CDK Phosphorylation Inhibits the DNA-binding and ATP-hydrolysis Activities of the Drosophila Origin Recognition Complex*

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Dirk Remus 1, Marco Blanchette 2, Donald C. Rio, and Michael R. Botchan 3

From the Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, University of California, Berkeley, California 94720-3204

Faithful propagation of eukaryotic chromosomes usually requires that no DNA segment be replicated more than once during one cell cycle. Cyclin-dependent kinases (Cdks) are critical for the re-replication controls that inhibit the activities of components of the pre-replication complexes (pre-RCs) following origin activation. The origin recognition complex (ORC) initiates the assembly of pre-RCs at origins of replication and Cdk phosphorylation of ORC is important for the prevention of re-initiation. Here we show that Drosophila melanogaster ORC (DmORC) is phosphorylated in vivo and is a substrate for Cdk2 in vitro. Cdk phosphorylation of DmORC subunits DmOrc1p and DmOrc2p inhibits the intrinsic ATPase activity of DmORC without affecting ATP binding to DmORC subunits. Moreover, Cdk phosphorylation inhibits the ATP-dependent DNA-binding activity of DmORC in vitro, thus identifying a novel determinant for DmORC-DNA interaction. DmORC is a substrate for both Cdk2-cyclin E and Cdk1-cyclin B in vitro. Such phosphorylation of DmORC by Cdk2-cyclin E, but not by Cdk1-cyclin B, requires an “RLX” motif in DmOrc1p. We also identify casein kinase 2 (CK2) as a kinase activity in embryonic extracts targeting DmORC for modification. CK2 phosphorylation does not affect ATP hydrolysis by DmORC but modulates the ATP-dependent DNA-binding activity of DmORC. These results suggest molecular mechanisms by which Cdks may inhibit ORC function as part of re-replication control and show that DmORC activity may be modulated in response to phosphorylation by multiple kinases.

Prevention of re-replication in eukaryotic cells is essential for the maintenance of genomic ploidy, and mechanisms preventing such re-initiation-associated events are needed because unscheduled or uncontrollable DNA replication initiation can lead to genomic instability (1–5). Re-replication control is achieved through (i) the prevention of repeated origin firing during one S phase and (ii) coordination of chromosomal replication with the segregation of the sister chromatids in mitosis. Genetic studies in the fission yeast Schizosaccharomyces pombe first demonstrated that cyclin-dependent kinases (Cdks) 4 play a prominent role in preventing re-replication during S phase and coupling S phase progression to chromosomal segregation in mitosis, by showing that inhibition of Cdk activity in G2/M can induce re-entry into S phase in the absence of mitosis (6–8). Additional studies in the budding yeast, Saccharomyces cerevisiae, substantiated these findings and demonstrated that some Cdk activity is required for one round of replication and that higher levels accumulating in S phase prevent re-initiation. Thus Cdks have both negative and positive roles in the initiation of DNA replication (9). It is now known that in all eukaryotes studied Cdks contribute to the prevention of re-replication through direct inactivation of pre-RC proteins via multiple redundant mechanisms. This redundancy ensures that deregulation of any single mechanism is not sufficient to induce genomic re-replication (1, 2, 10–14). For example, in S. cerevisiae, Cdk phosphorylation destabilizes Cdc6 and induces nuclear exclusion of Cdt1 and the minichromosome-maintenance 2–7 complex.

Initiation of DNA replication involves the ordered assembly of large protein complexes at origins of replication. Pre-replication complex (pre-RC) assembly in late M/G1, when Cdk activity is low, is essential for proteins associated with an origin of replication to acquire competence to initiate DNA synthesis in the ensuing S phase (1, 2). This “licensing” process involves the binding of the hetero-hexameric origin recognition complex (ORC) in its ATP-bound form to an origin of replication, followed by a stepwise assembly of proteins, including Cdc6 and Cdt1, and finally, the presumed replicative helicase, the hetero-hexameric minichromosome-maintenance 2–7 complex. At the G1/S transition, pre-RCs are somehow activated by the action of both Cdk and the Cdc7/Dbf4 kinase, allowing for the recruitment of additional protein complexes into pre-initiation complexes at the licensed origin. This assembly and remodeling ultimately result in the loading of the replicative DNA polymerases and the formation of functional replisomes at replication forks. How these modifications actually change the pre-initiation complex, or allow for the activity of additional factors required in subsequent steps, and thus lead to the engagement of an active replisome is unknown.

In metazoans, other levels of regulation that involve the repressive component geminin, and that do not involve direct phosphorylation of pre-RC components, significantly increased the complexity of re-replication control (1, 2). In addition, regulation of pre-RC components themselves is divergent throughout eukaryota. For example, phosphorylation by Cdk2-cyclin A negatively regulates Cdc6 activity in Xenopus egg extracts via nuclear exclusion rather than degradation, and Cdk phosphorylation contributes to increased protein instability of human ORC, rDmORC, recombinant DmORC; λPPase, λ protein phosphatase; TBB, 4,5,6,7-tetrabromobenzotriazole; EMSA, electrophoretic mobility shift assay; pre-RC, pre-replication complex; ORC, origin recognition complex; TBS, Tris-buffered saline; MS/MS, tandem mass spectrometry; ATP γS, adenosine 5’-O-(thiotriphosphate); CK2, casein kinase 2; ScORC, Saccharomyces cerevisiae ORC.

[Athens, N. 1995. J. Cell. Biochem. 58:471–478. 1 Current address: Cancer Research UK London Research Institute, Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, UK.
2 Recipient of a Human Frontier Science Program long term fellowship.
3 To whom correspondence should be addressed: Dept. of Molecular and Cell Biology, 16 Barker Hall #3204, University of California, Berkeley, CA 94720-3204. Tel.: 510-642-7057; Fax: 510-643-1729; E-mail: mbotchan@uclink.berkeley.edu.
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and Drosophila Cdt1 rather than regulating their subcellular localization (15–17).

Although progress has been made in the elucidation of mechanisms regulating the activities of Cdc6, Cdt1, and the minichromosome-maintenance 2–7 complex, little is known about how ORC activity itself may be regulated by the Cdk activities during the cell cycle (18). In S. cerevisiae, binding of Cdk1-Cib5 to Orc6 following origin activation has been shown to contribute to the prevention of origin re-firing (19). A similar mechanism may operate in Drosophila, where ORC-dependent association of Cdc2-Cdc13 with replication origins prevents re-entry into S phase in the absence of mitosis (20). Cdk phosphorylation of Orc2 and Orc6 in S. cerevisiae and Orc2 in Drosophila contributes to re-replication control (14, 21). However, neither ORC stability nor ORC-chromatin association appear to be regulated in the yeasts, leaving open the question of what aspect of ORC activity is regulated by phosphorylation (22, 23).

