Chromatin decondensation in S-phase involves recruitment of Cdk2 by Cdc45 and histone H1 phosphorylation

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Cdc45 is required for initiation of DNA replication and fork progression, but its function in these processes remains unknown. We show that targeting Cdc45 to specific chromosomal sites in mammalian cells results in large-scale chromatin decondensation that strongly correlates with histone H1 phosphorylation. Cdk2 is recruited to sites of Cdc45 decondensation, and Cdk2 inhibitors reduce the level of decondensation. Targeting wild-type Cdk2, but not kinase-defective Cdk2, to chromatin is also effective at inducing decondensation involving phospho-H1. Cdc45, Cdk2, Cyclin A, and phospho-H1 associate with chromatin during S-phase, and Cdc45, Cdk2, and an active H1 kinase physically interact. Replicating DNA and phospho-H1 foci colocalize in vivo, and S-phase progression and H1 phosphorylation are directly related and Cdk2 dependent. Because Cdk2 colocalizes with replication foci and H1 regulates higher-order chromatin, we suggest a model in which Cdc45 recruits Cdk2 to replication foci, resulting in H1 phosphorylation, chromatin decondensation, and facilitation of fork progression.

Introduction

Replication of eukaryotic genomes involves the regulated and timely assembly of multiprotein complexes on chromatin templates in vivo. The origin recognition complex recruits Cdc6 and Cdt1, which support the loading of minichromosome maintenance (MCM) complexes onto origins, followed by Cdc45 recruitment at the G_1–S transition (Bell and Dutta, 2002). Once DNA replication begins, Cdc45 and the MCM complex progress with the replication forks (Aparicio et al., 1997; Labib et al., 2000; Tercero et al., 2000; Zou and Stillman, 2000). Although the MCM complex is proposed to act as the replicative helicase (Bell and Dutta, 2002), the role of Cdc45 in the initiation and elongation processes has yet to be established.

In eukaryotes, the genome is packaged into nucleosomes, the basic unit of chromatin. Chromatin structure is dynamic, and nucleosome–nucleosome interactions lead to higher levels of chromatin compaction (Belmont et al., 1999). Higher-order chromatin compaction presents a formidable barrier to trans-acting factors that must access the underlying DNA. It is clear that the transcription apparatus modifies chromatin at promoters and within transcribed regions as transcription forks progress (Strahl and Allis, 2000; Belotserkovskaya et al., 2003). It is also likely that the DNA replication machinery is effective at chromatin remodeling, owing to the need for efficient access to the entire genetic complement. At present, very little is known about chromatin remodeling during S-phase.

An important player in higher-order chromatin packaging is histone H1, which binds to the linker DNA between nucleosome particles and stabilizes higher-order structure (Wolffe, 1997). H1 has been shown to affect nucleosome spacing and restrict nucleosome mobility (Dou et al., 2002). An important modification of H1 is phosphorylation (Roth and Allis, 1992). Initially, it was thought that H1 phosphorylation played a role in mitotic chromosome condensation because of elevated levels of hyperphosphorylated H1 during mitosis (Gurley et al., 1978; Bradbury, 1992). However, it has since been shown that neither H1 nor its phosphorylated derivative is required for mitotic chromosome condensation (Dou et al., 1999). H1 phosphorylation increases the dynamic exchange of H1 with chromatin (Dou et al., 2002) and has been shown to activate or repress specific genes (Dou and Gorovsky, 2000). Thus, these and other data have led to the proposal that H1 phosphorylation promotes decondensation, perhaps transiently, during which time other factors involved in various events gain access to DNA in chromatin (Roth and Allis, 1992).

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Abbreviations used in this paper: DHFR, dihydrofolate reductase; HSR, homogenously staining region; lacO, lac operator; MCM, minichromosome maintenance.

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H1 is in its lowest phosphorylation state in G_1 and becomes phosphorylated during S-phase, reaching a maximum in mitosis (Gurley et al., 1978; Lu et al., 1994). Unphosphorylated H1 added to *Xenopus laevis* extracts is capable of inhibiting DNA replication in vitro (De et al., 2002), whereas phospho-H1 extracted from S-phase cells and reconstituted with SV40 minichromosomes supports a higher level of replication than phospho-H1 from mitotic extracts (Halmer and Gruss, 1996). Furthermore, a correlation between defects in H1 phosphorylation and incomplete DNA replication has been detected in mammalian cells (Yasuda et al., 1981). These studies suggest that H1 phosphorylation may play an important role in S-phase.

It has been directly demonstrated in vivo that large-scale chromatin decondensation occurs before, or coincident with, replication through a chromosomal region (Li et al., 1998). However, the mechanisms involved in this decondensation process are poorly understood. One possibility is that proteins functioning at DNA replication forks interact with chromatin remodeling complexes to facilitate fork progression, as occurs during transcription. To test this proposal, we have used an in vivo chromatin remodeling system (Li et al., 1998; Tumbar et al., 1999; Ye et al., 2001) to determine if targeting Cdc45 (which is required for replication fork progression) to specific chromosomal sites leads to higher-order chromatin decondensation. We find that Cdc45 promotes a dramatic decondensation of chromatin in the targeted region. We show that decondensation correlates with the presence of phospho-H1 and that Cdk2 is likely involved. We further show that the effects observed in this chromatin remodeling assay system are likely to be physiologically relevant during DNA replication. We propose a model in which Cdc45 recruits Cdk2 to replication foci, leading to phosphorylation of histone H1, decondensation of chromatin, and facilitation of DNA replication.

Results

Cdc45 targeting promotes large-scale chromatin decondensation in mammalian cells

To investigate the mechanisms involved in allowing access to chromatin templates by the DNA replication machinery, we used an in vivo chromatin remodeling assay system to test the ability of selected replication proteins to promote decondensation of compacted chromatin at a targeted chromosomal locus (Li et al., 1998; Tumbar et al., 1999). In this system, multiple copies of a lac operator (lacO)/dihydrofolate reductase (DHFR) vector were stably integrated into a CHO-derived cell line, followed by amplification of the DHFR-containing chromosomal segment by selection on methotrexate (Li et al., 1998). The resulting cell line (A03_1) contains a 90-Mb homogenously staining region (HSR) consisting of ~60 amplicons, each containing ~400 kb of tandemly arrayed 14-kb lacO/DHFR vectors and ~1,000 kb of flanking coamplified genomic DNA.

The HSR can be directly visualized in either fixed or living A03_1 cells by providing the LacI protein or LacI-GFP derivative in trans. In either case, when visualized microscopically via an anti-LacI antibody or the fluorescent tag itself, the HSR assumes a condensed dot-like structure and is heterochromatic (Fig. 1 A, pNYE4-LacI and pRcLac; Li et al., 1998; Tumbar et al., 1999). However, the expression of certain proteins fused to LacI (e.g., VP16 or BRCA1) induces a dramatic decondensation of the HSR via recruitment of chromatin remodeling complexes by the LacI fusion partner (Fig. 1 A, pNYE4-LacI-VP16 and pRcLac-BRCA1(6c-w); Tumbar et al., 1999; Ye et al., 2001).

To determine whether or not proteins involved in initiation of replication can promote large-scale decondensation of the HSR, Chinese hamster Cdc45 and Cdc6 cDNAs were fused to LacI and transfected into AO3_1 cells. Immunoblotting showed similar levels of expression of both proteins (Fig. S1 C, available at http://www.jcb.org/cgi.content/full/jcb.200409055/DC1). As shown in Fig. 1 B, LacI-Cdc45 promotes a dramatic decondensation of the HSR in AO3_1 cells, with morphologies very similar to those induced by LacI-VP16 or LacI-BRCA1 (Fig. 1 A). Multiple examples are presented in Fig. 1 B to illustrate the heterogeneity of decondensed structures observed. As shown in Fig. 1 (A and B), the decondensed HSRs often display a punctate beads-on-a-string staining pattern consisting of lacO sites bound by the cognate fusion proteins alternating with coamplified, nonstaining, genomic sequences between the lacO cassettes (Tumbar et al., 1999; Ye et al., 2001). Note that most of the LacI-Cdc45–transfected cells display both nuclear and cytoplasmic staining, as do cells transfected with HA-Cdc45 (unpublished data), consistent with previous studies on native Cdc45 in vivo (Saha et al., 1998). These data suggest that A03_1 cells regulate the localization of the Cdc45 fusion protein by physiologically relevant mechanisms.

