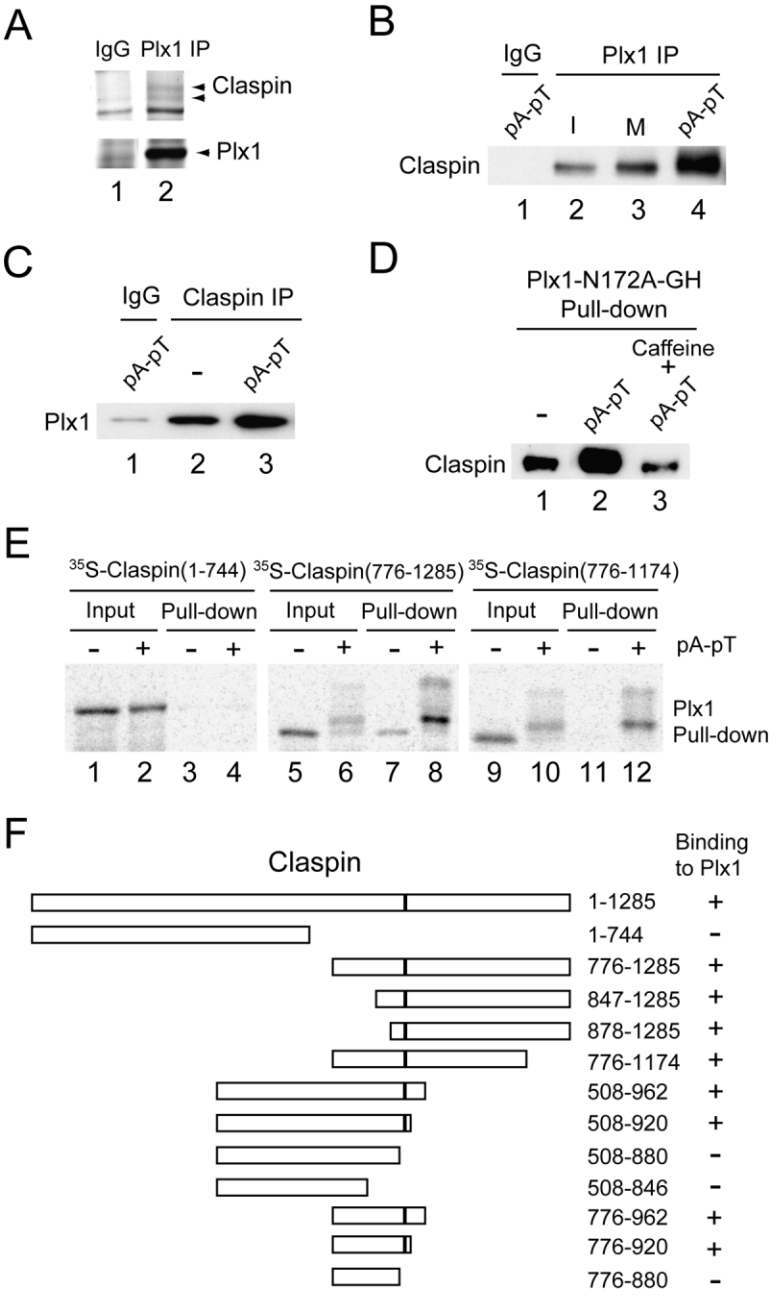

Figure 1. Claspin Interacts Specifically with Plx1

(A) Control (lane 1) and anti-Plx1 (lane 2) immunoprecipitates from interphase egg extracts containing pA-pT were subjected to SDS-PAGE and silver staining. Two bands at approximately 180 kDa (corresponding to those marked with arrowheads in top panel) were excised from another, large-scale preparation for mass spectrometry (see Experimental Procedures). The upper band contained Claspin. The bottom panel depicts the area of the gel containing Plx1.

(B) Interphase extracts (lane 2), M phase extracts (lane 3), and interphase extracts containing pA-pT (lanes 1 and 4) were immunoprecipitated with control (lane 1) or anti-Plx1 antibodies (lanes 2–4). Immunoprecipitates were immunoblotted for Claspin.

(C) Extracts containing no DNA (lane 2) or pA-pT (lanes 1 and 3) were immunoprecipitated with control (lane 1) or anti-Claspin antibodies (lanes 2 and 3). Immunoprecipitates were immunoblotted for Plx1.

(D) Plx1-N172A-GH on nickel beads was incubated in extracts containing no DNA (lane 1), pA-pT (lane 2), or pA-pT plus caffeine (lane 3). The beads were reisolated and immunoblotted for Claspin.

(E) ^{35}S-Labeled Claspin(1–744) (lanes 1–4), Claspin(776–1285) (lanes 5–8), and Claspin (776–1174) (lanes 9–12) were incubated in extracts containing Plx1-N172A-GH on nickel beads in the absence (lanes 1, 3, 5, 7, 9, and 11) or presence of pA-pT (lanes 2, 4, 6, 8, 10, and 12). The beads were reisolated, and bound ^{35}S-labeled proteins were detected by SDS-PAGE and phosphorimaging (lanes 3, 4, 7, 8, 11, and 12). Lanes 1, 2, 5, 6, 9, and 10 depict initial extract aliquots.

(F) Abilities of the indicated fragments of Claspin to interact with Plx1.

To establish explicitly that T906 undergoes phosphorylation in egg extracts, we prepared antibodies against a synthetic peptide from Claspin that contains phosphorylated T906 (anti-P-T906) (see Figure 3D). As shown in Figures 3E and 3F, the anti-P-T906 antibodies reacted well with both GST-Claspin(878–962) and full-length His6-Claspin that had been incubated in extracts in the presence but not the absence of pA-pT. The anti-P-T906 antibodies did not recognize T906A mutant versions of these polypeptides. Furthermore, treatment with caffeine abolished the reactivity of Claspin with the anti-P-T906 antibodies. Taken together, these results establish that phosphorylation of T906 in Claspin is essential for the binding of Plx1. Significantly, the sequence containing this residue (ST^{906}Q) fits the consensus established for the recognition of Plk substrates by the PBD, which is Ser-(pThr/pSer)-(Pro/X) (Elia et al., 2003). In a sequence alignment, human Claspin also contains a related motif (ST^{955}P) in a similar location (Figure 3D).

The PBD of Plx1 lies in its C-terminal regulatory region (amino acids 316–598). To evaluate whether this region interacts with phosphorylated Claspin, we prepared recombinant forms of the N-terminal and C-terminal domains of Plx1, which we named N-Plx1-GH and C-Plx1-GH, respectively (see Figure 3G). As depicted in Figure 3H, Claspin bound well to full-length Plx1-GH and C-Plx1-GH but not to N-Plx1-GH or a control His6-GST protein. These results, along with the known specificity of the PBD, suggest strongly that T906-phosphorylated Claspin interacts with the PBD of Plx1.