In contrast to the yeasts, metazoan cells may control ORC function through differential regulation of the chromatin-association of ORC or ORC subunits (18). Xenopus laevis ORC is phosphorylated in a cell cycle-dependent manner in egg extracts, and chromatin association of X. laevis ORC varies during the cell cycle in an inverse relationship to its phosphorylation status (24–30). Like X. laevis ORC, human ORC appears to be displaced from chromatin in metaphase when chromatin is most condensed (31, 32).

Evidence from mammalian systems and Drosophila indicates that the selective elimination of the essential Orc1 subunit via regulated proteolysis or transient dissociation from the Orc2–6 subunits may control ORC function (18). For example, in Drosophila imaginal disks, the bulk of Orc1 persists from late G1 into metaphase at which time most of the protein is abruptly degraded before entry into the next G1 phase (33). These and other studies suggest that, while the protein levels and chromatin association of Orc2–6 remain constant throughout the cell cycle, Orc1 is selectively removed following origin activation until the next cell cycle. However, it is not clear how the biochemical or cytological behavior of the bulk population of ORC contributes to DNA replication regulation (34).

In the present study we examined the effect of phosphorylation on the biochemical activities of Drosophila melanogaster ORC (DmORC). We found that DmORC is phosphorylated in vivo on subunits DmOrc1p and DmOrc2p and that DmORC is a substrate for Cdns both in vivo and in vitro. Cdk hyperphosphorylation of DmORC inhibits the ability of DmORC to hydrolyze ATP and to form ATP-dependent complexes with DNA in vitro, suggesting possible mechanisms for ORC inactivation by Cdk phosphorylation without affecting DmORC integrity. Unexpectedly, we also found that DmORC is a substrate for CK2 in vivo and in vitro. Hyperphosphorylation by CK2 has no effect on the ATPase activity of DmORC but affects its DNA-binding activity. In each case, phosphorylation and the associated effects on the activities of DmORC were reversed by removal of the inhibitory phosphates. These results suggest molecular mechanisms for how Cdk phosphorylation can regulate ORC activity and indicate that DmORC is a target for multiple kinases.

**Materials and Methods**

*In Vivo Labeling of Drosophila Tissue Culture Cells—* L2 cells were grown to 75% confluency on ten T-150 flasks in M3 medium/5% fetal calf serum. Cells were split into 3 equal fractions, washed subsequently 1× with TBS (25 mM Tris-HCl, pH 7.4, 2.7 mM KCl, 140 mM NaCl), 1× with phosphate-free M3 medium (M3 lacking yeast extract, Bactopeptone, and NaH₂PO₄)/5% fetal calf serum (dialyzed against TBS), 1× with phosphate-free M3/5% fetal calf serum supplemented with 0, 10, or 50 μM TBB (0.1% Me₃SO final), respectively, resuspended in 3 ml of phosphate-free M3/5% fetal calf serum with or without TBB, respectively, and incubated in a 6-well dish at 25 °C. After 1.5 h, 1.5 ml of 32P₀ (1 Ci/mM, 6000 Ci/mmol) was added, and cells incubated for another 2.5 h at 25 °C. Cells were washed 2× with 10 ml of ice-cold TBS, resuspended in 1 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 200 mM LiCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (Roche Applied Science), incubated on ice for 10 min, and then centrifuged for 10 min at 14,000 rpm in a microfuge (Eppendorf). The resulting supernatant constituted the whole cell extract.

DmORC was immunoprecipitated for 2 h at 4 °C from 0.3 ml of labeled whole cell extract with 20 μl of protein A-Sepharose beads coupled to affinity-purified polyclonal αDmOrc2p antibody. The immunoprecipitate was washed 2× in TBS/0.05% Nonidet P-40, 2× in TBS (0.3 mM NaCl)/0.05% Nonidet P-40, and resuspended in 20 μl of TBS plus SDS sample buffer. Half of each sample was fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. After exposure of the membrane to autoradiography film, the membrane was probed with antibodies against DmOrc1p, DmOrc2p, and DmOrc6p.

*In Vitro 32P Labeling of DmORC—* Nuclear extract was prepared from 0–12 h Drosophila embryos as described previously (35). The ammonium sulfate-precipitated extract pellet was redissolved in buffer A (25 mM Hepes-KOH, pH 7.6, 1 mM EDTA, 1 mM EGTA, 0.05% Nonidet P-40, 10 mM glycerol)/0.15 M KCl/5 mM MgCl₂/1 mM dithiothreitol/0.2 mM phenylmethylsulfonyl fluoride/protease inhibitor mixture. 0.2 ml of extract were supplemented with 10 μCi of [γ-32P]ATP (10 mCi/ml, 6000 Ci/mmol, Amersham Biosciences) and incubated for 30 min at 25 °C. Following the labeling period extracts were diluted 1:4 with TBS/0.1% Nonidet P-40, 10 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture, and DmORC immunoprecipitated for 3 h at 4 °C using either polyclonal αDmOrc2p or polyclonal αDmOrc1p antibody. The immunoprecipitate was fractionated by SDS-PAGE and transferred to nitrocellulose membrane, and the membrane was exposed to autoradiography film.

*In vitro* kinase reactions with purified CK2 or Cdk were performed in 30 μl of buffer A (0.15 M KCl/5 mM MgCl₂/0.1 mg/ml bovine serum albumin, including 50 μM ATP, 1 μCi of [γ-32P]ATP (10 mCi/ml, 6000 Ci/mmol, Amersham Biosciences), and 200 ng of DmORC (~16.5 nm) as indicated. Reactions were incubated for 10 min at 25 °C and stopped by adding 10 μl of 4× SDS sample buffer. Half of each sample was fractionated by SDS-PAGE, the gel was stained with silver, dried on Whatman paper, exposed to autoradiography film, and radiolabeled bands quantitated on a PhosphorImager.

**Phosphoamino Acid Analysis**—Radiolabeled DmORC was fractionated by SDS-PAGE, transferred to a polyvinyllidene difluoride membrane, the membrane exposed to autoradiography film, and radiolabeled protein bands corresponding to DmOrc1p or DmOrc2p excised from the membrane. The membrane pieces were pre-wet in methanol, incubated for 60 min at 110 °C in 0.1 ml of constant boiling (6 n) HCl (Pierce), the resulting supernatant was lyophilized, and phosphoamino acids were analyzed by two-dimensional thin layer chromatography as described before (36).