In contrast, the LacI-Cdc6 fusion protein does not induce detectable decondensation, with the HSR displaying a compact dot-like structure (Fig. 1 C, left). These patterns closely mimic those observed in cells expressing the LacI DNA-binding domain alone (Fig. 1 A, pNYE4-LacI and pRcLac) or when fused with certain BRCA1 mutants (Tumbar et al., 1999; Ye et al., 2001). Many of the LacI-Cdc6–expressing cells also display staining throughout the nucleus, with no apparent targeting to the HSR (Fig. 1 C, right), which is consistent with previous data on endogenous and ectopically expressed Cdc6 in mammalian cells (Alexandrow and Hamlin, 2004). The latter result again suggests that the fusion proteins are regulated by normal physiological mechanisms.

We have quantified the data from six different transfected samples in Table I. Patterns were classified as open and decondensed (labeled O in Fig. 1 B), closed and condensed (labeled C in Fig. 1 B), or indeterminate (labeled I in Fig. 1 B). The HSR was considered to be condensed if the fluorescent image occupied 2–5% of the nuclear area, decondensed if it occupied >10% of the nuclear area, and indeterminate if the HSR occupied 5–10% of the nuclear area (Tumbar et al., 1999). In this analysis, ~75% of LacI-Cdc45–expressing cells displayed open structures, whereas only 7% of LacI-Cdc6– or LacI-expressing cells did so. With LacI-Cdc6, 41% displayed dots, whereas 36% displayed complete nuclear staining patterns. In agreement with previous work, LacI-VP16 and LacI-BRCA1(6c-w) displayed

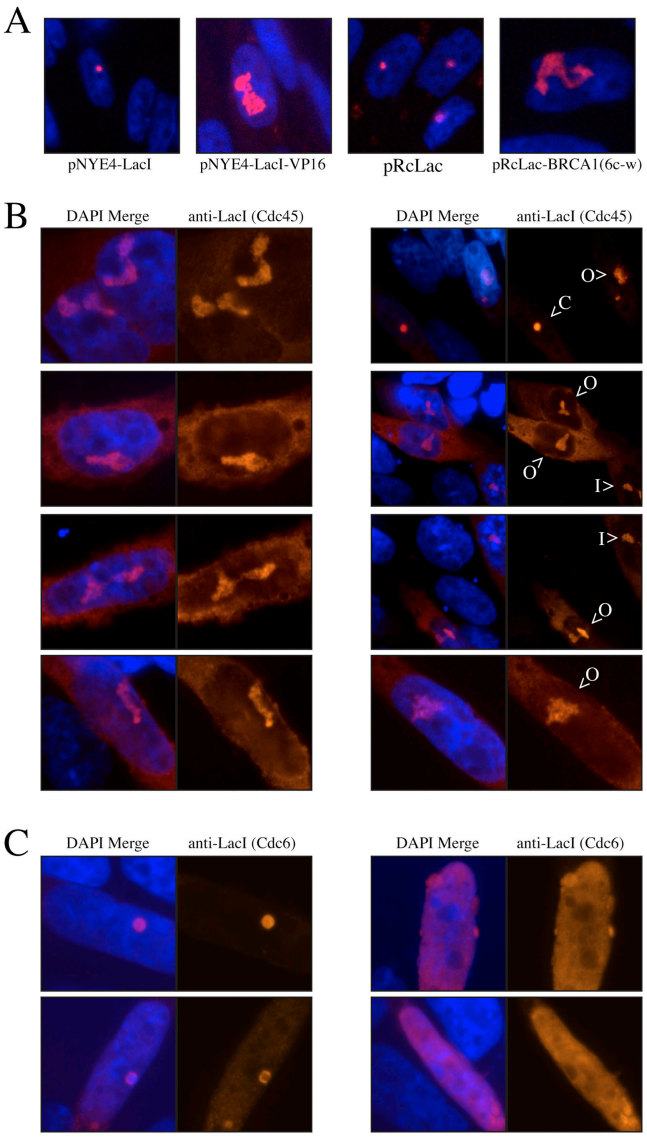

Figure 1. **Cdc45, but not Cdc6, targeting promotes large-scale chromatin decondensation.** (A) Control A03_1 cells were transfected with vectors expressing LacI alone, LacI-VP16, LacI alone, or LacI-BRCA1(6c-w). Immunohistochemistry was performed using rabbit anti-LacI (with Texas red) to detect open or closed chromatin. DAPI was used for nuclei. (B) A03_1 cells were transfected with pRcLac-Cdc45 and chromatin decondensation (or lack thereof) was detected as in A. O, open chromatin; C, closed; I, indeterminate. (C) A03_1 cells were transfected with pRcLac-Cdc6 and chromatin decondensation structures were detected. Representative pictures from seven similar experiments are shown.

about equal numbers of cells with open and closed configurations (Tumbar et al., 1999; Ye et al., 2001).

Histone modifications associated with Cdc45-promoted decondensation

By analogy to changes that occur in transcriptionally active chromatin (Strahl and Allis, 2000), chromatin decondensation promoted by Cdc45 is likely to be mediated by recruitment of one or more chromatin remodeling enzymes. In turn, these would lead to modification of either the core or linker histones by acetylation, methylation, or phosphorylation. To address this possibility, we asked if any modified histones colocalize with Cdc45-promoted open structures.

Table I. **Quantification of open and closed chromatin structures**

Protein Expressed	Decondensed (open) chromatin	Condensed (closed) chromatin	Indeterminate	Number of cells examined
	%	%	%	
CgCdc45	75	8	17	206
CgCdc6	7	41	36[a], 16	134
Lac-DBD	7	69	24	169
Lac-DBD(2)	12	73	15	138
VP16	45	37	18	190
BRCA1(6c-w)	34	44	22	210

Proteins were expressed from pRcLac, except VP16 and Lac-DBD(2) (DNA-binding domain), which were from pNYE4.
[a]The number of LacI-Cdc6–expressing cells displaying a homogenous nuclear stain with no apparent closed or open structures.

Using modification-specific antibodies, we were not able to detect an increase in H3 or H4 acetylation or methylation at sites of Cdc45-decondensed chromatin (Fig. 2 A, top; and Table II). An important caveat is that failure to demonstrate colocalization of modified core histones with Cdc45-decondensed chromatin could result from the possible transience of such alterations or to the particular choice of antisera. However, colocalizing core histone modifications were detected when VP16 was targeted to the HSR (Fig. 2 A, bottom; and Table II), which is consistent with other published observations (Tumbar et al., 1999; Ye et al., 2001). Thus, it appears that Cdc45-induced chromatin decondensation does not involve these particular modifications to core histones. Importantly, the latter data argue against the possibility that tethering Cdc45 to LacI unmasks a cryptic transactivation domain that is actually responsible for chromatin decondensation.

In contrast, confocal imaging showed that staining with an antibody specific for phospho-H1 (H1P; Lu et al., 1994) frequently colocalizes with decondensed chromatin after Cdc45 targeting (Fig. 2, B and C). Colocalization is sometimes dramatic (Fig. 2 B, bottom; and Fig. 2 C), whereas in other examples, it is less dramatic but still apparent (Fig. 2 B, top and middle). In one example, it appears that decondensation was occurring at the time of sample preparation because the HSR is only moderately decondensed but stains strongly with anti-H1P (Fig. 2 B, bottom). In total, ~65% of the HSRs in transfected cells that displayed decondensation after Cdc45 targeting also costained for H1P (Table II). Colocalization with H1P was not detected in the HSRs of cells transfected with LacI or LacI-VP16 (Fig. S1 D and Table II), nor was it detected in the minor population of condensed HSRs observed when Cdc45 was targeted to the HSR (Table II). We conclude that chromatin decondensation promoted by Cdc45 is associated with H1 phosphorylation. Furthermore, because H1 phosphorylation normally occurs during S-phase, the data suggest that H1P-associated decondensation of chromatin after Cdc45 targeting may be relevant to DNA replication.