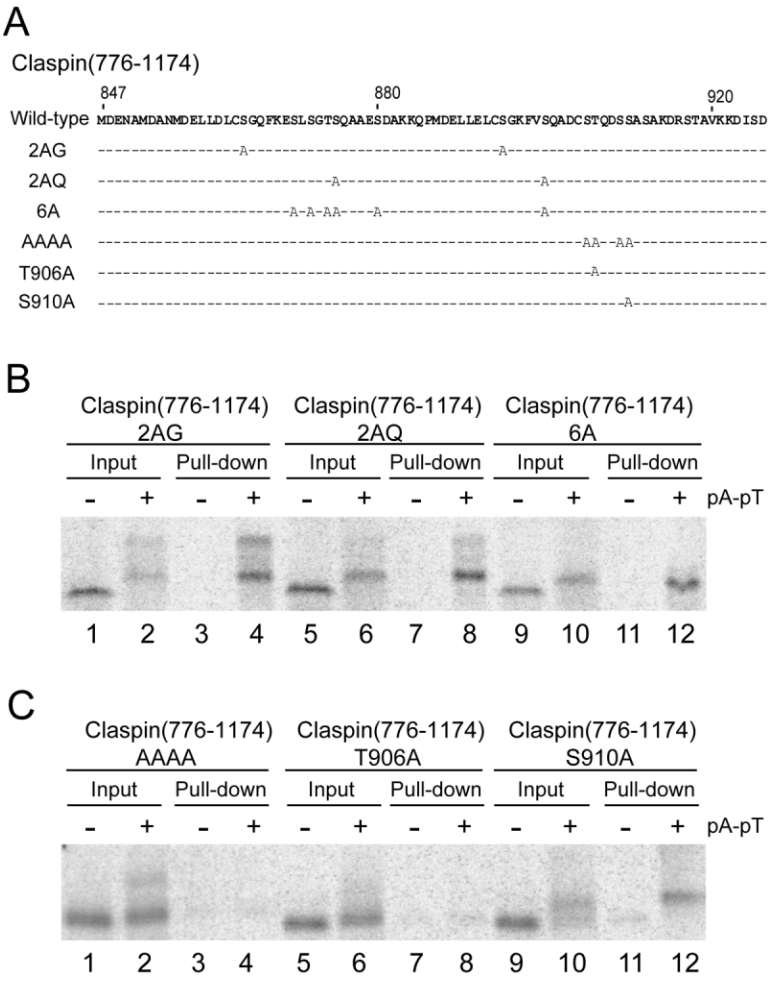

Figure 2. T906 of Claspin Is Required for Binding to Plx1

(A) Mutants of Claspin(776–1174) used for the Plx1 binding studies.

(B) ^{35}S-Labeled 2AG, 2AQ, and 6A mutants were analyzed for binding to Plx1 as described in Figure 1E.

(C) ^{35}S-Labeled AAAA, T906A, and S910A mutants were analyzed for binding to Plx1.

Phosphorylation of T906 on Claspin Is Checkpoint Regulated and Depends upon ATR

Thus far, we have shown that the DNA template pA-pT stimulates the binding of Plx1 to Claspin. In *Xenopus* egg extracts, this template can trigger the phosphorylation of both Xchk1 and Xchk2, the *Xenopus* homolog of Chk2 (Guo and Dunphy, 2000; Kumagai and Dunphy, 2000). In general, Xchk1 is activated primarily in response to DNA replication blocks. By contrast, the activity of Xchk2 is increased by the presence of double-stranded DNA breaks.

To examine the relationship between Claspin and Plx1 during a checkpoint response to stalled replication forks in chromosomal DNA, we added demembranated sperm nuclei and aphidicolin to egg extracts. After 100 min, we isolated chromatin fractions from the extracts and performed immunoblotting with antibodies against Claspin, P-T906 of Claspin, and Xorc2 (to monitor recovery of chromatin) (Figure 4A). As described previously, Claspin binds in elevated amounts to aphidicolin-treated chromatin (Lee et al., 2003). The Claspin that had associated with chromatin in aphidicolin-treated extracts reacted strongly with the anti-P-T906 antibodies. Likewise, T906 became phosphorylated on UV-damaged chromatin, which also accumulates replication blocks. By contrast, Claspin from control extracts did not react with the anti-P-T906 antibodies.

To investigate whether Claspin and Plx1 also form a complex in the presence of aphidicolin-induced replication blocks, we extracted Claspin from chromatin fractions with 0.5 M NaCl and then immunoprecipitated the salt eluates with anti-Plx1 antibodies. We found that Claspin was present in the anti-Plx1 immunoprecipitates from aphidicolin-treated chromatin but not in immunoprecipitates from samples that lacked aphidicolin or contained aphidicolin plus caffeine (Figure 4B). These results suggest that Plx1 associates with Claspin at stalled replication forks.

We also asked what kinase regulates phosphorylation of Claspin on T906. Xatr seemed a plausible candidate because phosphorylation on T906 increases in the presence of DNA replication blocks, and the sequence around T906 resembles a consensus site for ATR. We found that the aphidicolin-dependent phosphorylation of Claspin on T906 was abolished in the Xatr-depleted extracts (Figures 4C and 4D). Therefore, it appears that Xatr or some Xatr-dependent kinase phosphorylates T906. Taken together, these results suggest that the

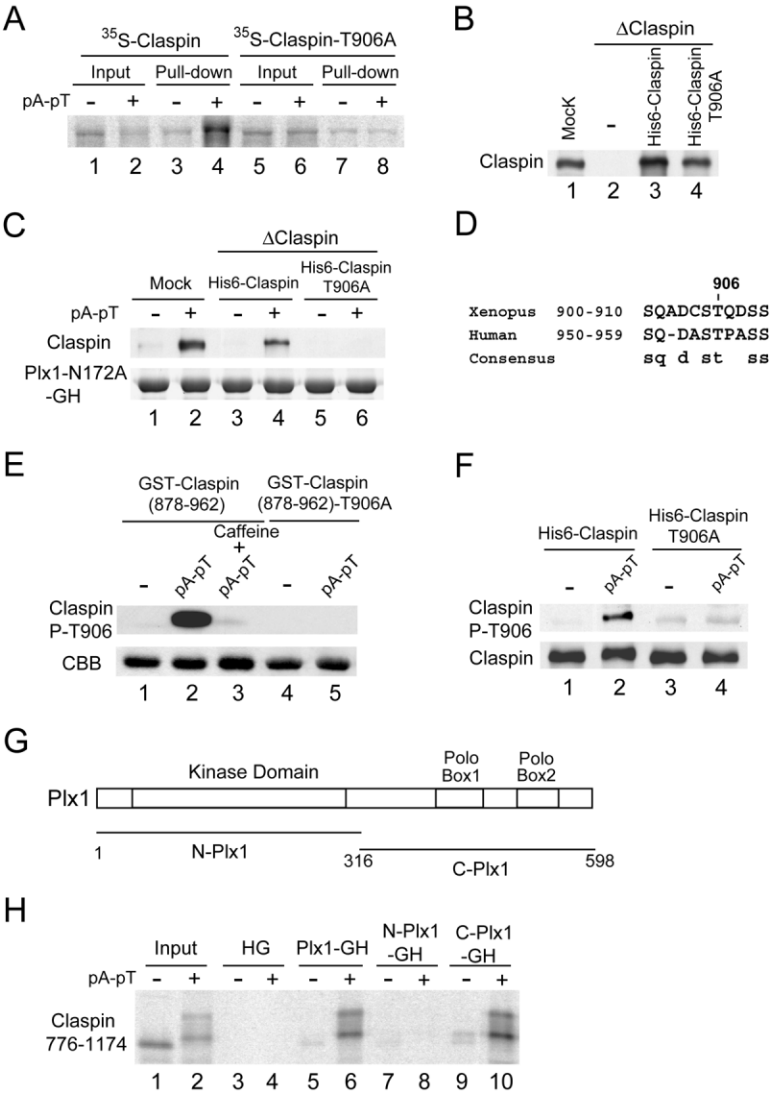

Figure 3. Phosphorylation of Claspin on T906 Is Required for Binding to Plx1

(A) Binding of ^{35}S-labeled full-length Claspin (lanes 1–4) and Claspin-T906A (lanes 5–8) to recombinant Plx1 was determined in the absence (lanes 1, 3, 5, and 7) or presence of pA-pT (lanes 2, 4, 6, and 8).

(B) Egg extracts were mock depleted with control antibodies (lane 1) or immunodepleted with anti-Claspin antibodies (lanes 2–4). Subsequently, no recombinant protein (lane 2), wild-type His6-Claspin (lane 3), or His6-Claspin-T906A (lane 4) was added back to the depleted extracts. Extracts were immunoblotted for Claspin.

(C) Mock-depleted (lanes 1 and 2) and Claspin-depleted extracts containing His6-Claspin (lanes 3 and 4) or His6-Claspin-T906A (lanes 5 and 6) were incubated with Plx1-N172A-GH in the absence (lanes 1, 3, and 5) or presence of pA-pT (lanes 2, 4, and 6). Plx1-N172A-GH was reisolated with glutathione beads, and bound Claspin was detected by immunoblotting. Plx1 was stained with Coomassie blue.

(D) Alignment of residues 900–910 from *Xenopus* Claspin with human Claspin.