**Purification of Drosophila melanogaster CK2—** Nuclear extract was prepared from 200 g of 0–12 h Drosophila embryos. One-seventh of ammonium sulfate-precipitated extract was redissolved in 50 ml of buffer A (0.1 M KCl/2 mM β-mercaptoethanol/0.2 mM phenylmethylsulfonyl fluoride, centrifuged for 30 min at 35,000 rpm and 4 °C in a Ti45 rotor, the resulting supernatant dialyzed for 3 h at 4 °C against the same buffer A.
buffer, re-centrifuged for 30 min at 35,000 rpm and 4°C using a Ti45 rotor, and 20 ml of the dialyzed extract (0.1 M KCl) fractionated on a 10-ml Poros heparin column. The fraction eluting at 0.4–0.6 M KCl was subjected to ion-exchange and gel-filtration chromatography as outlined in Fig. 5B. In the final glycerol sedimentation step, 150 μl of the pooled peak fractions off the Mono Q column were loaded onto a 4-ml 15–35% glycerol gradient in buffer A/0.2 M KCl, and the gradient was spun for 16 h at 42,000 rpm and 4°C using a SW60 rotor.

Kinase assays directed against rDmORC were performed in 50 μl of buffer A/0.2 M KCl/5 mM MgCl₂/0.1 mg/ml bovine serum albumin/2 mM β-mercaptoethanol containing 2 μg of purified rDmORC, 5 μl of respective fraction, 50 μM ATP, including 5 μCi of [γ-32P]ATP. Reactions were incubated for 30 min at 25°C, subsequently chilled on ice, diluted with 350 μl of buffer A/0.2 M KCl/10 mM imidazole, and the rDmORC re-purified on nickel-nitrilotriacetic acid-agarose using His-Microspin columns (Amersham Biosciences). Reactions were rotated for 30 min in pre-spun columns at 4°C, spun for 1 min at 3,000 rpm and 4°C, the beads washed 1 × with 500 μl of Ni-buffer (50 mM Tris-HCl, pH 7.6/0.3 M KCl/0.05% Nonidet P-40/10% glycerol/10 mM imidazole, and bound material eluted with 50 μl of Ni-buffer/200 mM imidazole. 20% of the eluate was fractionated by SDS-PAGE, and the gel stained with silver, dried on Whatman paper, and exposed to autoradiography film. Antibodies against CK2α and CK2β were kind gifts of Dr. Cai- borne V. C. Glover III (University of Georgia).

**Histone H1 Kinase Assay**—Reactions were carried out for 30 min at 25°C in 50 μl of buffer A/0.2 M KCl/5 mM MgCl₂/0.1 mg/ml bovine serum albumin/50 μM ATP/1 mM dithiothreitol containing 1 mg/ml histone H1 (Upstate), 5 μl of respective fraction, and 5 μCi of [γ-32P]ATP. Reactions were stopped by adding SDS sample buffer, and 10% of each reaction was fractionated by SDS-PAGE. The gel was Coomassie-stained, dried on Whatman paper, and subsequently exposed to autoradiography film.

**Purification of Modified rDmORC**—Untreated rDmORC was purified as described (37). Baculovirus expressing ORC-1-LRN was generated by PCR-based mutagenesis from DmOrc1-wt containing vectors using the following primer pair: 5’-CCGGGCCTCG-GGACCCGCCTCCCGCAAACTCTAAGTGCTGAGATCG-3’ and 5’-CGATCCAGGCTAGATTGTCGGAGGCGGGCTGCCG-AGGCCGCG-3’. To obtain a concentrated stock of dephosphorylated rDmORC (ORC<sup>APase</sup>), a preparation of rDmORC (~500 μg) was diluted before the final Mono S chromatography step with buffer A to reduce the salt concentration to 0.15 M KCl, supplemented with 2 mM MnCl₂, and incubated for 30 min at 25°C with 8,000 units of APase (New England Biolabs). The reaction was subsequently chilled on ice and then fractionated on Mono S.

To generate ORC<sup>CK2</sup>, ORC<sup>CK2/cyclinE</sup>, and ORC<sup>CK2/Cdk2/cyclinE</sup> respectively, 15 μg of ORC<sup>APase</sup> was incubated for 45 min at 25°C in 100 μl of buffer A/0.2 M KCl/5 mM MgCl₂/1 mM ATP with either ~5 μg of purified CK2, Cdk2/cyclin E, or both. Phosphorylated rDmORC was re-purified by centrifugation of the kinase reactions for 16 h at 42,000 rpm and 4°C through a 4-ml 15–35% glycerol gradient containing buffer A/0.3 M KCl. Baculoviruses expressing His<sub>6</sub>-tagged human cyclin B, glutathione S-transferase-tagged human cyclin E, and untagged human Cdc2 and Cdk2, were kindly provided by David Morgan (University of California, San Francisco) and Brian Dynlacht (New York University). Cyclin/Cdk complexes were purified from whole cell extracts of SF-9 insect cells using nickel-chelate or glutathione affinity chromatography, respectively, followed by conventional chromatography.

**Electrophoretic Mobility Shift Assay**—Binding reactions were carried out as described (37, 38), except that 2 mM MnCl₂ was included in the reaction buffer to allow for simultaneous APase treatment. Where indicated APase was included at 200 units per reaction. When 6-dimethylaminopurine (Sigma) was tested for its ability to inhibit Cdk, binding reactions included 3 μl (~100 ng) of glycerol gradient-purified DmORC, 1 mM ATP, 1.5 mM, 5 mM, or 10 mM 6-dimethylaminopurine, and ~50 ng of purified Cdk2/cyclin E.

**APase Assays**—Reactions were carried out at 25°C in 50 μl of buffer A/0.15 M KCl/0.02% Nonidet P-40/0.5 mM dithiothreitol, including 3 μl of 8-azidoadenosine [α-32P]triphosphate (2–10 mCi/mm, MP Biomedicals) and ~35 mM glycerol gradient-purified rDmORC as indicated. The reaction mixture was incubated for 10 min at 25°C followed by 2 min of UV (254 nm) irradiation using a hand-held UV lamp. Proteins were precipitated using trichloroacetic acid to remove unbound nucleotide, and fractionated by SDS-PAGE. The gel was silver-stained, dried on Whatman paper, and exposed to autoradiography film. Radiative bands were quantitated on a PhosphorImager.