Note that in those transfected cells that clearly exhibited a decondensed HSR after Cdc45 targeting (Fig. 2 C, arrows), endogenous H1P staining was only slight to moderate throughout

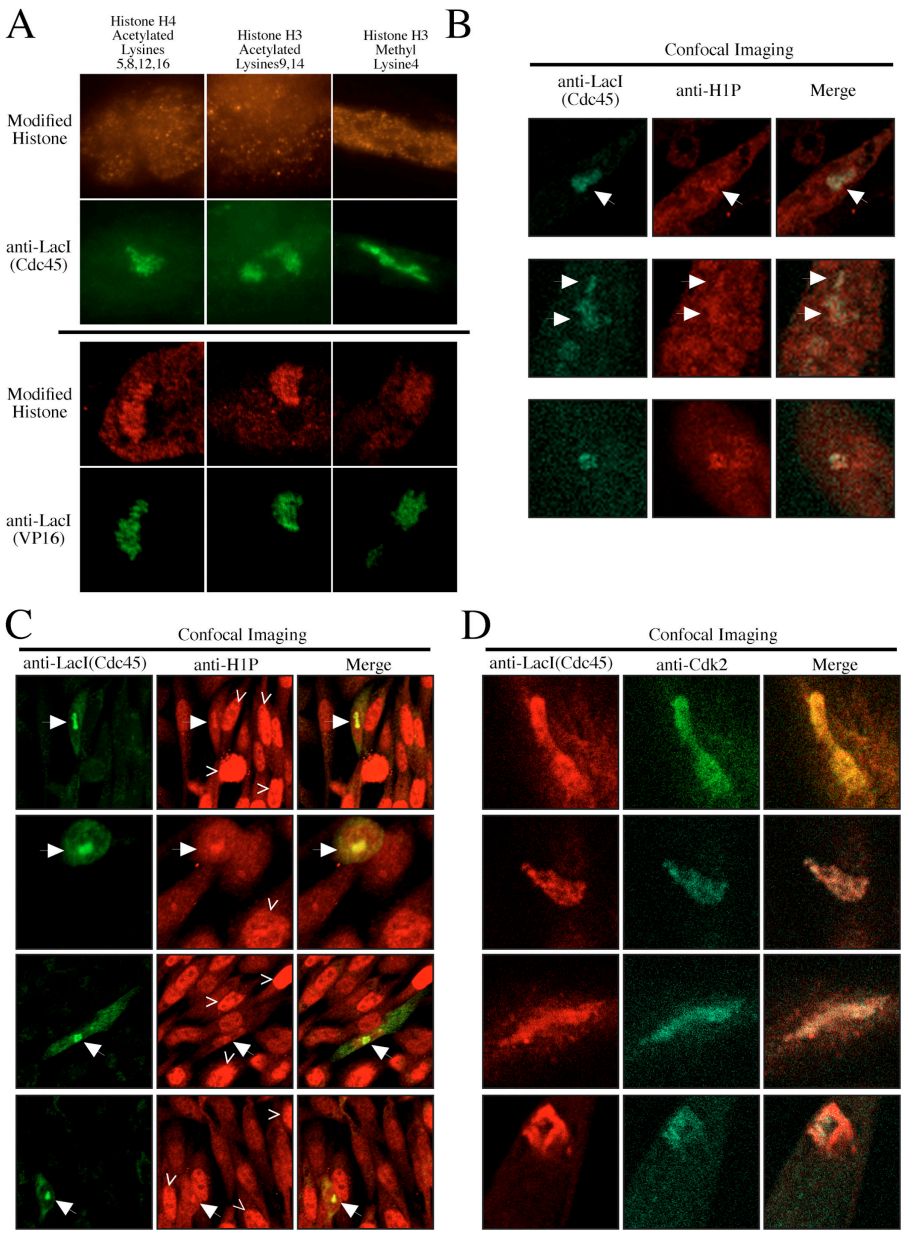

Figure 2. **Cdc45-promoted chromatin decondensation is associated with H1 phosphorylation and Cdk2 recruitment.** (A) A03_1 cells expressing LacI-Cdc45 or LacI-VP16 were analyzed for open structures and colocalizing modified core histones. Open structures were detected with mouse anti-LacI (and FITC), and modified histones with anti–acetyl-H3/H4 and anti–methyl-K4 (and Texas red). (B and C) Open chromatin structures promoted by Cdc45 were analyzed as in A. H1P colocalization was detected with anti-H1P (and Texas red). Arrows indicate open chromatin structures. Arrowheads indicate nuclei displaying a high level of H1P, suggesting G_2 or M staging. (D) Open chromatin structures promoted by Cdc45 were visualized with rabbit anti-LacI (and Texas red). Cdk2 colocalization was detected with mouse anti-Cdk2 (and FITC). Colocalization is shown in the figure by yellow in merge.

the rest of the nucleus; in no case was the entire nucleus brightly stained for H1P (i.e., such as those nuclei indicated with arrowheads in Fig. 2 C). Because phosphorylation of H1 is lowest in early G_1, moderate in late G_1 and S-phase, and highest in G_2/M cells (Gurley et al., 1978; Lu et al., 1994), chromatin unfolding after Cdc45 targeting probably occurred only in those cells that were in G_1 and S-phase when the LacI-Cdc45 protein was transiently expressed. This interpretation is consistent with the idea that Cdc45 functions at the G_1–S transition and in S-phase (Bell and Dutta, 2002) and further suggests that H1P-associated decondensation promoted by Cdc45 is physiologically relevant.

Cdk2 mediates decondensation promoted by Cdc45

We reasoned that the H1P-associated chromatin decondensation promoted by Cdc45 targeting would be indirect and would involve recruitment of an H1 kinase to the targeted region. The most likely candidate is Cdk2 (Roth and Allis, 1992). Cdk2 is required at the G_1–S transition, is active enzymatically throughout S-phase in a complex with Cyclin A (Cardoso et al., 1993; Van den Heuvel and Harlow, 1993), and is required for S-phase progression (Ogryzko et al., 1997; Schutte et al., 1997). In addition, Cdk2 and Cyclin A localize in vivo to DNA replication foci during S-phase (Cardoso et al., 1993). It is thought that Cdk2 phosphorylates DNA replication substrates at the G_1–S transition (Bell and Dutta, 2002), but its role during S-phase progression is unknown.

We found that Cdk2 colocalizes with Cdc45-promoted decondensed HSRs in ~25% of cells that showed a decondensed structure (Fig. 2 D and Table II), but was not detectable in the small percentage of cells with condensed HSRs (Table II). Because this value is lower than the number of open HSRs dis-

Table II. **Frequency of colocalizing proteins/modifications with targeted fusion proteins**

LacI fusion targeted		Colocalizing protein/modif.	Percent observed
Cdc45	+	H1P	65%
Cdc45 (closed)	+	H1P	0%
Cdc45	+	Cdk2	25%
Cdc45 (closed)	+	Cdk2	0%
Cdc45	+	AcH3	0%
Cdc45	+	AcH4	0%
Cdc45	+	MeK4	0%
VP16	+	H1P	0%
LacI (closed)	+	H1P	0%
VP16	+	AcH3	75%
VP16	+	AcH4	75%
VP16	+	MeK4	50%
Cdk2-wt	+	H1P	>80%[a]
Cdk2-DN (open or closed)	+	H1P	0%[a]

Unless noted, colocalizations were observed with open chromatin structures; 50–60 cells were examined in each case.
[a]The number of cells with very strong H1P colocalization.

playing H1P costaining, it is conceivable that the kinase associates only transiently with Cdc45 during the decondensation process. We also found that coexpression of LacI-Cdc45 with the Cdk2 inhibitor p21 reduced Cdc45-dependent chromatin unfolding to less than half its normal value and concomitantly increased the number of condensed HSRs detected (Fig. 3 A). Additionally, the Cdk2-specific inhibitor olomoucine (Schutte et al., 1997) reduced the ability of Cdc45 to promote decondensation (Fig. 3 A, time 0). These observations are consistent with a direct role for Cdk2 in mediating chromatin unfolding promoted by Cdc45 targeting.

To test whether indirect cell cycle blocking effects of olomoucine and p21 might interfere with chromatin decondensation, a MEK1-specific inhibitor that affects cell cycle progression (U0126; Favata et al., 1998) was added during transfection with the LacI-Cdc45 vector. However, little effect on Cdc45-promoted decondensation was observed (Fig. 3 A). As an additional control, olomoucine was added 6 h after transfection of the LacI-Cdc45 vector, a time when the fusion protein first becomes detectable on Western blots (unpublished data). Therefore, the cell cycle was allowed to progress normally until the time when LacI-Cdc45 should be able to induce chromatin decondensation. With this regimen, olomoucine blocked Cdc45-promoted chromatin unfolding even more effectively than when added earlier (Fig. 3 A, compare the T0 to the T6 sample). Importantly, LacI-VP16–induced decondensation was unaffected by p21 or by drug treatment at either time point (Fig. 3 A). Thus, Cdk2 inhibitors appear to reduce chromatin decondensation promoted by Cdc45 targeting directly and not through perturbations of cell cycle progression.

We additionally asked whether or not Cdk2 could remodel chromatin when targeted directly to the HSR. As shown in Fig. 3 (B and C, left), expression of LacI-Cdk2 causes large-scale chromatin decondensation on its own: ~54% of cells receiving Cdk2-wt displayed open chromatin structures. Furthermore, a kinase-defective Cdk2 (Cdk2-DN) that is enzymatically inactive but able to bind cyclins (Van den Heuvel and Harlow, 1993) is less effective at chromatin decondensation than wild type (Fig. 3, B and C, right). In cells expressing Cdk2-DN, only 29% displayed open configurations, with a significantly higher number exhibiting a closed configuration (Fig. 3 C). Note that the expression of LacI-Cdk2-wt and LacI-Cdk2-DN was similar in these experiments (Fig. S1 C). Importantly, the majority of decondensed HSRs that result from Cdk2-wt targeting were strongly associated with H1P costaining when compared with Cdk2-DN–targeted HSRs (both condensed and decondensed), which showed only a very small amount of H1P colocalization (Fig. 3 D and Table II). Note that in Fig. 3 D we exposed the images equally to allow a qualitative comparison between the two Cdk2 alleles. The small amount of H1P staining observed with Cdk2-DN targeting may be due to recruitment of other enzymes/complexes by Cdk2-DN that are capable of low level H1 phosphorylation, which could explain some of the observed unfolding activity of Cdk2-DN.