(E) Specificity of anti-P-T906 antibodies. Interphase extracts containing no DNA (lanes 1 and 4), pA-pT (lanes 2 and 5), or pA-pT plus caffeine (lane 3) were incubated with GST-Claspin(878–962) (lanes 1–3) or GST-Claspin(878–962)-T906A (lanes 4 and 5). The GST-tagged fragments were reisolated with glutathione beads, subjected to SDS-PAGE, and either immunoblotted with anti-P-T906 antibodies (top) or stained with Coomassie brilliant blue (CBB) (bottom).

(F) His6-Claspin (lanes 1 and 2) or His6-Claspin-T906A (lanes 3 and 4) on nickel beads was incubated in egg extracts in the absence (lanes 1 and 3) or presence of pA-pT (lanes 2 and 4). His6-tagged proteins were reisolated and immunoblotted with anti-P-T906 (top) or anti-Claspin antibodies (bottom).

(G) Diagram of kinase and Polo-box domains of Plx1.

(H) ^{35}S-Claspin(776–1174) was incubated in extracts containing control His6-GST protein (HG) (lanes 3 and 4), full-length Plx1-GH (lanes 5 and 6), N-Plx1-GH (lanes 7 and 8), or C-Plx1-GH (lanes 9 and 10) in the absence (lanes 1, 3, 5, 7, and 9) or presence of pA-pT (lanes 2, 4, 6, 8, and 10). The beads were reisolated, and binding of radiolabeled Claspin was determined. Lanes 1 and 2 depict initial extract aliquots.

Xatr-dependent phosphorylation of Claspin on T906 recruits Plx1 to chromatin during a DNA replication checkpoint response in egg extracts.

Claspin Appears Not to Be Involved in Negative Regulation of Plx1

In order to evaluate the functional significance of the interaction between Claspin and Plx1, we considered various models. For example, Claspin could be involved in the negative regulation of Plx1. In this case, one might predict that Plx1 would be downregulated in checkpoint-activated egg extracts. To examine this issue, we isolated Plx1 at various times from interphase extracts lacking or containing aphidicolin and assayed kinase activity toward Cdc25C. We observed that the kinase activity of Plx1 increased steadily in both the absence and presence of aphidicolin (Figure 4E). By 100 min, the activity of Plx1 in both cases approached that of Plx1 from M phase extracts. By contrast, the activity of Plx1 in interphase extracts did not increase in the presence of cycloheximide, which prevents the synthesis of cyclin B. In conclusion, it appears that Plx1 is not kept inactive in the presence of incompletely replicated DNA.

We also examined the activity of Plx1 in extracts containing pA-pT (Figure 4E). We observed that pA-pT blocked the activation of Plx1 very efficiently, which is consistent with reports that double-stranded DNA ends downregulate Plk1 in irradiated human cells (Smits et al., 2000). These observations raised the possibility that Claspin might be necessary for the negative regulation of Plx1 in response to double-stranded DNA ends. However, we found that Plx1 is still downregulated efficiently by the presence of pA-pT in Claspin-depleted egg extracts (data not shown). Taken together, these various observations make it seem unlikely that binding of Claspin to Plx1 reflects negative regulation of Plx1. Inter-

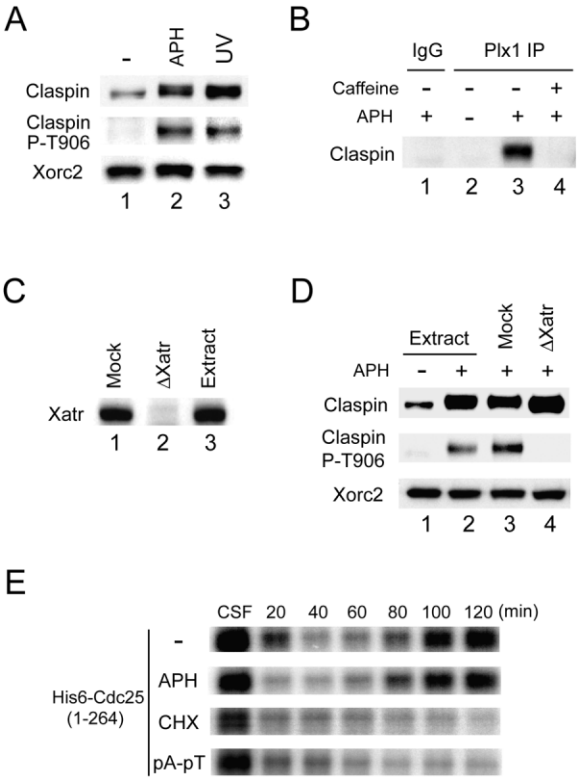

Figure 4. Characterization of the Claspin-Plx1 Interaction in Aphidicolin-Containing Extracts

(A) Extracts were incubated for 100 min with 1000 sperm nuclei/μl alone (lane 1), sperm nuclei plus aphidicolin (APH) (lane 2), or UV-damaged sperm nuclei (lane 3). Chromatin fractions were isolated and immunoblotted with anti-Claspin, anti-P-T906, and anti-Xorc2 antibodies.

(B) Extracts were incubated for 100 min with no drug (lane 2), aphidicolin (lanes 1 and 3), or aphidicolin plus caffeine (lane 4). Chromatin fractions were isolated and extracted with 0.5 M NaCl. Control (lane 1) and anti-Plx1 (lanes 2–4) immunoprecipitates from salt eluates were immunoblotted for Claspin.

(C) Immunodepletion of Xatr. Extracts were treated with control (lane 1) or anti-Xatr antibodies (lane 2) and immunoblotted for Xatr. Lane 3 shows untreated extract.

(D) The indicated extracts from (C) were incubated in the absence (lane 1) or presence of aphidicolin (lanes 2–4). Nuclear fractions were isolated and immunoblotted with anti-Claspin, anti-P-T906, and anti-Xorc2 antibodies.

(E) Kinase activity of Plx1 in checkpoint-activated extracts. M phase extracts from cytostatic factor (CSF)-arrested eggs were activated with calcium and incubated with no further addition (top), 1000 sperm nuclei/μl and 100 μg/ml aphidicolin, 100 μg/ml cycloheximide (CHX), or 50 μg/ml pA-pT. At the indicated times, Plx1 was immunoprecipitated and assayed for kinase activity by ^{32}P incorporation into His6-Cdc25(1-264) as described in Supplemental Data.

estingly, however, Plx1 responds differently to DNA replication blocks versus double-stranded DNA ends.

Plx1 Phosphorylates Claspin on S934

The docking of Plx1 onto Claspin could facilitate the phosphorylation of Claspin by Plx1. Consistent with this possibility, Plx1 is active in aphidicolin-treated extracts, in which Plx1 binds well to Claspin. Therefore, we examined directly whether Claspin could serve as a substrate for Plx1. We observed that full-length His6-Claspin underwent significant phosphorylation upon incubation with ^{32}P-ATP and anti-Plx1 immunoprecipitates from M phase extracts of *Xenopus* eggs (data not shown). In mapping experiments, GST peptides containing amino acids 847–962 and 878–962 of Claspin served as excellent substrates for immunoprecipitated Plx1 (Figure 5A). By contrast, GST-Claspin(847–920) did not become phosphorylated in this assay, which suggests that residues 921–962 contain a phosphorylation site(s) for Plx1. We also found that recombinant wild-type Plx1 but not the kinase-inactive Plx1-N172A mutant could phosphorylate the GST-Claspin(847–962) substrate efficiently (Figure 5B). Consistent with the results described above, recombinant Plx1 did not phosphorylate the GST-Claspin(847–920) peptide.

In the 921–962 region of Claspin, the sequence containing S934 resembles a consensus Plk phosphorylation site (Nakajima et al., 2003). We prepared a version of GST-Claspin(847–962) in which S934 was changed to alanine. As shown in Figure 5C, GST-Claspin(847–962)-S934A could not function as a substrate for Plx1. We performed tryptic phosphopeptide mapping to establish that S934 becomes phosphorylated in egg extracts (see Supplemental Figure S1 at http:/www.cell.com/cgi/content/full/117/5/575/DC1). We also prepared anti-phosphopeptide antibodies against an S934-phosphorylated peptide from Claspin (anti-P-S934). These anti-P-S934 antibodies reacted well with Claspin in aphidicolin-treated extracts (Figure 5D). Furthermore, immunodepletion of Plx1 abolished the reactivity of Claspin with the anti-P-S934 antibodies.