**Tandem Mass Spectrometry Analysis**—Purified ORC was subjected to a triple digest protocol as described (39) with the difference that chymotrypsin (Roche Diagnostic) was used instead of subtilisin. The three digested protein samples containing 5% formic acid were pooled and desalted on separate fused silica capillary desalting columns containing 3 cm of Polaris C18-A packed into a 250-μm inner diameter capillary with a 2-μm filtered union (UpChurch Scientific, Oak Harbor, WA). The desalting columns were washed with 5% MeCN, 5% formic acid and eluted with 80% MeCN, 5% formic acid. The samples were lyophilized and stored dried. The dried samples were resuspended in 5% MeCN, 5% formic acid and loaded onto a biphasic 100-μm inner diameter micropipillary column packed with 10 cm of 5-μm Polaris C18-A material and 1 cm of 5-μm Partisphere strong cation exchanger. After loading the peptide digests, analysis was performed using a 9-step multidimensional separation as described previously (40) using 0.02% heptafluorobutyric acid as the counter ion. The MS/MS spectra were analyzed sequentially. First, the MS/MS spectra were searched by using a parallel virtual machine version of SEQUEST running on a 16-node IBM computing cluster against the Drosophila complete protein data base generated from GadFly version 4.0. The resulting SEQUEST output files were filtered using the program DTASelect (41). A subset data base was made containing just the proteins identified. This subset data base was then used to expedite all subsequent differential modification searches (42). The MS/MS data were searched against the database to look for phosphorylation (+80 on STY) and oxidation (+16 on MWY).

**RESULTS**

*DmORC Phosphorylation in Vivo and in Vitro*—In our previous DNA binding studies of DmORC, we used gel-shift assays to measure the formation of an ATP-dependent complex between DmORC and various DNA fragments (37, 38). Curiously, we noticed variations in the binding efficiencies between different protein preparations and that...
DmOrc2p staining after SDS-PAGE displayed a characteristically fuzzy appearance. Such heterogeneity in electrophoretic mobility can be indicative of phosphorylation variations.

Consistent with this hypothesis we found that in the steady state, DmORC2p is the major subunit labeled in vitro by [γ-32P]ATP in 0- to 12-h embryonic extracts (72) (Fig. 1A). In vivo labeling of proliferating L2 or Kc Drosophila cells substantiated this finding (Fig. 1B and data not shown). Under these conditions we found serines were predominantly phosphorylated on DmORC2p, both in embryonic nuclear extract and in tissue culture cells in vivo. Overexpression of recombinant DmORC (rDmORC) in baculovirus-infected insect cells (43) also resulted in the phosphorylation of rDmORC, predominantly on DmORC2p and to a lesser extent on DmORC1p, as suggested by immunoblot examinations of purified rDmORC using phosphoserine- or phosphothreonine-specific antibodies (data not shown).

DmOrc1p and DmOrc2p contain seven and four consensus Cdk sites ((S/T)P) and Cdk consensus sites ((S/T)P) respectively, and these sites are the only consensus Cdk consensus sites in DmOrc1p: A bromo adjacent homology (BAH) domain at the N terminus; an ATP-binding domain (AAA+), and winged-helix domain (WH). RNL indicates an RXL motif at position 314–316 that is important for Cdk2-cyclin E interaction. Upper vertical bars indicate potential Cdk sites (IS/TP) and Cdk consensus sites (IS/TPXR(R)). DmORC2p was subjected to PAA as described in A (small panels). Cdk2-cyclin E and -2p staining after SDS-PAGE displayed a characteristically fuzzy appearance. Such heterogeneity in electrophoretic mobility can be indicative of phosphorylation variations. In vivo labeling of proliferating L2 or Kc Drosophila cells substantiated this finding (Fig. 1B and data not shown).

Based on sequence homology, three structural domains have been identified in DmOrc1p: A bromo adjacent homology (BAH) domain at the N terminus; an ATP-binding domain of the AAA+ type in the C-terminal half of the protein; and a potential winged-helix (WH) DNA-binding domain at the C terminus (Fig. 1E) (44–46). Interestingly, all Cdk consensus sites were found in a large linker region between the BAH and AAA+ domains. We used mass spectrometry to identify phosphorylated residues in purified embryonic DmORC and rDmORC, and compared these data to that obtained from rDmORC that was first dephosphorylated by a protein phosphatase (APPase) and then phosphorylated by Cdk2-cyclin E in vitro (Fig. 1E).

All of the identified phosphorylated residues in DmOrc1p from the three sources were found in the linker region between the BAH and AAA+ domains and corresponded to both Cdk consensus sites, or sites with minimal homology to the consensus sequence. Six modified residues in DmOrc1p were phosphorylated by Cdk2-cyclin E in vitro (Ser-263, Ser-293, Thr-341, Thr-346, Thr-401, and Thr-519). Strikingly, only two sites, Ser-263 and Thr-401, corresponded to Cdk consensus sites. In DmOrc1p from purified rDmORC, five phosphorylation sites were
identified (Ser-293, Ser-481, Thr-516, Thr-519, and Ser-533), consisting of only one Cdk consensus site, Ser-481. Two of the remaining four sites were identical to sites phosphorylated by Cdk2/cyclin E in vitro (Ser-293 and Thr-519). Finally, two phosphorylation sites (Thr-516 and Thr-519) were found in DmOrc1p from embryonic DmORC, both of which overlapped rDmORC phosphorylation sites, and one of which, Thr-519, was targeted by Cdk2/cyclin E in vitro.

Six residues in rDmOrc2p were phosphorylated by Cdk2/cyclin E in vitro (Thr-136, Thr-151, Thr-154, Thr-157, Thr-160, and Thr-258), including only one Cdk consensus site, Thr-136. Strikingly, none of the six phosphorylated residues identified in purified rDmOrc2p (Thr-24, Ser-26, Ser-91, Ser-92, Ser-102, Ser-230, or Ser-231) were Cdk consensus or close-to-consensus sites, indicating that DmORC is also a target for kinases other than Cdks. No phosphorylation sites were identified in embryonic DmOrc2p, perhaps due to low coverage of the protein (40–50%) during mass spectrometric analysis. Due to incomplete coverage in the mass spectrometric analysis for all DmORCs tested, none of the above phosphorylation maps are necessarily complete.