The fact that Cdk2 inhibitors and p21 do not completely abolish the chromatin remodeling promoted by Cdc45, together with the fact that Cdk2-DN still shows some residual unfolding activity (apparently not involving significant H1P), suggests that other unknown remodeling enzymes must also be involved. However, Cdk2 (and its kinase activity) is at least one mediator of the chromatin unfolding because Cdk2-DN is clearly reduced in its ability to unfold chromatin and because Cdk2 inhibitors block a significant amount of the decondensation promoted by LacI-Cdc45 targeting. Thus, our data support a model in which Cdk2 is one of the enzymes recruited to replication foci (by Cdc45 or other fork proteins), followed by H1 phosphorylation and chromatin unfolding. In addition, because Cdk2 is normally active during S-phase and is localized to replication foci (Cardoso et al., 1993), identification of Cdk2 as a mediator of Cdc45-promoted decondensation suggests that our results are likely to be physiologically relevant to S-phase.

Chromatin association of Cdc45, Cdk2, Cyclin A, and H1P

Mediation of Cdc45-promoted chromatin decondensation by Cdk2 and H1P predicts that all of these proteins should be associated with chromatin during S-phase. To test this prediction, CHO cells were synchronized by arrest in G_0 and then released into G_1 and S-phases. Total cell extracts, detergent-sensitive soluble cytoplasmic and nucleoplasmic proteins (S1), and detergent-resistant (P3) proteins (operationally defined as chromatin-bound) were collected at the times indicated in Fig. 4. Antitubulin and antilamin immunoblotting showed that the fractionation was effective (Fig. 4 A, S1 and P3 samples), whereas anti–Cyclin A verified the effectiveness of the synchronization regimen (Fig. 4 A, TCE samples for Cyclin A) as did BrdU labeling of S-phase cells (not depicted). After verifying that two different anti-Cdc45 antisera recognize endogenous Cdc45 (as a doublet) as well as Cdc45 tagged with the LacI domain (Fig. S1, A and B; and not depicted), we determined the kinetics of Cdc45 chromatin binding.

As shown in Fig. 4 A (6–18 h), Cdc45 and Cdk2 are both associated with the chromatin fraction during S-phase. Interest-

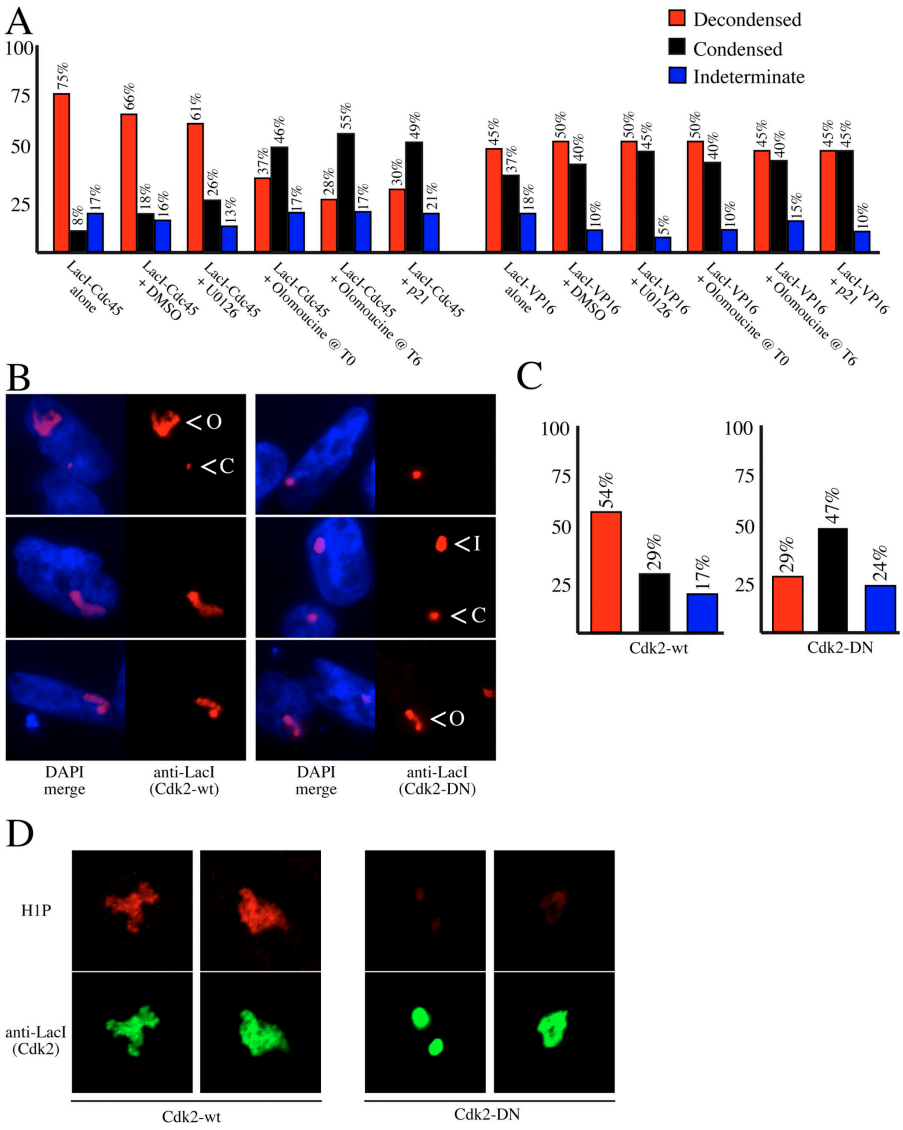

Figure 3. **Cdk2 mediates Cdc45-promoted chromatin decondensation.** (A) A03_1 cells expressing LacI-Cdc45 or LacI-VP16 were analyzed as in Fig. 1. 25 μM U0126, 250 μM olomoucine, or DMSO were added at the time of transfection, or 6 h afterward, and all cells were fixed 24 h after transfection. p21 inhibitor was cotransfected at a 3:1 ratio with LacI-Cdc45 or -VP16 (far right). Chromatin structures were quantified from 110–180 cells per condition. (B and C) A03_1 cells expressing LacI-Cdk2-wt (left) or LacI-Cdk2-DN (right) were analyzed for open chromatin structures with rabbit anti-LacI (and Texas red). Chromatin structure percentages were determined after examining more than 140 cells for each Cdk2 allele, and the data plotted in C. O, open chromatin; C, closed; I, indeterminate. (D) Confocal imaging of H1P and Cdk2-wt or -DN was performed using mouse anti-LacI (FITC) and anti-H1P (Texas red). Equal exposures were obtained, and lack of bleed-through of emission signals was verified as described in Materials and methods. (A–C) O and red columns, open; C and black columns, closed; I and blue columns, indeterminate.

ingly, Cdc45 appears to associate with chromatin before Cdk2, which would be predicted if Cdc45 recruits Cdk2 to chromatin targets. In addition, Cyclin A associates with chromatin with kinetics nearly identical to those of Cdk2 (Fig. 4 A). The Cdk2 and Cyclin A chromatin association data are in agreement with other studies (Pagano et al., 1993).

H1P is also associated with chromatin during S-phase, appearing around the G_1–S transition (Fig. 4 A, 6–18 h). This behavior is consistent with S-phase–dependent phosphorylation of H1 (Lu et al., 1994). Thus, our data suggest a model in which Cdc45 recruits Cdk2/Cyclin A to chromatin at the G_1–S transition, leading to S-phase–dependent H1 phosphorylation and decondensation of chromatin in replication foci.

Cdc45, Cdk2, and an H1 kinase interact in vivo

The aforementioned colocalization of Cdc45 and Cdk2 suggests that these proteins should interact physically. To test this, immunoprecipitations were performed followed by immunoblotting with antibodies to human Cdc45 and Cdk2. HeLa cells were used in these studies to optimize reactions. Two different Cdc45 antibodies (but not nonspecific serum) immunoprecipitated endogenous Cdc45 protein (Fig. 4 B, lanes 1, 2, and Ser of the Cdc45 Western), and, as predicted, endogenous Cdk2 was coprecipitated with Cdc45 (Fig. 4 B, lane 2 of the Cdk2 Western). Although the amount of Cdk2 bound to Cdc45 was relatively small, the amount of immunoprecipitated Cdc45 was also small, indicating that the goat anti-Cdc45 may not be optimal in this assay. It is also apparent that the Cdk2 bound to Cdc45 migrates somewhat more slowly than the majority of Cdk2 (Fig. 4 B, compare lanes 2 and 3 of the Cdk2 Western; a small shoulder is clearly evident on shorter exposures). Experiments with rabbit anti-Cdc45 were not successful due to high background during the immunoblotting (unpublished data).