These results indicate that Plx1 phosphorylates Claspin on S934, which is relatively close to the Plx1-docking site at T906. Human Claspin also contains a serine at position 984 in a homologous sequence (Figure 5F). As one step to evaluate whether phosphorylation of Claspin on T906 facilitates phosphorylation of S934 by Plx1, we performed a time course experiment. For this purpose, we immunoblotted chromatin fractions from egg extracts at different times with anti-P-T906 and anti-P-S934 antibodies (Figure 5E). The results demonstrated that phosphorylation of T906 significantly precedes phosphorylation of S934.

The T906A and S934A Mutants of Claspin Are Defective in Checkpoint Adaptation

In order to explain the interaction between Plx1 and Claspin, we considered the possibility that Plx1 could negatively regulate Claspin, for example, by promoting the termination of a checkpoint arrest. Typically, control egg extracts enter mitosis after approximately 90–120 min of incubation. By contrast, in the presence of aphidicolin, the egg extracts remain arrested in interphase for at least 180 min. Nonetheless, aphidicolin-treated extracts also eventually undergo mitosis. Typically, this process begins at approximately 180–210 min. This appears to be a genuine mitosis on the basis of several criteria. There is a sharp rise in Cdc2-associated H1 kinase activity at this time (see below). Concomitantly, Cdc25C undergoes the characteristic hyperphosphorylation that is a marker for its activation at mitosis (data

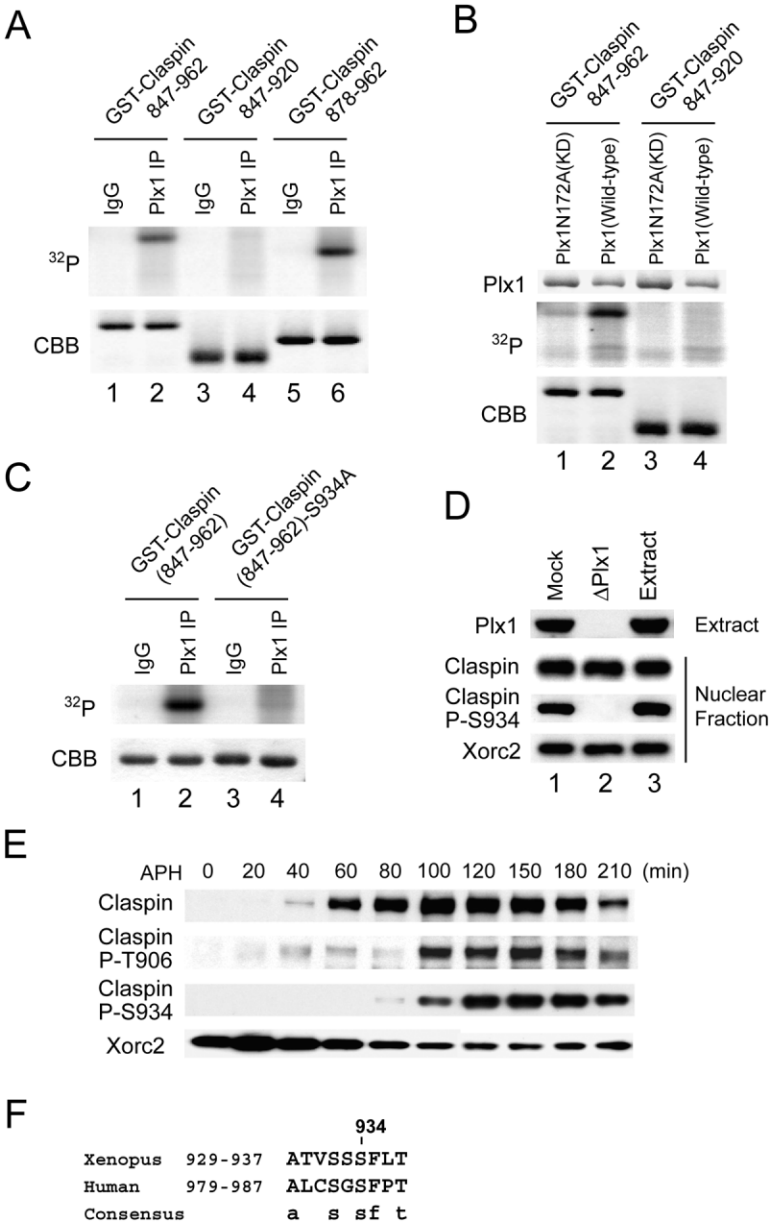

Figure 5. Plx1 Phosphorylates Claspin on S934

(A) GST-Claspin(847–962) (lanes 1 and 2), GST-Claspin(847–920) (lanes 3 and 4), and GST-Claspin(878–962) (lanes 5 and 6) were incubated with ^{32}P-ATP and either control (lanes 1, 3, and 5) or anti-Plx1 immunoprecipitates (lanes 2, 4, and 6) from M phase extracts. GST-tagged fragments were stained with Coomassie blue (bottom). Incorporation of ^{32}P was detected with a phosphorimager (top).

(B) Plx1-GH (lanes 2 and 4) and Plx1-N172A-GH (lanes 1 and 3) on nickel beads were incubated in M phase egg extracts as described in Supplemental Data. The beads were reisolated and incubated with Claspin(847–962) (lanes 1 and 2) or Claspin(847–920) (lanes 3 and 4) in the presence of ^{32}P-ATP. Recombinant Plx1 proteins (top) and GST-tagged Claspin fragments (bottom) were stained with Coomassie blue. Incorporation of ^{32}P was detected with a phosphorimager (middle).

(C) GST-Claspin(847–962) (lanes 1 and 2) and GST-Claspin(847–962)-S934A (lanes 3 and 4) were incubated in the presence of ^{32}P-ATP with control (lanes 1 and 3) or anti-Plx1 immunoprecipitates (lanes 2 and 4) from M phase extracts. GST-Claspin fragments were stained with Coomassie blue (bottom). Incorporation of ^{32}P was detected with a phosphorimager (top).

(D) Effect of immunodepletion of Plx1 on phosphorylation of S934. Egg extracts were treated with control antibodies (lane 1), anti-Plx1 antibodies (lane 2), or no antibodies (lane 3) and incubated with sperm nuclei and aphidicolin for 120 min. Extracts were immunoblotted for Plx1 (top panel). Nuclear fractions were isolated and immunoblotted with anti-Claspin, anti-P-S934, and anti-Xorc2 antibodies (bottom three panels).

(E) Time courses for phosphorylation on T906 and S934. Chromatin fractions were isolated from aphidicolin-treated extracts at the times shown and immunoblotted with anti-Claspin, anti-P-T906, anti-P-S934, and anti-Xorc2 antibodies.

(F) Alignment of residues 929–937 from *Xenopus* Claspin with human Claspin.

not shown). Finally, the condensed chromatin that forms in such extracts is morphologically very similar to the chromatin in mitotic extracts that had never been exposed to aphidicolin (data not shown).

In order to assess whether regulation of Claspin by Plx1 is involved in adaptation of the DNA replication checkpoint, we first removed the endogenous Claspin from egg extracts by immunodepletion with anti-Claspin antibodies (Figures 6A and 6B). In parallel, we prepared mock-depleted extracts by using control antibodies. Next, we added back wild-type His6-Claspin, His6-Claspin-T906A, or His6-Claspin-S934A to the Claspin-depleted extracts at very similar concentrations. Finally, we added sperm chromatin and aphidicolin to the extracts and monitored nuclear envelope breakdown (NEB) as an indicator for mitosis.