In summary, these data revealed that DmORC is a target for Cdks in vitro and likely in vivo. In addition, DmORC, as inferred from the sequence of the modified sites, is likely to be a substrate for one or more unidentified kinases. Cdks phosphorylated a large number of residues in both DmOrc1p and DmOrc2p, including simple (S/T)P sequences in addition to Cdk consensus sites. We, therefore, asked what the effects were of DmORC hyper- and hypophosphorylation on the biochemical activities of DmORC in vitro.

Dephosphorylation Increases the DNA-binding and ATP-hydrolysis Activities of DmORC—We dephosphorylated embryonic DmORC (DmORCembryo) by APPase treatment and assayed its efficiency side by side with untreated purified DmORCembryo to form an ATP-dependent DNA complex with a radiolabeled ori-β DNA fragment (Fig. 2A). As reported previously, DmORCembryo formed a discrete complex with the radiolabeled DNA fragment in the presence of ATP (Fig. 2A, lanes 2 and 3), and this binding was stimulated ~3–5 fold when APPase was included in the binding reaction (Fig. 2A, lanes 5 and 6).

We then tested whether APPase treatment also affected the DNA-binding activity of rDmORC. To ensure that any effect on the DNA-binding activity of APPase-treated rDmORC was solely due to removal of phosphates from rDmORC, we re-purified rDmORC either before (rDmORCppase) or after dephosphorylation (rDmORCppase) using glycerol gradient sedimentation (Fig. 2B). APPase treatment did not change the sedimentation profile of the complex but led to a more homogenous appearance and increased electrophoretic mobility of DmOrc2p after SDS-PAGE, whereas the mobility of DmOrc1p appeared only marginally increased and other subunits remained unaffected (Fig. 2B, and Fig. 3A, lanes 1–2 and 6–7). Comparison of the DNA-binding activities of the two complexes in the presence of ATP or ATPS revealed that ORCppase bound ~3–5 fold more efficiently to DNA than ORCunppase (Fig. 2C, compare lanes 6–7 to lanes 3–4). Taken together, these results demonstrated that DmORC phosphorylation, as modified in insect cells either from recombinant baculoviral vectors or as made in Drosophila eggs, reversibly reduces the ability of proteins to form ATP-dependent complexes with DNA.

We previously showed that DmORC, like ScORC, contains an intrinsic ATPase activity that is inhibited upon binding to DNA (37, 38). Unlike ScORC, whose ATPase activity is inhibited ~8 fold in the presence of DNA (47), only a ~2-fold inhibition of the ATPase activity of DmORC had been observed in the presence of DNA. We asked whether the ATPase activity of dephosphorylated DmORC was inhibited more strongly in the presence of DNA than mock-treated DmORC. Both ORCppase and ORCunppase exhibited the same low rates of ATP hydrolysis in the presence of ori-β-containing DNA, indicating that dephosphorylation of DmORC does not further enhance the inhibitory effect of excess DNA (Fig. 2D). Unexpectedly, the rate of ATP hydrolysis by
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FIGURE 3. Cdk phosphorylation inhibits ATP-dependent DNA binding of rDmORC. A, rDmORC was incubated with XPPase (lanes 2 and 7), Cdk2-cyclin E (lanes 4 and 9), Cdk1-cyclin B (lanes 5 and 10), or respective buffers as controls (lanes 1 and 6 and lanes 3 and 8), re-purified by glycerol gradient centrifugation, and analyzed by SDS-PAGE and silver-staining. DmORC1p-3p-containing regions of lanes 1–5 are depicted enlarged in lanes 6–10. The positions of rDmORC subunits as well as dephosphorylated and phosphorylated DmORC1p and -2p are indicated. B, glycerol gradient-purified rDmORC, mock-treated with buffer (lanes 3 to 8), or Cdk2-cyclin E (lanes 9 to 14), or Cdk1-cyclin B (lanes 15–20), was tested for binding to ori-β in the absence (lanes 3–5, 9–11, and 15–17) or presence (lanes 6–8, 12–14, and 18–20) of XPPase. Binding was monitored in the absence of ATP or in the presence of 0.5 mM ATP or 0.5 mM ATP*5 as indicated. Controls: lane 1, no protein; lane 2, XPPase alone. C, binding of untreated rDmORC to ori-β was tested in the absence (lanes 3 and 4) or presence of Cdk2-cyclin E (lanes 5–8), and in the absence (lanes 3 and 5) or presence of 1.5 mM DNA (lane 6), 5 mM (lane 7), and 10 mM (lanes 4 and 8) 6-dimethylaminopurine (6-DMAP). All reactions contained 1 mM ATP. Controls: lane 1, no protein; lane 2, Cdk2-cyclin E alone.

Orc<sup><sub>APase</sub></sup> in the absence of DNA was increased 1.8-fold compared with that of Orc<sub>mut</sub>. Thus, although the ATPase activity of Orc<sub>mut</sub> was inhibited 2.8-fold in the presence of DNA, Orc<sup><sub>APase</sub></sup> was inhibited 5-fold by DNA. This suggests that phosphorylation inhibits the intrinsic ATPase activity of DmORC, and this reduced ground-state activity of phosphorylated DmORC combined with an overall lower affinity for DNA may explain the differences in DNA-dependent inhibition of ATPase activity between DmORC and ScORC.

Cdk Phosphorylation Inhibits ATP-dependent DmORC-DNA Binding in Vitro—Because DmORC is a substrate for Cdks, we were interested to learn how the DNA-binding activity of DmORC might be affected upon hyperphosphorylation by Cdk. We performed in vitro phosphorylation reactions using rDmORC and Cdk2-cyclin E or Cdk1-cyclin B and repurified the phosphorylated DmORC by glycerol gradient centrifugation. Cdk treatment did not affect the integrity of the complex as judged from the unaltered sedimentation profile during glycerol gradient sedimentation (not shown). Electrophoretic mobility changes of rDmOrc2p in response to both APase and Cdk treatment (Fig. 3A, lanes 2–4 and 7–9) indicated that rDmORC was only partially phosphorylated in the baculovirus system.