We next determined if antibodies to Cdk2 could reciprocally coprecipitate Cdc45. We detected a Cdc45 doublet (also observed in total cell extracts) in association with endogenous Cdk2 (Fig. 4 C, lanes 4 and 5 of the Cdc45 Western). To corroborate these data, CHO cells were also transfected with HA-Cdc45, and anti-HA immunoprecipitates were analyzed for the

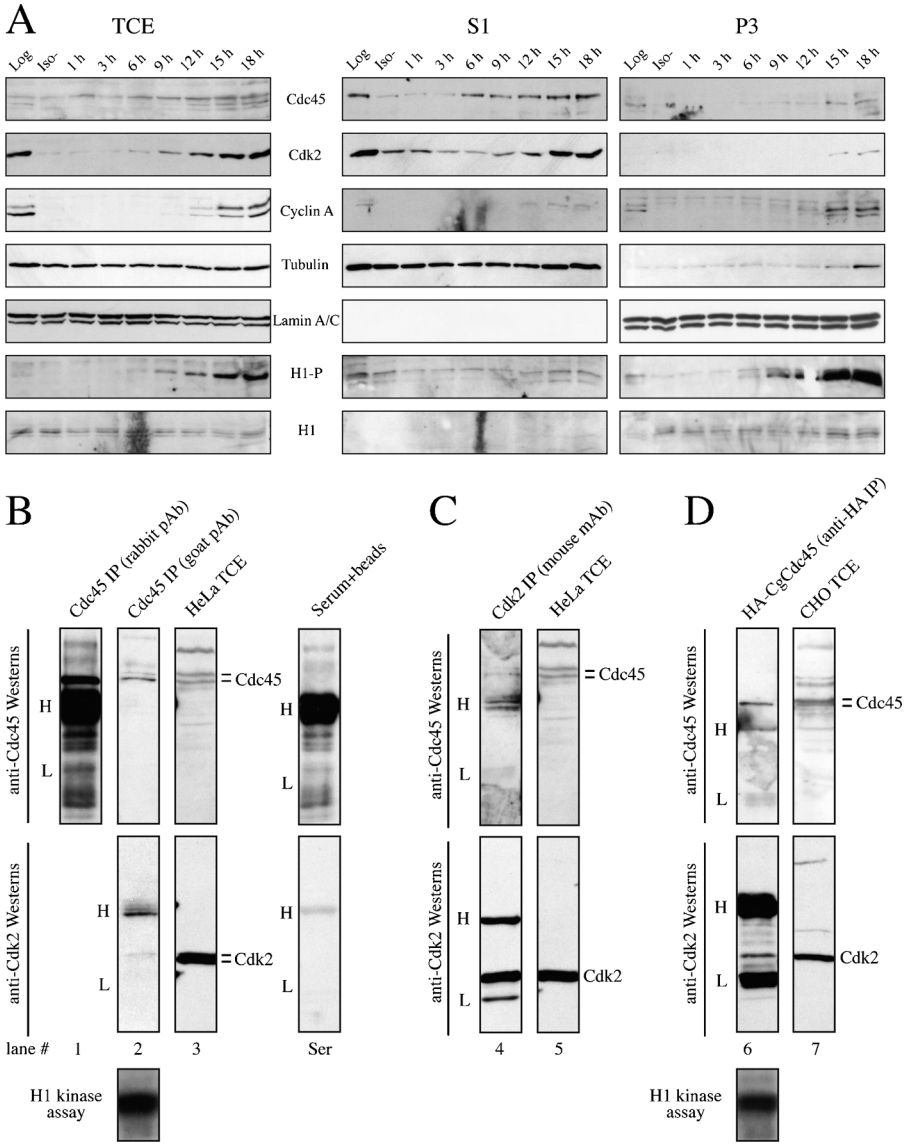

Figure 4. **Cdc45, Cdk2, Cyclin A, and H1P associate with chromatin during G_0-, G_1-, and S-phases, and Cdc45 interacts with Cdk2 in cells.** (A) CHO cells were synchronized in G_0 by isoleucine deprivation and released into G_1 and S. Total cell extract (TCE), S1, and P3-chromatin fractions were isolated. Log CHO cells were used as a reference. BrdU labeling (30 min) at each time point verified synchrony and indicated that S-phase occurred from 9 to 18 h (not depicted). Immunoblotting was performed with indicated antibodies. (B) HeLa cells were subjected to immunoprecipitation with rabbit anti-Cdc45 (lane 1), goat anti-Cdc45 (lane 2), or nonspecific serum + beads (Ser). Immunoprecipitation (lanes 1, 2, and Ser) and HeLa TCE (lane 3) samples were immunoblotted with rabbit anti-Cdc45 (top) or mouse anti-Cdk2 (bottom). An H1 kinase assay was performed in parallel (lane 2, far bottom). Heavy (H) and light (L) chains of immunoprecipitating antibodies are indicated. (C) HeLa cells were subjected to immunoprecipitation with mouse anti-Cdk2 (lane 4). Immunoprecipitation (lane 4) and HeLa TCE (lane 5) samples were immunoblotted with mouse anti-Cdk2 (top) or rabbit anti-Cdc45 (bottom). (D) CHO cells expressing HA-Cdc45 were immunoprecipitated with mouse anti-HA. HA-Cdc45 (lane 6) and endogenous Cdc45 from CHO TCE (lane 7) were detected with rabbit anti-Cdc45 (top). Coimmunoprecipitating (lane 6) and endogenous (lane 7) Cdk2 were detected with mouse anti-Cdk2 (bottom). An H1 kinase assay was performed in parallel (lane 6, far bottom).

presence of endogenous Cdk2. As shown in Fig. 4 D (lane 6 of the Cdk2 Western), endogenous Cdk2 was effectively immunoprecipitated with HA-Cdc45.

We predicted from these data that an H1 kinase should also be present in Cdc45 immunoprecipitates, and this prediction was confirmed. Data in Fig. 4 (B and D, H1 kinase panels) show that an active H1 kinase is indeed associated with both endogenous and transfected Cdc45. As a control for the kinase assays, we found that CycB1/Cdc2 immunoprecipitates from S-phase samples produced no significant activity, whereas assays on mitotic samples produced kinase activity similar to that observed with Cdc45 immunoprecipitates (unpublished data). We conclude that Cdc45 and Cdk2 form a complex in cells and that Cdc45 is associated with an active H1 kinase, probably Cdk2.

H1P foci and replicating foci colocalize in vivo

An important concern is whether or not the chromatin remodeling system recapitulates what normally occurs in vivo. To address this issue, we asked if H1P foci colocalize with replication foci during S-phase. As shown by confocal imaging in Fig. 5 (and in additional examples shown in Fig. S1 E), there is significant colocalization between replicating DNA and H1P foci at all five stages of S-phase (indicated by the yellow signal in the merge column; Alexandrow and Hamlin, 2004). This is most obvious in late S-phase (stages 4 and 5) when the replication foci are distinctive, and the number of foci is small (Fig. 5, A–C, note arrowheads). In middle S-phase (stages 2 and 3) when the number of replication foci is larger and displays a perinuclear pattern, the H1P signals are visible in the regions of the nucleus undergoing BrdU labeling, particularly at the periphery (Fig. 5 D, arrowheads). Also evident in early S-phase nuclei (stage 1) are similar spatial patterns of some groups of foci, and distinct shapes of some of the individual foci, with the H1P and BrdU stains (Fig. 5 F, circled region [an enlargement of the region shown by arrowhead in E]).