For the first 150–180 min, the mock-depleted extracts, as well as Claspin-depleted extracts containing His6-Claspin, His6-Claspin-T906A, or Claspin-S943A, all behaved similarly. In particular, these extracts all arrested in interphase, which indicates that a checkpoint-induced delay of the cell cycle had been triggered successfully. For comparison, mock-depleted extracts lacking aphidicolin underwent NEB at 100–120 min. Therefore, the T906A and S934A mutants are proficient at triggering a checkpoint delay. Consistent with this observation, we observed that Xchk1 underwent checkpoint-dependent phosphorylation normally in extracts containing the T906A and S934A mutants (data not shown; also see Figure 7). However, the various aphidicolin-treated extracts displayed different behaviors at later times. We observed that mock-depleted extracts and Claspin-

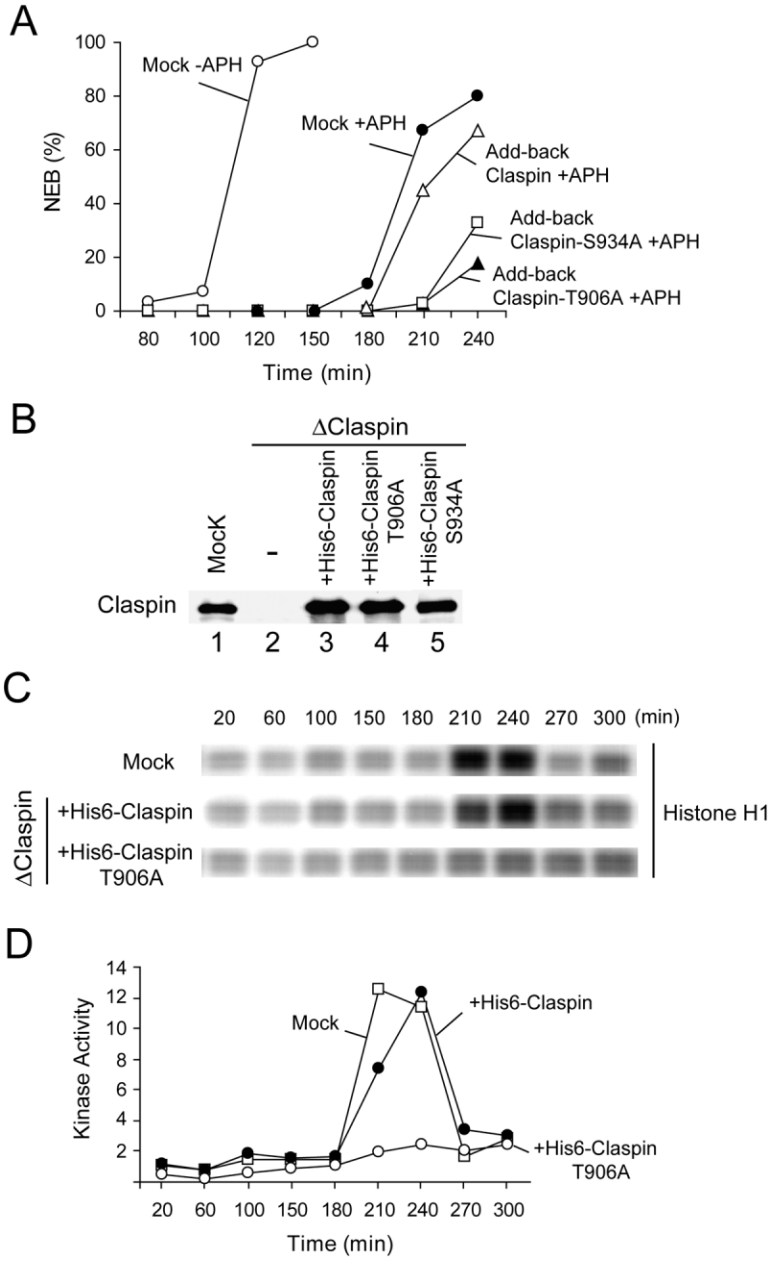

Figure 6. Egg Extracts Containing the T906A and S934A Mutants of Claspin Are Defective for Checkpoint Adaptation

(A) Extracts were subjected to mock immunodepletion (open circles and closed circles) or immunodepletion of Claspin (open triangles, closed triangles, and open squares). His6-Claspin (open triangles), His6-Claspin-T906A (closed triangles), and His6-Claspin-S934A (open squares) were added back to the Claspin-depleted extracts. The extracts were incubated with sperm nuclei in the absence or presence of aphidicolin, as indicated. Nuclear envelope breakdown (NEB) was determined at the indicated times by microscopy.

(B) Anti-Claspin immunoblots of extracts used for the experiment in Figure 6A. Mock-depleted (lane 1) and Claspin-depleted extracts containing no added protein (lane 2), His6-Claspin (lane 3), His6-Claspin-T906A (lane 4), and His6-Claspin-S934A (lane 5) were immunoblotted with anti-Claspin antibodies.

(C) Assay of Cdc2-associated H1 kinase activity. Aliquots from mock-depleted (top) and Claspin-depleted extracts containing either His6-Claspin (middle) or His6-Claspin-T906A (bottom) were removed at the indicated times and incubated with histone H1 and ^{32}P-ATP. Incorporation of ^{32}P was detected with a phosphorimager.

(D) Quantitation of H1 kinase activity from Figure 6C (in arbitrary units).

depleted extracts containing wild-type His6-Claspin underwent NEB at 210–240 min. By contrast, the timing of NEB was significantly delayed in extracts containing the His6-Claspin-T906A or His6-Claspin-S934A mutants.

In order to pursue these observations further, we also measured phosphorylation of histone H1 as a marker for Cdc2-associated kinase activity. As shown in Figures 6C and 6D, there was also a steep rise in H1 kinase activity at 210–240 min coincident with NEB in mock-depleted extracts and Claspin-depleted extracts containing wild-type His6-Claspin, both of which were treated with aphidicolin. However, there was no rise in H1 kinase activity even by 300 min in aphidicolin-treated extracts containing the T906A mutant (Figures 6C and 6D). We obtained similar results for the S934A mutant (data not shown). Upon further examination, we found that the NEB that occurs at later times in extracts containing the T906A and S934A mutants is due to the occurrence of apoptosis rather than entry into mitosis (data not shown). Apoptosis also occurs in untreated extracts lacking aphidicolin with similar timing, which has been well documented (Newmeyer et al., 1994). Therefore, it is unclear whether there is a connection between failure of adaptation and occurrence of apoptosis. Taken together, these results indicate that extracts containing the T906A and S934A mutants of Claspin are unable to undergo adaptation and entry into mitosis after a prolonged checkpoint arrest. Eventually, the nuclei in extracts containing these mutants do undergo breakdown, but this process represents apoptotic disintegration and not mitotic disassembly.

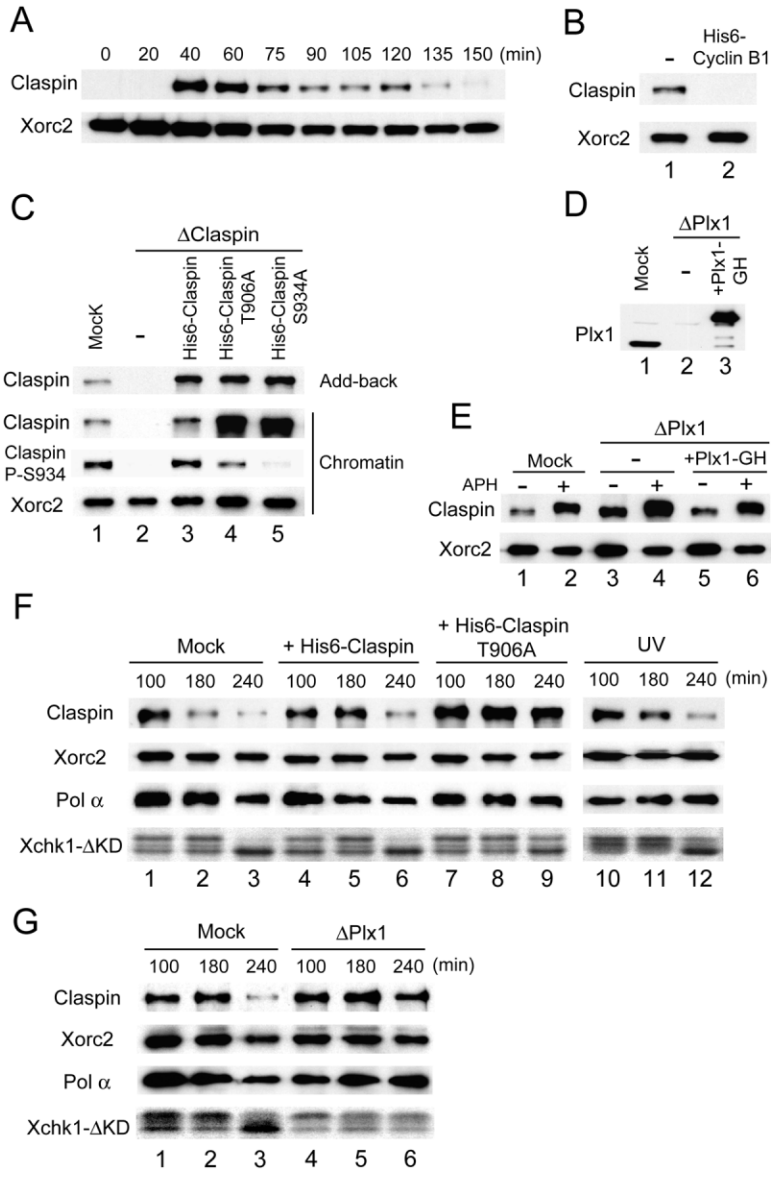

Figure 7. The T906A and S934A Mutants of Claspin Display Elevated Binding to Aphidicolin-Treated Chromatin