As observed before with embryonic DmORC, the efficiency of ATP-dependent DNA binding by mock-treated rDmORC was increased ~4-fold when APase was included in the DNA binding reaction (Fig. 3B, lanes 4, 5, 7, and 8). Both Cdk2-cyclin E- and Cdk1-cyclin B-treated rDmORC showed greatly diminished ATP-dependent DNA-binding activity (Fig. 3B, lanes 10, 11, 16, and 17) that was restored by APase treatment after Cdk phosphorylation (Fig. 3B, lanes 13, 14, 19, and 20). Simultaneous incubation of untreated rDmORC with both DNA and Cdk2-cyclin E in the presence of ATP also inhibited the DNA-binding activity of rDmORC (Fig. 3C, compare lanes 3 and 5), and increasing amounts of the kinase inhibitor 6-dimethylaminopurine reversed the inhibitory effect of Cdk treatment (Fig. 3C, lanes 5–8). Alanine substitutions at the phosphoacceptor residues of all Cdks consensus sites present in DmOrc1p and DmOrc2p, whereas reducing the extent of phosphorylation of DmORC by Cdk did not abolish the ability of the Cdk to inhibit DmORC-DNA binding (data not shown). Consistent with the phospho-mapping studies described above, this indicates that minimal sites contributed to Cdk-dependent inhibition of DmORC-DNA binding.

Cdk Phosphorylation Inhibits the ATPase Activity of DmORC—To assess the effect of Cdk phosphorylation in the absence of any other phosphorylation on DmORC activity, rDmORC was subjected to a two-step modification protocol (Fig. 4A). In the first step, phosphates present on purified rDmORC were removed by APase treatment, followed by re-purification of the dephosphorylated rDmORC by ion-exchange chromatography, yielding Orc<sup><sub>APase</sub></sup>. Orc<sup><sub>APase</sub></sup> was then either mock treated or phosphorylated by Cdk and re-purified from the kinase reaction using glycerol gradient sedimentation. This sequential treatment resulted in the generation of rDmORC complexes that were either dephosphorylated (Orc<sup><sub>APase</sub></sup>) or that contained only Cdk2-cyclin E-dependent (Orc<sup><sub>Cdk2-cyclinE</sub></sup>) or only Cdk1-cyclin B-dependent (Orc<sup><sub>Cdk1-cyclinB</sub></sup>)-phosphorylated residues (Fig. 4B).

We first tested whether DmORC phosphorylation interfered with the ability of DmOrc1p to bind to ATP by cross-linking to the photoreactive ATP analog, 8-azido-ATP. As a control, the ATP binding-deficient complex, DmORC-1A (37), was analyzed in parallel (Fig. 4C). The specific cross-linking signal to DmOrc1p was normalized in each case to the nonspecific cross-linking signal for DmOrc2p (Fig. 4C, inset bar graph). Comparison of the cross-linking efficiencies between the wild-type complexes and the DmORC-1A mutant demonstrated that ATP cross-linking to DmOrc1p was dependent on an intact P-loop in the ATP-binding domain of DmOrc1p. Importantly, these data demon-
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We then asked whether Cdk phosphorylation affected the ATPase activity of rDmORC, as we observed previously with phosphorylated embryonic and rDmORC (Fig. 2D). Indeed, Cdk phosphorylation reduced the ATPase activity of rDmORC to background levels either in the presence or absence of DNA (Fig. 4D, compare ORC<sub>Cdk2-cyclinE</sub> and ORC<sub>Cdk1-cyclinB</sub> to ORC<sub>ΔPPase</sub>). Consistent with the reversibility of the phosphorylation-dependent inhibition of the DNA-binding activity of DmORC, treatment with APPase after Cdk phosphorylation restored the intrinsic ATPase activity of rDmORC, and DNA was again able to inhibit the ATPase activity of rDmORC 4- to 5-fold (Fig. 4D, ORC<sub>Cdk2-cyclinE</sub> × APPase and ORC<sub>Cdk1-cyclinB</sub> × APPase). Thus, Cdk phosphorylation strongly inhibited the ATPase activity of DmORC by specifically interfering with the ATP hydrolysis but not the ATP-binding step in the ATPase cycle of DmORC1p.

DmORC Interacts with Cdk2-cyclin E via an RXL Motif in DmORC1p—Despite some functional redundancy, Cdk2-cyclin complexes exhibit substrate specificity both in vivo and in vitro (48–50). How this specificity is achieved is not entirely clear. One mechanism involves a bipartite binding mode of the Cdk to its substrate, in which the cyclin subunit serves as a docking module that recruits the Cdk to a specific substrate by binding to a short sequence motif on the substrate surface known as the cyclin-binding (Cy) or RXYL sequence motif, which comprises the signature sequence RXL or KXL (49, 51, 52).

Visual inspection of the amino acid sequence for all six DmORC subunits identified multiple RXL and KXL sequence motifs. DmOrc2p contains a single RXL (amino acids 556–558) and two KXL motifs. DmOrc1p contains seven RXL and five KXL motifs, with one RXL (amino acids RNL 314–316) and one KXL (amino acids 389–391) motif found within the large linker region (Fig. 1E). We tested whether RXL314–316 in DmOrc1p and RXL389–391 in DmOrc2p were required for phosphorylation by either Cdk2-cyclin E or Cdk1-cyclin B in vitro.

We found that a peptide spanning RXL314–316 in DmOrc1p did not affect the phosphorylation of DmORC by either Cdk1-cyclin B or Cdk2-cyclin E (data not shown). However, a peptide spanning amino acids 306–322 of DmOrc1p specifically inhibited the phosphorylation of DmORC by Cdk2-cyclin E (but not Cdk1-cyclinB), with a 5- to 10-fold more efficient inhibition of DmOrC1p phosphorylation than DmOrc2p phosphorylation (data not shown). The inhibitory effect of the DmOrc1p peptide was dependent on the RXL sequence, because a peptide containing LRN in place of RXL had no effect on Cdk2-cyclin E phosphorylation of DmORC (data not shown).

We generated rDmORC bearing the RNL314–316LRN mutation, which we termed ORC-1-LRN. This complex purified with the same characteristics as wild-type rDmORC and displayed wild-type ATP-dependent DNA-binding activities (supplemental Fig. S1). To test the mutant complex for potential phosphorylation defects, we purified the complex following dephosphorylation by APPase and tested the mutant complex for potential phosphorylation defects, we purified the complex following dephosphorylation by APPase and compared the efficiency of its phosphorylation by Cdk1-cyclin B or Cdk2-cyclin E (data not shown). However, a peptide spanning amino acids 306–322 of DmOrc1p specifically inhibited the phosphorylation of DmORC by Cdk2-cyclin E (but not Cdk1-cyclinB), with a 5- to 10-fold more efficient inhibition of DmOrC1p phosphorylation than DmOrc2p phosphorylation (data not shown). The inhibitory effect of the DmOrc1p peptide was dependent on the RXL sequence, because a peptide containing LRN in place of RXL had no effect on Cdk2-cyclin E phosphorylation of DmORC (data not shown).