The distinctiveness of spatial patterns and individual shapes of foci in H1P and BrdU stains argues against colocalization

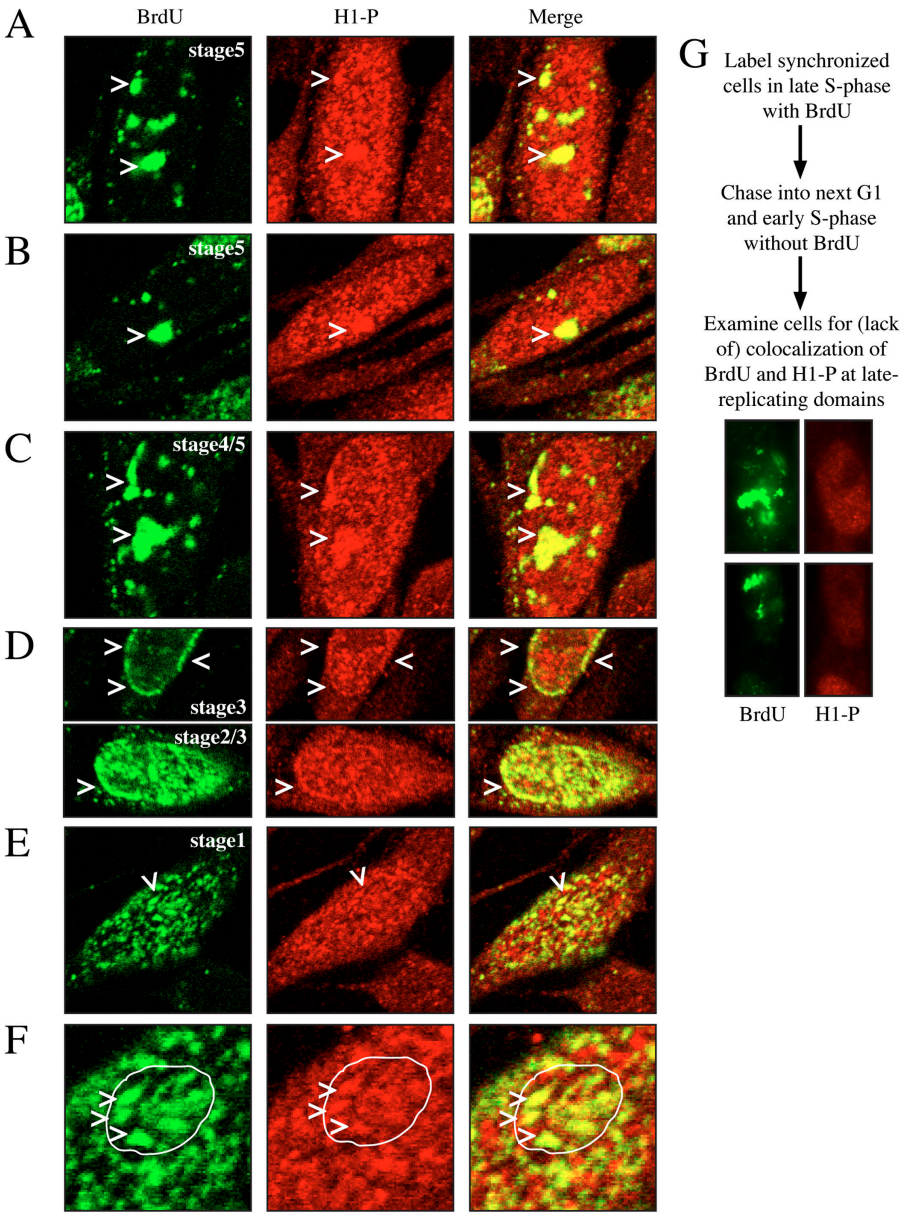

Figure 5. **Replication foci and H1P foci colocalize in vivo.** (A–F) Log CHO cells were labeled with BrdU for 30 min and processed for immunohistochemistry. Confocal imaging of BrdU (with FITC) and H1P foci (Texas red) was performed on various stages of S-phase nuclei. Arrowheads in A–F note particular regions of biologically interesting overlap (see text). The arrowhead in E also points to the region enlarged and circled in F. (G) CHO cells were synchronized with mimosine at the G_1–S transition, followed by release into S-phase and pulse-labeling with BrdU for 30 min in late S-phase (8 h after release). Control cells were fixed immediately to verify that the population was in late S-phase by the presence of stage 5 patterns (not depicted). Experimental samples were chased for 8 h without BrdU, putting them in late G_1/early S-phase (verified by BrdU pulse-labeling a control sample after the chase; ~85% of cells in early S-phase and 15% unlabeled/G_1 [not depicted]). Immunohistochemistry was performed as in A–F.

being a chance occurrence. In addition, DAPI staining was homogeneous throughout the nuclei except for the nucleoli, as evident in Figs. 1 and 3 (not depicted). Therefore, H1P signals were not enriched simply because of increased DNA density. Clearly, however, colocalization is most obvious in late S-phase and much of the H1P signal does not overlap with obvious replication foci, the physiological implications of which will be discussed in the Discussion.

H1 phosphorylation is lowest in G_1-phase, increases significantly during S-phase, reaches a maximum by G_2/M, and is removed at the end of mitosis (Lu et al., 1994). Thus, the H1P that colocalizes with BrdU in the aforementioned experiment should not be present before S-phase entry, but should appear during the process of replication. To test this prediction, late S-phase cells were pulsed with BrdU to label late-replicating DNA, followed by a chase without BrdU into the next G_1/early S-phase (Fig. 5 G). It is evident that late-replicating regions marked in the previous S-phase with BrdU do not significantly colocalize with H1P in the next G_1/early S-phase. Note that the cells shown here are probably in G_1 because the level of H1P is very low relative to other cells on the plate, ~85% of which were in early S-phase (unpublished data). Thus, the H1P modification that colocalizes with late-replicating regions in Fig. 5 (A–C) appears to be added to H1 de novo at the time of replication in late S-phase.

H1 phosphorylation and DNA replication are temporally coordinated and Cdk2 dependent

To further investigate the correlation between H1P and replication, we examined the kinetic relationship between the two processes. It has been shown that Cdk2 is required for cells to progress through S-phase (Ogryzko et al., 1997; Schutte et al., 1997), which is consistent with a model in which Cdk2 helps to

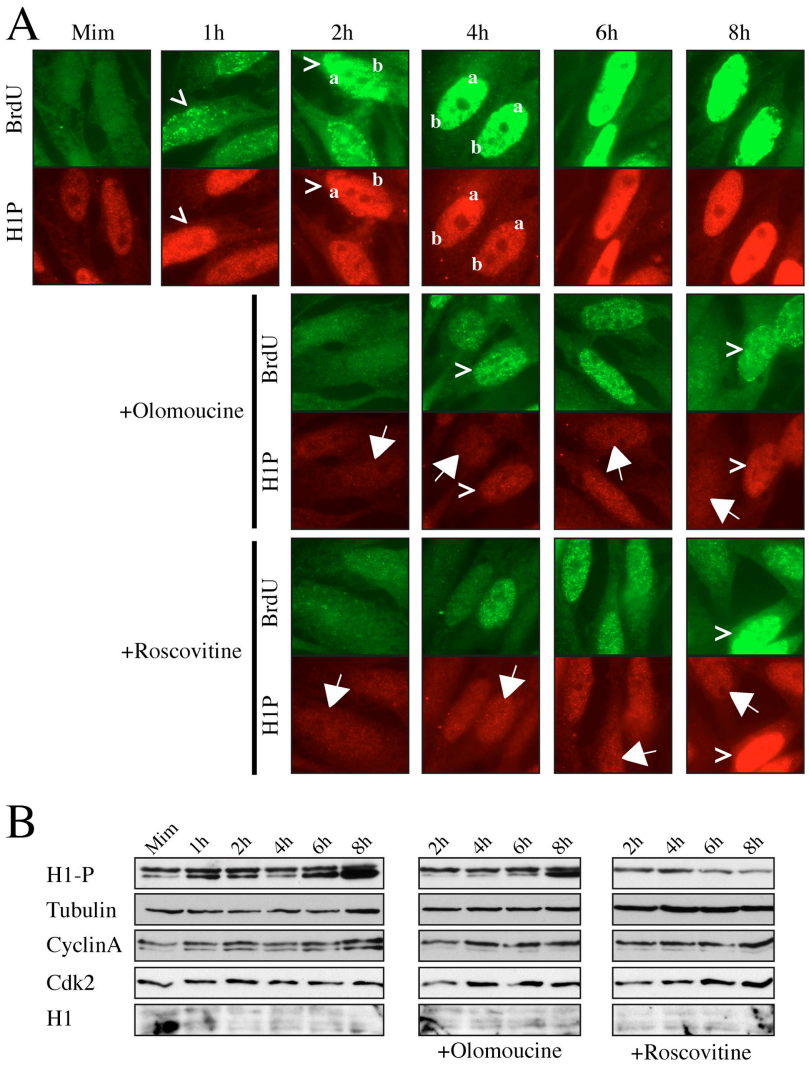

Figure 6. **DNA replication progression and H1P are directly related and dependent on Cdk2.** CHO cells were synchronized at G_1–S using isoleucine and mimosine. (A) Cells were released into S-phase, and 1 h later 250 μM olomoucine, 40 μM roscovitine, or DMSO (top, BrdU) was added. Cells were pulsed with BrdU (15 min) at each time point, followed by analysis of BrdU (FITC) and H1P (Texas red) levels. Equal (long) exposures were obtained for each field of cells. Arrowheads indicate nuclei with coelevated BrdU and H1P levels versus other nuclei in the field. Arrows indicate nuclei that show low H1P levels. a, strong regions; b; weaker regions. (B) Parallel samples were immunoblotted. Normalization was to total protein.

regulate chromatin accessibility. In the present work, we compared the levels of BrdU incorporated during a pulse and the overall H1P signal in control cells to that seen in cells treated with two efficient Cdk2 inhibitors, olomoucine and roscovitine (Schutte et al., 1997). To aid comparison of immunohistochemical signals, each sample field was exposed so that the cytoplasmic background was approximately the same and could serve as an internal reference.