(A) Claspin is present in low amounts on chromatin at M phase. At the indicated times, chromatin fractions were isolated from extracts lacking cycloheximide and immunoblotted for Claspin (top) and Xorc2 (bottom). This extract entered mitosis at 105–120 min.

(B) Interphase extracts were incubated with cycloheximide for 60 min, and then buffer (lane 1) or human His6-cyclin B1 (lane 2) was added. After 10 min (when mitosis had occurred in the cyclin-treated extract), chromatin fractions were isolated and immunoblotted for Claspin (top) and Xorc2 (bottom).

(C) Chromatin binding properties of mutant Claspin proteins. Egg extracts were treated with control (lane 1) or anti-Claspin antibodies (lanes 2–5). To the Claspin-depleted extracts, no recombinant protein (lane 2), wild-type His6-Claspin (lane 3), His6-Claspin-T906A (lane 4), and His6-Claspin-S934A (lane 5) were added back. Aliquots of the extracts were immunoblotted for Claspin (top). These extracts were incubated for 100 min. At this time, chromatin fractions were isolated and immunoblotted with anti-Claspin, anti-P-S934, and anti-Xorc2 antibodies (bottom three panels).

(D) Immunodepletion of Plx1. Egg extracts were treated with control (lane 1) or anti-Plx1 antibodies (lanes 2 and 3). Recombinant Plx1-GH was added back to an aliquot of Plx1-depleted extract (lane 3). The extracts were immunoblotted for Plx1.

(E) Binding of Claspin to chromatin in Plx1-depleted extracts. Mock-depleted extracts (lanes 1 and 2), Plx1-depleted extracts (lanes 3 and 4), and Plx1-depleted extracts containing Plx1-GH (lanes 5 and 6) from panel D were incubated for 100 min in the absence (lanes 1, 3, and 5) or presence of aphidicolin (lanes 2, 4, and 6). Chromatin fractions were isolated and immunoblotted for Claspin (top) and Xorc2 (bottom).

(F) Mock-depleted extracts (lanes 1–3), Claspin-depleted extracts containing His6-Claspin (lanes 4–6), and Claspin-depleted extracts containing His6-Claspin-T906A (lanes 7–9) were incubated with sperm nuclei and aphidicolin. In addition, mock-depleted extracts were incubated with UV-damaged sperm chromatin (lanes 10–12). At the indicated times, chromatin fractions were isolated and immunoblotted for Claspin (top panel), Xorc2 (second panel from top), and the p70 subunit of Pol α (third panel from top). For the experiment in the bottom panel, ^{35}S-labeled Xchk1-ΔKD was synthesized in the TnT system, separated from unincorporated ^{35}S methionine by gel filtration, and added to the indicated extracts. At the times shown, whole extracts were subjected to SDS-PAGE, and the electrophoretic mobility of ^{35}S-Xchk1-ΔKD was assessed by phosphorimaging.

(G) Immunodepletion of Plx1 prevents adaptation. Mock-depleted (lanes 1–3) and Plx1-depleted extracts (lanes 4–6) were treated with aphidicolin. At the indicated times, binding of Claspin, Xorc2, and Pol α to chromatin and phosphorylation of ^{35}S-Xchk1-ΔKD in whole extracts were determined as in Figure 7F.

The T906A and S934A Mutants of Claspin Display Enhanced Binding to Aphidicolin-Treated Chromatin

To investigate the mechanism by which Plx1 might control Claspin during checkpoint adaptation, we examined whether regulation by Plx1 affects the chromatin binding properties of Claspin. As described previously, Claspin binds to chromatin during a normal S phase in *Xenopus* egg extracts in a manner that requires the pre-RC, Cdc45, and Cdk2 (Lee et al., 2003). This binding increases substantially in the presence of aphidicolin. All of these previous experiments were performed in the presence of cycloheximide, which blocks the entry into mitosis. Cycloheximide inhibits the synthesis of cyclin B and also prevents the activation of Plx1 (Figure 4E). For this reason, we initially characterized the binding of Claspin to chromatin in extracts that proceed into mitosis. First, we examined binding in extracts that did not contain either cycloheximide or aphidicolin (Figure 7A). Consistent with previous findings, binding of Claspin to chromatin peaked at 40–60 min and then declined as DNA replication approached completion. At 120 min, when this particular extract had entered mito-

sis, there was relatively little Claspin on chromatin. In another type of experiment, we incubated chromatin in egg extracts containing cycloheximide for 60 min and then added a high concentration of recombinant human His6-cyclin B1, which induced mitosis in 5–10 min. We observed that addition of cyclin B1 triggered the release of Claspin from chromatin (Figure 7B). Altogether, these results indicate that there is little if any Claspin on chromatin at mitosis when Cdc2-cyclin B and Plx1 are maximally active.

We proceeded to evaluate whether Plx1 affects the binding of Claspin to chromatin during an aphidicolin-induced arrest. To examine this possibility, we first immunodepleted the endogenous Claspin from egg extracts and replaced it with wild-type His6-Claspin, His6-Claspin-T906A, or His6-Claspin-S934A (Figure 7C). Next, we added sperm chromatin and aphidicolin to the various extracts. Finally, we reisolated chromatin from the extracts after 100 min and performed immunoblotting with anti-Claspin antibodies (Figure 7C). As expected, we could readily detect Claspin on aphidicolin-treated chromatin from mock-depleted extracts and Claspin-depleted extracts containing wild-type His6-Claspin. However, there was highly elevated binding of both the T906A and S934A mutants of Claspin to chromatin under these conditions. We also examined the chromatin fractions by immunoblotting with anti-S934-P antibodies. These antibodies reacted well with endogenous Claspin and wild-type His6-Claspin. By comparison, the anti-P-S934 antibodies interacted weakly with the chromatin bound His6-Claspin-T906A mutant (note that amount of His6-Claspin-T906A on chromatin greatly exceeds that of wild-type His6-Claspin). These observations imply that phosphorylation of Claspin on T906 facilitates subsequent phosphorylation of S934.

These findings suggest that Plx1 might negatively regulate the association of Claspin with chromatin. To investigate this possibility further, we removed Plx1 from egg extracts with anti-Plx1 antibodies (Figure 7D). We could restore Plx1 by adding back a recombinant Plx1-GH protein. As shown in Figure 7E, immunodepletion of Plx1 resulted in enhanced binding of Claspin to chromatin in both the absence and presence of aphidicolin. Furthermore, this increase was abrogated by the addition of recombinant Plx1-GH back to the Plx1-depleted extracts.