We generated rDmORC bearing the RNL314–316LRN mutation, which we termed ORC-1-LRN. This complex purified with the same characteristics as wild-type rDmORC and displayed wild-type ATPase and ATP-dependent DNA-binding activities (supplemental Fig. S1). To test the mutant complex for potential phosphorylation defects, we purified the complex following dephosphorylation by APPase (ORC-1-LRN<sub>ΔPPase</sub>) and compared the efficiency of its phosphorylation by Cdk2-cyclin E or Cdk1-cyclin B to that of dephosphorylated wild-type complex (ORC-wt<sub>ΔPPase</sub>, Fig. 5). We found that DmOrc1p was 4- to 5-fold less efficiently phosphorylated, and DmOrc2p was 2- to 3-fold
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Figure 5. DmORC Interacts with Cdk2-cyclin E via an RXL motif in DmORC1p. rDmORC carrying the mutation RNL314–316LRN in its subunit DmOrclp (ORC-1-LRN) was dephosphorylated using xPase, and repurified by ion-exchange chromatography, yielding ORC-1-LRN\textsubscript{PPase}. Equal amounts of mutant and wild-type dephosphorylated rDmORC were analyzed by SDS-PAGE and silver staining (upper left panel). In vitro kinase titration reactions in the presence of [γ\textsuperscript{32}P]ATP were carried out with increasing amounts of Cdk2-cyclin E (center panels) or Cdk1-cyclin B (bottom panels) versus constant amounts of either wild-type or mutant dephosphorylated rDmORC complexes as indicated. The reactions were analyzed by SDS-PAGE and silver staining, the dried gel exposed to autoradiography film, and \textsuperscript{32}P incorporation into DmOrclp or DmOrc2p quantitated using a PhosphorImager. Phosphorylation efficiencies are plotted as signal intensity over the amount of kinase added to each reaction.

DmORC Is a Substrate for Multiple Kinases—Our mass spectrometric phospho-mapping studies of rDmORC identified multiple phosphorylation sites in rDmOrc2p that did not conform to characteristic Cdk sites, indicating that rDmORC was a substrate for kinases other than Cdns. Thus, we sought to identify the kinase(s) phosphorylating DmORC in 0- to 12-h embryonic extracts by fractionation and purification. For our assay we treated rDmORC with the various extract fractions and [γ\textsuperscript{32}P]ATP, repurified rDmORC from the reactions, and monitored \textsuperscript{32}P incorporation of individual rDmORC subunits following SDS-PAGE and autoradiography. We found two peaks of kinase activity in POROS-heparin fractions that targeted only DmOrclp and -2p (Fig. 6A). Inspection of the load and flow-through fractions showed that all potential kinase activities directed against DmORC were retained on the heparin resin at 0.1 M KCl.

The first detectable kinase activity eluted at 0.2–0.3 M KCl and required prior dephosphorylation of rDmORC, indicating that rDmORC was already phosphorylated on sites that coincided with some of those being targeted in these fractions. Several lines of evidence suggested that the activity eluting at 0.2–0.3 M KCl was due to Cdk(s) present in these fractions: (i) the activity co-fractionated with histone H1 kinase activity (Fig. 6A, "H1 Coomassie + autoradiogram"); (ii) cyclin B was present in these fractions (Fig. 6A, Western α cyclin B); (iii) the activity is highly sensitive toward the Cdk-specific inhibitor, purvalanol A (Fig. 6C, left panels and right graph) (53); and the activity preferred DmOrclp as a substrate to DmOrc2p, as was observed with Cdk5 in vitro (see Fig. 1C).

A second and more predominant kinase activity eluted at 0.6 M KCl (Fig. 6A) and strongly preferred DmOrc2p to DmOrclp. After multiple chromatography steps and a final glycerol gradient sedimentation, we found the activity to co-fractionate with a complex of two proteins of apparent molecular masses of ~40 and ~25 kDa, respectively (Fig. 6B). Mass spectrometric analysis identified the larger protein as the catalytic subunit of casein kinase 2, CK2α, and the smaller protein as the regulatory CK2β subunit. Immunoblot analysis confirmed that both CK2 subunits fractionated almost exclusively at 0.6 M KCl (Fig. 6A, Western α CK2 and CK2 β). In addition, purvalanol A did not inhibit this kinase activity even at concentrations as high as 1 mM (Fig. 6C, center panel and right graph), consistent with the notion that CK2, and not another Cdk, is the kinase present in the 0.6 M KCl fraction.

Intrigued by the prominent CK2 phosphorylation of DmORC in embryo extracts, the preference of CK2 for DmOrc2p over DmOrclp as a substrate, and the fact that CK2 phosphorylated rDmORC on serine residues only (supplemental Fig. S2), we asked whether CK2 was responsible for DmORC phosphorylation in tissue culture cells in vivo. We repeated the labeling experiments in L2 cells in the absence or presence of increasing amounts of the CK2 inhibitor, TBB (Fig. 7) (54). As before, \textsuperscript{32}P incorporation during the 2- to 3-h labeling period was predominantly found on DmOrc2p. We found that a 10 mM TBB concentration reduced the extent of DmOrc2p phosphorylation 5-fold, and 50 mM TBB resulted in a reduction of ~13-fold in DmOrc2p phosphorylation. These data indicated that CK2 is involved in the phosphorylation of DmORC in vivo.

Having identified DmORC as a substrate for both Cdk and CK2 in Drosophila cells, we asked whether rDmORC was also phosphorylated by these two kinases in the baculovirus expression system. We compared the phosphorylation efficiencies of ORC\textsubscript{baculo} and ORC\textsubscript{PPase} in vitro kinase reactions using purified CK2 or Cdk2-cyclin E (supplemental Fig. S2). We found that DmOrclp from ORC\textsubscript{PPase} was the only subunit affected by increasing amounts of Cdk2-cyclin E, demonstrating that rDmORC is phosphorylated on at least some of the same sites in the baculovirus system as are phosphorylated by Cdk2-cyclin E in vitro. However, DmOrc2p from ORC\textsubscript{PPase} was a much better substrate for CK2 in vitro, indicating that rDmORC was phosphorylated on the same sites in DmOrc2p as were targeted by CK2 in vitro. Thus, expression in the baculovirus system resulted in phosphorylation on DmOrc2p sites predominantly in rDmOrclp and on CK2 sites predominantly in rDmOrc2p, consistent with the mass spectrometric phospho-mapping studies described above.
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CK2 and Cdk Phosphorylation Affect DmORC-DNA Binding Activity Differently—To compare the effects of CK2 and Cdk phosphorylation on the biochemical activities of DmORC, rDmORC was either dephosphorylated using APase (ORC<sup>APase</sup>) or phosphorylated following dephosphorylation by purified CK2 (ORC<sup>CK2</sup>) or Cdk2-cyclin E (ORC<sup>C2cyclinE</sup>), or phosphorylated using a combination of both CK2 and Cdk2-cyclin E (ORC<sup>C2K2/C2cyclinE</sup>), and repurified as described above (Fig. 8A and B, left panels). Although the ATPase activity of ORC<sup>C2cyclinE</sup> was strongly reduced upon phosphorylation (Fig. 4D), the ATPase activity of ORC<sup>CK2</sup> was unaffected (supplemental Fig. S3), indicating that inhibition of the ATPase activity of DmORC required the phosphorylation of specific residues targeted by Cdk and not by CK2.