Cells were synchronized in G_0 by isoleucine deprivation and were released into G_1 in the presence of mimosine to arrest cells at the G_1–S transition (Fig. 6, mim time point). As expected, virtually no BrdU was incorporated during the pulse period in the presence of mimosine (Fig. 6 A, mim sample in top panels). However, control cells released into S-phase after mimosine removal efficiently incorporated BrdU as S-phase progressed (Fig. 6 A, top). In contrast, cells treated with olomoucine or roscovitine at 1 h into S-phase did not efficiently incorporate BrdU at any time thereafter (Fig. 6 A, middle and bottom, samples 2, 4, 6, and 8 h), consistent with Cdk2 being required for S-phase progression (Schutte et al., 1997).

Cdk2 activity could be required during S-phase for initiation at late-firing origins, for replication fork progression, or for both. Although it has been shown that Cdk2 is not necessary for polymerization during replication of plasmids in the *X. laevis* system (Walter and Newport, 2000), two observations suggest that Cdk2 may be required for efficient elongation during chromosomal replication in mammalian cells. Within 1 h after drug treatment (i.e., at 2 h into S-phase), at a time when replication forks are clearly very active in control cells, drug-treated cells show virtually no BrdU incorporation (Fig. 6 A, 2 h). Therefore, the drugs acutely block even established replication forks. In addition, the very small amount of replication in late time points in the presence of drugs (seen only at these exposures) displays a BrdU labeling pattern indicative of early stage S-phase nuclei (Fig. 6 A, 8 h), at a time when control cells show exclusively late stage BrdU patterns (not depicted). This finding suggests that cells with reduced Cdk2 activity have initiated replication but are unable to progress efficiently out of early S-phase. Thus, these data are consistent with Cdk2 playing an important role in facilitating elongation through restrictive higher-order chromatin. However, we cannot exclude a role for Cdk2 in regulating late-firing origins or possible checkpoint effects of the drugs.

Examination of the H1P levels in the same experiment showed that the H1P signal in control cells noticeably increased during S-phase (Fig. 6 A, H1P). However, inhibitor-treated cells showed dramatically reduced levels of H1P at all time points examined (Fig. 6 A, middle and bottom, arrows), which is consistent with H1P being dependent on Cdk2 activity. Immunoblotting data also showed that the lower H1P band was reduced relative to controls or completely absent in cells treated during S-phase with olomoucine or roscovitine, respectively (Fig. 6 B). The top H1P band (Fig. 6 B) is present even in the mimosine sample, suggesting that it appears before S-phase. Cdk2, Cyclin A, tubulin, and total H1 levels are unaffected during drug treatment (Fig. 6 B).

There was also a strong correlation between the intensity of BrdU incorporation in individual nuclei and the intensity of the H1P signal in the same nuclei (Fig. 6 A, compare nuclei with arrowheads to other nuclei in the same field). In particular, a small number of nuclei (<0.1%) was resistant to the drugs and displayed both intense BrdU labeling and dramatic H1P signals (Fig. 6 A, arrowhead in roscovitine 8 h time point). Interestingly, spatial examination of some of the overexposed nuclei on a gross scale showed that the most strongly labeled BrdU regions of the nuclei were also the most strongly labeled H1P regions (Fig. 6 A, top, compare strong regions indicated with an "a" to weaker regions indicated with "b"), which is consistent with the results of Fig. 5 and again suggests a direct relationship between H1P and BrdU. Altogether, these data suggest that active DNA replication and increasing H1 phosphorylation are tightly linked processes and are Cdk2 dependent in vivo.

Discussion

Roles for Cdc45 and Cdk2 in chromatin decondensation

We have demonstrated that tethering Cdc45 to a chromosomal locus promotes large-scale chromatin decondensation. H1P becomes highly concentrated in the decondensed chromatin after Cdc45 targeting. Cdk2 is recruited to Cdc45-promoted open chromatin, and direct tethering of Cdk2 to the chromatin also induces unfolding coincident with the appearance of H1P. These data suggest that Cdk2 is a mediator of the decondensation process. In support of this proposal, kinase-defective Cdk2 is less effective at decondensing chromatin, and Cdk2 inhibitors reduce the ability of Cdc45 to promote decondensation. Because previous studies have suggested that H1P is involved in decondensing chromatin and allowing transient access of proteins to DNA (Roth and Allis, 1992), the simplest interpretation of these data is that recruitment of Cdk2 to replication foci leads to H1 phosphorylation, chromatin decondensation, and facilitation of DNA replication.

Such a role for Cdk2 in chromatin remodeling during DNA replication is not mutually exclusive with a role for Cdk2 during initiation and explains why Cdk2 and Cyclin A are found at replication foci throughout S-phase in vivo (Cardoso et al., 1993) and why its kinase activity is detectable and required throughout S-phase (Ogryzko et al., 1997; Schutte et al., 1997). This model also explains why H1P increases during S-phase in mammalian cells (Gurley et al., 1978; Lu et al., 1994). Because G_2 and mitotic cells display even greater H1P signals (Lu et al., 1994), H1P incorporation during S-phase may be cumulative. Although we did not analyze cyclin participation in this paper, it is possible that Cdk2-dependent H1P may also involve Cyclin E because Cyclin E has recently been shown to become chromatin-bound during S-phase progression in *X. laevis* (Furstenthal et al., 2001).

Interestingly, Cdk2 is not required for fork progression on plasmids replicating in *X. laevis* extracts (Walter and Newport, 2000); additionally, Cdk2 knockout mice are viable but display S-phase entry defects (Berthet et al., 2003). A role for Cdk2 in chromatin remodeling is compatible with such evidence because plasmids in the *X. laevis* system are unlikely to have higher-order chromatin configurations and would not require Cdk2 to facilitate elongation. Furthermore, it has been shown that Cdk4 can interact with Mcm7 (Gladden and Diehl, 2003), and Cdk3 is also required for S-phase entry (Van den Heuvel and Harlow, 1993), suggesting that other Cdks could function redundantly in mammals at replication foci in the absence of Cdk2.

Cdc6, which interacts with Cdk2 (Alexandrow and Hamlin, 2004), does not appear to be capable of remodeling chromatin, and we were unable to detect colocalization of Cdk2 with LacI-Cdc6 (unpublished data). The reasons for this are unclear. Cdc45 binds at origins and moves with the replication fork, whereas Cdc6 is bound to origins (Bell and Dutta, 2002). Cdc6 is a Cdk2 substrate, leading to apparent negative effects on its function (Alexandrow and Hamlin, 2004). It may be that these differences contribute to the way in which Cdk2 interacts with Cdc6 or Cdc45 and ultimately functions in replication foci (i.e., Cdc6 may be a transient substrate, whereas Cdc45 may be a scaffold-like factor at forks).

Physiologic relevance to S-phase

Several lines of evidence suggest that the results obtained with the decondensation assays are physiologically relevant to DNA replication. In vivo, H1P foci colocalize with active replication foci, suggesting that the remodeling detected in the decondensation assay is probably also occurring in replication foci. In addition, Cdk2 activity, H1P, and S-phase progression appear to be tightly linked, as predicted if chromatin remodeling by Cdk2 is necessary for fork progression during S-phase. Importantly, previous in vivo studies have shown that large-scale chromatin decondensation does occur before, or coincident with, replication through a chromosomal region (Li et al., 1998). All of these data suggest that chromatin decondensation is indeed a normal physiological process occurring during replication.

Many of the BrdU and H1P foci in Fig. 5 do not colocalize, which is predicted given the likely physiology of these two processes. Not all regions of chromatin contain H1 linker histone, which is often associated with heterochromatin (Wolffe, 1997), and it is only when a replication fork reaches an H1-containing region that H1P and BrdU should colocalize. Indeed, late-replicating heterochromatic regions in the stage 4/5 nuclei showed the most obvious and distinct colocalization patterns

(Fig. 5, A–C; and Fig. S1 E). Also, the replication forks acting before the BrdU pulse may have encountered H1 before the pulse period, producing H1P foci that no longer colocalize with newly incorporated BrdU. Thus, visible colocalization of H1P and BrdU will only occur when the two processes occur at the same time. Furthermore, it has been shown that H1 phosphorylation regulates transcription of certain genes in *Tetrahymena thermophila* (Dou and Gorovsky, 2000). Although it is unknown whether any genes are regulated by H1P in mammals, some H1P foci in cells may have acquired H1P during transcription.