Next, we examined the binding of Claspin to chromatin as a function of time in aphidicolin-treated extracts undergoing adaptation. As shown in Figure 7F, the amount of endogenous Claspin on chromatin in mock-depleted extracts declined at 180–240 min as these extracts underwent adaptation. A similar decrease occurred around the same time for wild-type His6-Claspin that had been added back to Claspin-depleted extracts. By contrast, the His6-Claspin-T906A mutant remained associated with chromatin at high levels during this period. In parallel, we also examined the checkpoint-dependent phosphorylation of Xchk1 (Figure 7F). For this purpose, we monitored the electrophoretic mobility of a ^{35}S-labeled form of the Xchk1-ΔKD protein (Michael et al., 2000), whose phosphorylation can be detected in whole egg extracts without the need for isolation of nuclear fractions. At 240 min, in both mock-depleted and Claspin-depleted extracts containing wild-type His6-Claspin, the aphidicolin-induced phosphorylation of Xchk1-ΔKD was reversed. By contrast, in Claspin-depleted extracts containing the T906A mutant, Xchk1-ΔKD remained in its hyperphosphorylated form at 240 min. As a control, we showed that, in the absence of aphidicolin, Xchk1-ΔKD does not undergo phosphorylation in extracts containing either the T906A or S934A mutants (see Supplemental Figure S2 on *Cell* website). This observation argues that the T906A and S934A mutants do not prolong a checkpoint response by disrupting replication forks.

A significant consideration in these experiments is whether the initiating checkpoint signal, namely aphidicolin-induced DNA replication blocks, is still present at the time of adaptation. We ruled out the trivial possibility that aphidicolin loses efficacy at later times by showing that there is no measurable DNA synthesis at 240 min in aphidicolin-treated extracts (data not shown). We also assessed the presence of stalled replication forks by immunoblotting chromatin fractions for Pol α, which accumulates in high amounts at stalled forks in aphidicolin-treated chromatin (Walter and Newport, 2000). Although there was some reduction at later times, the results showed that, at 240 min, Pol α remained in substantial amounts on chromatin from mock-depleted extracts and Claspin-depleted extracts containing either wild-type His6-Claspin or the Claspin-T906A mutant (Figure 7F). Thus, there appears not to be a general collapse of stalled DNA replication forks at this time. Extracts containing UV-damaged chromatin also undergo adaptation by 240 min, as indicated by dissociation of Claspin from chromatin, inactivation of Xchk1, and persistence of Pol α on the DNA (Figure 7F). Therefore, adaptation of the checkpoint response to DNA replication blocks occurs whether these blocks accumulate because of treatment with aphidicolin or infliction of UV damage.

Finally, we also assessed the role of Plx1 in adaptation by immunodepleting this kinase from the extracts. As shown in Figure 7G, Claspin remained on chromatin, and Xchk1 did not undergo inactivation at 240 min in Plx1-depleted extracts containing aphidicolin. Overall, these results suggest that Plx1 promotes both the dissociation of Claspin from chromatin and inactivation of Xchk1 in aphidicolin-treated extracts that undergo adaptation after a prolonged interphase arrest. This process corresponds to adaptation rather than recovery, because stalled replication forks persist upon mitotic entry.

Discussion

Signal transduction pathways commonly undergo adaptation in the continued presence of an activating signal or ligand. In this report, we have found that the DNA replication checkpoint response in *Xenopus* egg extracts eventually undergoes adaptation, so that biochemical events of mitosis occur despite the presence of incompletely replicated DNA. This process depends upon Plx1, which negatively regulates Claspin, a critical mediator of the DNA replication checkpoint (see Figure

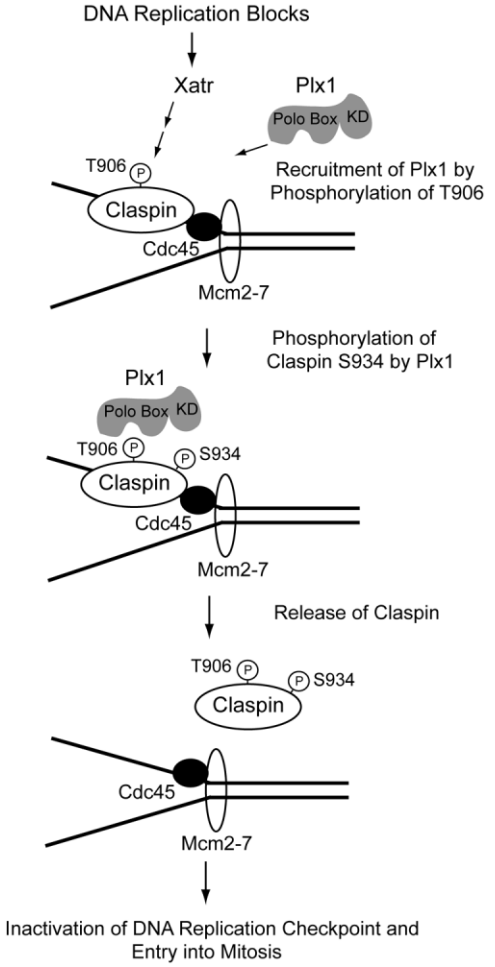

Figure 8. Model for Regulation of Claspin by Plx1 during a DNA Replication Checkpoint Response

8). This regulation involves docking of Plx1 onto a phosphopeptide motif in Claspin containing T906, which undergoes phosphorylation during the course of the checkpoint response. After docking, Plx1 phosphorylates a nearby residue, namely S934, in Claspin. This sequence of events leads to dissociation of Claspin from chromatin, inactivation of Xchk1, and release of the cell cycle arrest.

Targeting of a Checkpoint Mediator Protein for Adaptation

Claspin is an attractive target for inactivation of a DNA replication checkpoint response, because this protein is essential for imposition of this response. In principle, there could be other targets of Plx1 as well as Plx1-independent mechanisms for checkpoint adaptation. However, our observations appear to argue that Claspin is the major if not exclusive target of this pathway. It is difficult to envision how single point mutations in Claspin (e.g., T906A and S934A) would compromise the ability of Plx1 to interact with other targets or affect Plx1-independent mechanisms. Xchk1 undergoes dephosphorylation and inactivation when Claspin dissociates from chromatin. The identity of the Xchk1-inactivating phosphatase is unknown, but our data argue that this enzyme is either constitutively active or subject to negative regulation by the Claspin-dependent pathway.

Claspin accumulates on chromatin at high levels when control by Plx1 is compromised. One model would be that phosphorylation of Claspin by Plx1 lowers its affinity for chromatin and that dissociation of Claspin from the replication fork consequently leads to ablation of checkpoint signaling. Another possibility is that Plx1 inactivates Claspin by some other mechanism—for example, by inhibiting its ability to mediate Xatr-dependent activation of Xchk1. In this case, Claspin may dissociate from chromatin as a result of abolished checkpoint signaling. The fact that adaptation appears to lag behind phosphorylation on S934 suggests that some additional step(s) is involved in this process.

According to the definition of checkpoint adaptation (Sandell and Zakian, 1993; Toczyski et al., 1997), a checkpoint-inducing signal should be present at the time that arrest of the cell cycle is alleviated. Therefore, a significant consideration is whether aphidicolin-induced DNA replication blocks remain at the time that Plx1 promotes mitotic entry. Pol α remains associated in large amounts with chromatin at the time of adaptation, which argues that this process is not due to a general collapse of DNA replication forks. It remains possible that there is some less drastic alteration of replication forks that compromises checkpoint signaling at this time. Nonetheless, since extracts containing the T906A and S934A mutants of Claspin cannot enter mitosis, one would have to argue that such an alteration could not occur in the presence of these mutants. Interestingly, Mrc1, a Claspin homolog, has been implicated in replication pausing in budding yeast, which suggests that this protein regulates the replication fork in some manner (Katou et al., 2003). It is possible that Claspin could control the structure of replication forks, in addition to its known function in mediating the activation of Xchk1 by Xatr. If so, our results would suggest that Plx1 negatively regulates the ability of Claspin to control replication fork structure. In this scenario, adaptation would occur because Claspin cannot maintain the appropriate replication fork structure for checkpoint signaling after phosphorylation by Plx1.