Chromatin decondensation after Cdc45 targeting appears to occur in late G_1 and S-phase, the time in the cell cycle when such an effect is predicted to occur. Furthermore, the large-scale decondensation observed in response to targeting Cdc45 to chromatin identified mediators that are themselves physiologically relevant to S-phase, namely Cdk2 and H1P. In addition, Cdk2 is recruited to unfolded chromatin by Cdc45, which is normally localized to replication forks (Tercero et al., 2000), and Cdk2 itself is also localized to replication foci in vivo (Cardoso et al., 1993). Consistent with an in vivo role for these proteins during S-phase, Cdc45, Cdk2, Cyclin A, and H1P are associated with chromatin during S-phase, and Cdk2 and Cdc45 (and an active H1 kinase) physically interact in cells. In sum, we believe that the experimental system used here has allowed a more detailed view of the sequence of events that occurs in replication foci.

Although we did not present direct evidence that H1 phosphorylation in S-phase causes increased replication efficiency, several lines of evidence suggest that this is the case. Addition of unphosphorylated H1 to *X. laevis* extracts significantly inhibits DNA replication in vitro (De et al., 2002), and H1P from S-phase cells that is reconstituted with SV40 minichromosomes supports a higher efficiency of replication than H1P from G_0 or mitotic extracts (Halmer and Gruss, 1996). In the latter study, sedimentation analyses also showed that S-phase H1P induces an open chromatin structure on SV40 minichromosomes. Finally, defects in H1 phosphorylation correlate directly with incomplete DNA replication in mammalian cells (Yasuda et al., 1981). Therefore, H1 phosphorylation and chromatin decondensation are likely to facilitate DNA replication. However, a more detailed understanding of the cause–effect relationship between H1P and fork progression will require development of in vitro chromatin-based replication systems.

Specificity of the chromatin remodeling system

The chromatin remodeling system used here was constructed by Li et al. (1998) and Tumbar et al. (1999) to investigate chromatin structure and compaction in cells by increasing the signal of a specific chromatin region to levels observable by light microscopy. It has since been used to investigate the ability of transcription factors to remodel chromatin and to investigate the ability of replication proteins to cause decondensation (the present study).

Several proteins are incapable of remodeling chromatin in this system, including Cdc6 (this study), geminin (unpublished data), LacI (Tumbar et al., 1999), and several domains of BRCA1 (Ye et al., 2001). Different histone modifications contribute to the decondensation observed with proteins that are effective. For example, VP16-, E2F1-, and p53-decondensed chromatin is associated with histone hyperacetylation and recruitment of HATs (Tumbar et al., 1999; Ye et al., 2001), whereas BRCA1 decondensation is associated with phospho-H2AX and the possible recruitment of COBRA1 (Ye et al., 2001). Cdc45 is shown here to be associated with Cdk2 and H1P. These data suggest that if a protein is capable of causing large-scale decondensation, either directly or indirectly, open structures will be observed in this system, but the mechanisms can clearly be different. The residual chromatin remodeling in some of our assays suggests that there are other enzymes involved in chromatin decondensation besides Cdk2. The identity of other players in this process are currently under investigation.

Materials and methods

Cell culture, synchronization, and transfections

CHO, 3T3, and HeLa cells were maintained in MEM (Invitrogen) containing 10% Fetal Clone II (Hyclone). The CHO-derived cell line A03_1 (Li et al., 1998) was maintained in MEM containing 10% Fetal Clone I and 0.3 μM methotrexate (Calbiochem). CHO cells were synchronized in G_0 by isoleucine deprivation for 36 h and released into complete medium or medium containing 200 μM mimosine (Alexandrow and Hamlin, 2004). Nascent DNA was labeled with 15 μM BrdU (Sigma-Aldrich). Transfections were performed for 24 h using FuGene-6 (Roche).

Antibodies

Antibodies were prepared in rabbits unless otherwise specified and were as follows: mouse anti-Lamin A/C (Calbiochem), 1:500; mouse anti-tubulin (Calbiochem), 1:1,000; mouse anti-BrdU (Roche), 1:20; anti–Cyclin A (Upstate Biotechnology), 1 μg/ml; goat anti-Cdc45 (Santa Cruz Biotechnology, Inc.), 1:500; anti-Cdc45 (Santa Cruz Biotechnology, Inc.), 1:300; anti-H1 (Santa Cruz Biotechnology, Inc.), 1:500; anti–H3-methylK4, 1:500; anti–acetyl-H3 and anti–H4-penta, 1:1,000 (anti–Ac-histone and anti–Me-histone antibodies were all provided by C.D. Allis, Rockefeller University, New York, NY); mouse anti-Cdk2 (Santa Cruz Biotechnology, Inc.), 1:500 (Westerns) and 1:100 (immunohistochemistry); mouse anti-Cdk2 (BD Biosciences), 1:3,000 (Westerns) and 1:100 (immunohistochemistry); anti-H1P, 1:1,000 (Westerns) and 1:100 (immunohistochemistry) (provided by C. Mizzen, University of Illinois, Urbana-Champaign, IL); anti-LacI (Stratagene), 1:10,000 (Westerns) and 1:20,000 (immunohistochemistry); mouse anti-HA (HA.11; Covance), 1:1,000 (Westerns) and 1:150 (immunohistochemistry); mouse anti-LacI, 1:2,000 (provided by A. Belmont, University of Illinois, Urbana-Champaign, IL); donkey anti–rabbit Texas red–conjugated or anti–mouse FITC-conjugated secondaries (Jackson ImmunoResearch Laboratories), 1:50.

Plasmids and cDNAs

Chinese hamster Cdc45 and Cdc6 (Alexandrow and Hamlin, 2004) were subcloned into pRcLac (provided by R. Li, University of Virginia, Charlottesville, VA; Ye et al., 2001) for expression with an NH_2-terminal LacI tag or into pc2HA for expression with two HA tags. Cdk2 alleles (provided by E. Harlow, Massachusetts General Hospital, Boston, MA; Van den Heuvel and Harlow, 1993) were ligated into pRcLac. In all cases, only three to five amino acids were inserted between LacI and fused cDNAs, and no nuclear localization signals were added. The p21 expression vector was provided by J. Nevins (Duke University, Durham, NC), the BRCA1(6c-w) expression vector was provided by R. Li, and the pNYE4 vector was provided by A. Belmont.

Immunoprecipitations and immunoblotting

Total cell extracts were lysed in loading dye. For immunoprecipitation-Westerns, lysis was in TNN (50 mM Tris, pH 7.4, 250 mM NaCl, 0.1% IGEPAL CA-630, 50 mM NaF, 100 μM Na-vanadate, 1μg/ml pepstatin A, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM PMSF). Insoluble debris was removed by centrifugation and lysates were precleared by incubation with purified mouse IgG (for monoclonals) or nonimmune serum (for polyclonals). After incubation with primary antibodies and beads, im-

mune complexes were pelleted and washed 3× with TNN. P3 and S1 fractions were collected as described previously (Alexandrow and Hamlin, 2004). Immunoblotting was performed by standard methods.

Kinase assays

After TNN washing, samples were washed with kinase buffer (KB; 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, and 1 mM DTT). Samples were resuspended in KB containing 30 μM of cold ATP, 5 μCi ^{32}P-γ-ATP, and 10 μg H1 (Roche). Reactions were incubated for 30 min at 37°C.

Immunohistochemistry

Cells were fixed in 2% formaldehyde on glass coverslips (Corning) and immunohistochemistry was performed as described previously (Ye et al., 2001). For BrdU labeling, cells were incubated with 1.5 N HCl for 30 min. Coverslips were mounted onto slides with Pro-Long Anti-Fade (Molecular Probes). Cells were examined with a Microphot-SA epifluorescence microscope (Nikon), and images were obtained with a Spot digital camera and Spot software. Confocal imaging used an LSM 510-Meta/FCS laser-scanning microscope (Carl Zeiss MicroImaging, Inc.), 100× oil objective (Carl Zeiss MicroImaging, Inc.; 40× objective in Fig. 2 C), and software (Carl Zeiss MicroImaging, Inc.). No further manipulations of images were performed. For confocal imaging, lack of bleed-through from the FITC emission into the Texas red emission during image acquisition was verified by observing that the red images could also be detected using the far-red excitation laser (albeit more weakly).

Online supplemental material

Fig. S1 shows the expression of Cdc45 and LacI fusion derivatives, lack of H1P colocalization with LacI or LacI-VP16, and additional H1P/BrdU colocalization examples. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200409055/DC1.

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