Diverse Regulation of Plk during Checkpoint Responses

These studies have also provided additional insight into the regulation of Plk during checkpoint responses. Human Plk1 is downregulated in response to ionizing radiation, which creates double-stranded DNA breaks (Smits et al., 2000). We have confirmed these observations in *Xenopus* egg extracts by showing that the free double-stranded DNA ends inhibit activation of Plx1. By contrast, substantial activation of Plx1 occurs in aphidicolin-containing extracts. The effect of aphidicolin (or hydroxyurea) on regulation of human Plk1 has not been reported. It appears that aphidicolin can induce an interphase arrest in egg extracts by triggering inhibition of the Cdc2-cyclin B pathway (and possibly one or more other pathways) without shutting off the activation of Plx1. It has been suggested that both active and inactive Plk can dock onto prospective substrates by means of

its PBD (Elia et al., 2003). In the latter case, such docking might promote the activation of inactive Plk. The fact that Plx1 is already active in aphidicolin-treated extracts would likely enhance its ability to phosphorylate Claspin.

Adaptation in Other Systems and Contexts

An important issue is whether the regulation of checkpoint mediator proteins such as Claspin by Plk is conserved in other systems. The budding yeast homolog of Plk is encoded by the *CDC5* gene. Previous studies have found that yeast cells harboring an allele of *CDC5* called *cdc5-ad* do not undergo adaptation of the DNA damage response (Toczyski et al., 1997). The budding yeast Cdc5-ad protein appears to have a very selective defect. For example, this protein is not compromised in general mitotic functions, because undamaged yeast cells containing this mutation appear to divide normally. Furthermore, budding yeast cells with the *cdc5-ad* mutation that have managed to repair damaged DNA also recover normally from a checkpoint arrest and undergo mitosis. The implication is that the Cdc5-ad protein has some deficiency in interactions with the checkpoint control machinery. By analogy with the studies in this paper, it is plausible that budding yeast Cdc5 may inactivate the checkpoint mediator protein Rad9. The downregulation of Rad9 would be expected to lead to inactivation of Rad53, which is known to occur during checkpoint adaptation in this organism (Pellicioli et al., 2001).

In fission yeast, Crb2, a homolog of budding yeast Rad9, is essential for the activation of Chk1 after DNA damage. Crb2 is a substrate of Cdc2, the archetypal cell cycle regulatory kinase (Esashi and Yanagida, 1999). Fission yeast cells with a mutant of Crb2 lacking a certain phosphorylation site for Cdc2 (Crb2-T215A) cannot exit a DNA damage arrest properly. More recent studies have provided evidence that Cdc2 may regulate a repair function of Crb2 during a G2 arrest (Caspari et al., 2002).

Notably, the sequences comprising the docking and phosphorylation sites for Plx1 in *Xenopus* Claspin are conserved in human Claspin. Treatment of mammalian cells with aphidicolin or hydroxyurea leads to a prolonged S phase arrest. No clear evidence for adaptation of this arrest has been provided, but this matter deserves further investigation. Aphidicolin-treated egg extracts that eventually undergo mitosis with stalled replication forks would most likely experience the equivalent of a mitotic catastrophe. A similar process in intact animal cells would almost certainly ensure cell death. Under such circumstances, adaptation may serve as a prelude to cell death and elimination of defective cells from the body. It should also be noted that nearly complete inhibition of replication, as occurs following treatment with aphidicolin, is not a situation that would normally be encountered in nature. It is conceivable that adaptation in animal cells with less extensive blockage of replication, for example, following UV exposure, could result in viable progeny with chromosomal aberrations. Although such events could be quite rare, their occurrence might be very detrimental to an organism. Cells with impaired death capabilities may be more susceptible to such problems.

A related question is whether Plk is involved in adaptation of DNA damage responses in mammalian cells. In this case, targeting by Plk could inactivate putative checkpoint mediator proteins that are involved in DNA damage responses (e.g., 53BP1 and Mdc1) (reviewed in Canman [2003]). Cells with extensive DNA damage undergo apoptosis. The downregulation of checkpoint mediator proteins could also help to determine the eventual fate of cells with damaged DNA.

Conclusion

We have established that Plx1 negatively regulates the checkpoint mediator protein Claspin during a DNA replication checkpoint response. As a consequence, Plx1 triggers adaptation of the checkpoint arrest of the cell cycle, so that mitosis occurs despite the presence of incompletely replicated DNA. It will be important to evaluate whether such adaptive processes can lead to cell death or genetic aberrations in multicellular organisms.

Experimental Procedures

Xenopus Egg Extracts

Xenopus egg extracts were prepared as before (Kumagai and Dunphy, 2000). To induce checkpoint responses, extracts were incubated with 100 μg/ml aphidicolin in the presence of demembranated sperm nuclei (1000–3000/μl), sperm nuclei (1000–3000/μl) irradiated with a UV dose of 1000 J/m^2, or 50 μg/ml pA-pT (Kumagai and Dunphy, 2000). Methods for preparation of nuclear and chromatin fractions from extracts are described in Supplemental Data.

Antibodies and Recombinant Proteins

The various antibodies used in this paper are described in Supplemental Data. Recombinant proteins were produced by expression in baculovirus-infected insect cells or in vitro translation as described in Supplemental Data.

Immunoprecipitation and Immunodepletion

For immunoprecipitations, extracts (100 μl) were incubated with Affiprep-protein A beads (Bio-Rad) containing 5 μg of anti-Plx1 or anti-Claspin antibodies for 45 min at 4°C. The beads were washed three times with buffer A (10 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 0.5% NP-40, 2.5 mM EGTA, and 20 mM β-glycerolphosphate) and once with HEPES-buffered saline (HBS; 10 mM HEPES-KOH [pH 7.5], and 150 mM NaCl). For immunodepletion of Plx1, interphase extracts (100 μl) were incubated with 15 μg of anti-Plx1 antibodies bound to 10 μl of Affiprep protein A beads at 4°C for 45 min. After the incubation, the beads were removed by centrifugation, and the supernatants were treated for another round of depletion. Claspin and Xatr were immunodepleted as described (Lee et al., 2003).

Identification of Plx1-Interacting Proteins by Mass Spectrometry

Anti-Plx1 immunoprecipitates were subjected to SDS-PAGE and stained with Coomassie blue. Two bands at approximately 180 kDa were excised and in-gel digested with trypsin as described previously (Shevchenko et al., 1996). Tryptic peptides were sequenced by nanoelectrospray tandem mass spectrometry on a QSTAR Pulsar *i* quadrupole time-of-flight mass spectrometer (MDS Sciex, Canada). Uninterpreted MS/MS spectra were first searched against a protein sequence database MSDB by Mascot v.1.8 software (Matrix Science Ltd., United Kingdom) installed on a local server. No restrictions on species of origin or protein molecular weight were imposed. All Mascot hits were further verified by manual inspection of matched tandem mass spectra. Spectra not matched by Mascot were manually interpreted de novo. The interpretation of each spectrum rendered a few degenerate, redundant, incomplete, and partly accurate candidate peptide sequences. All peptide sequence proposals obtained by the interpretation of all tandem mass spectra were assembled into a single query, as described (Shevchenko et al., 2001), which was submitted to Mass Spectrometry-driven BLAST (MS BLAST) (Shevchenko et al., 2003) search against a nonredundant

protein database nrdb95 on a web server at http://dove.embl-heidelberg.de/Blast2/msblast.html. Peptides from five proteins along with a few peptides from rabbit IgG were identified in the two bands. Three fragmented peptides exactly matched the corresponding peptide sequences from *Xenopus* Claspin. One additional peptide from Claspin was identified by sequence similarity because of deamidation of asparagine. Five sequenced peptides enabled crossspecies identification of a yet unknown *Xenopus* homolog of the transcription activator SNF2L4. Unknown *Xenopus* homologs of golgin-160 (nine matched peptides), polybromo-1/BAF180 (six matched peptides), and a human hypothetical protein AK094821 (one matched peptide) were also identified. We noted that, by combining stringent (Mascot) and sequence similarity (MS BLAST) identification approaches, all fragmented peptide precursors were matched, which ensured that no unknown *Xenopus* proteins were missed.

Assay for Binding of Claspin Fragments to Plx1 in Egg Extracts

Recombinant Plx1 (2 μg) bound to nickel agarose beads (10 μl) and ^{35}S-labeled Claspin fragments were incubated for 100 min in egg extracts (50 μl) containing 100 μg/ml cycloheximide in the absence or presence of 50 μg/ml pA-pT. The beads were isolated by centrifugation and washed three times with buffer A and once with HBS. Bound proteins were subjected to SDS-PAGE and detected with a phosphorimager.

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