We then examined the ATP-dependent DNA-binding activity of the differentially phosphorylated rDmORC in the presence of increasing amounts of competitor DNA (Fig. 8, A and B). The overall amount of radiolabeled probe bound by ORC<sup>CK2</sup> was only slightly reduced when compared with that bound by ORC<sup>APase</sup> at all competitor concentrations tested. However, ORC<sup>CK2</sup>-DNA complexes differed significantly in the electrophoretic mobilities of the observed DNA complexes (Fig. 8A, compare lanes 8–13 and 2–7). Best visualized at low (1–5 μg/ml) competitor concentrations, ORC<sup>CK2</sup>-DNA complexes appeared smeared, indicative of increased structural heterogeneity among ORC<sup>CK2</sup>-DNA complexes as compared with ORC<sup>APase</sup>-DNA complexes. In addition, whereas the main population of ORC<sup>CK2</sup>-DNA complexes (indicated by an arrow) was reduced 2–4-fold at the various concentrations of competitor tested (Fig. 8A, graph), the most dramatic qualitative change induced by CK2 phosphorylation was the increased appearance of higher electrophoretic mobility complexes. Although the reason for the increased heterogeneity in ORC<sup>CK2</sup>-DNA complexes is currently unclear, all of the detectable gel shifts were ATP-dependent and could be supershifted with a rDmOrc2p antibody (Supplementary Fig. 4), suggesting that the shifts are due to structurally distinct DmORC-DNA complexes.

Despite a slightly more fuzzy appearance, ORC<sup>C2K2 cyclinE</sup>-DNA complexes appeared as the same two major shifted populations that were observed with ORC<sup>APase</sup> (Fig. 8A, lanes 14–19). However, the overall abundance of DmORC-DNA complexes was reduced up to 10–15-fold upon Cdk phosphorylation depending on the competitor concentration used, indicating that the main effect of Cdk phosphorylation was a significant reduction in the affinity of DmORC for DNA.

**FIGURE 6.** DmORC is a substrate for Cdns and CK2 in 0- to 12-h embryo nuclear extracts. A, Poros heparin fractionation of 0–12 h embryo nuclear extract. Each fraction was tested for DmORC-directed kinase activity by incubating purified rDmORC with an extract fraction in the presence of [γ-<sup>32</sup>P]ATP, re-purifying the rDmORC from the kinase reaction using nickel-nitrilotriacetic acid-agarose, and analyzing the re-purified rDmORC for <sup>32</sup>P incorporation using SDS-PAGE, silver staining (upper panel), and autoradiography (center panel). Fractions were analyzed for the presence of CK2, CK2, and cyclin B by immunoblot as indicated (lower panels), and for histone H1 kinase activity as described under "Experimental Procedures." B, purification scheme of the DmORC-directed kinase activity from the 0.6M KCl heparin fraction. A silver-stained gel of the glycerol gradient fractions is depicted in the upper right panel. Glycerol gradient fractions were tested for DmORC-directed kinase activity and the fractions analyzed by SDS-PAGE, silver staining (center right panel) and autoradiography (center right panel). C, DmORC-directed kinase activities in the 0.3M KCl (lanes 1–6), 0.4M KCl (lanes 7–13), and 0.6 M KCl (lanes 14–19) fractions were tested at increasing amounts of purvalanol A. The silver-stained gels of the reactions are shown on the left, and the corresponding autoradiograms are on the right. The [γ-<sup>32</sup>P] incorporation into DmOrc1p and DmOrc2p was plotted as the percentage of [γ-<sup>32</sup>P] incorporation in the absence of purvalanol A over the concentration of purvalanol A (graph in right panel).
Although CK2 phosphorylation changed the electrophoretic mobility, and Cdk phosphorylation strongly inhibited the ATP-dependent DmORC-DNA complex formation, simultaneous phosphorylation of DmORC with both CK2 and Cdk essentially abolished all ATP-dependent DNA-binding activity by DmORC (Fig. 8B), demonstrating that the two kinases have an additive effect on the inhibition of DmORC-DNA binding in vitro.

**DISCUSSION**

In all eukaryotes examined Cdk5 contribute to the inhibition of unscheduled pre-RC assembly at origins of replication through redundant and evolutionarily divergent mechanisms. However, the direct molecular mechanisms governing the control of ORC function following Cdk phosphorylation has not been previously examined. While the data presented in this study are discussed in light of the role of DmORC in the initiation of DNA replication, it has to be stressed that DmORC and its individual subunits also have functions in cytokinesis, heterochromatin formation, and at the neuromuscular junction, which are distinct and separable from their replication functions (55–58). We therefore anticipate that DmORC phosphorylation would also modulate activity of these other functions. We found that DmOrc1p and DmOrc2p are substrates for Cdns in vitro and that Cdk phosphorylation inhibits both ATP-hydrolysis and DNA-binding functions of the complex.

We do not know if these effects are linked to a single, or distinct and non-overlapping subgroup of modifications. Prior work has shown that ATP-hydrolysis and DNA-binding functions of ORC are un-linked (33, 48), and the structural basis for the current findings will need further study focusing on both the specific modifications required for the regulation and the structure of ORC.

ATP hydrolysis by ScOrc1p is essential for multiple rounds of loading of the minichromosome-maintenance 2–7 complex in an in vitro pre-RC assembly system with S. cerevisiae extracts (59). Therefore, it is possible that inhibition of the ATP-hydrolysis activity of ORC following Cdk phosphorylation might contribute to an inactivation of this ORC function in one aspect of re-replication control. The phosphorylation sites in DmOrc1p determined here concentrate to the linker region between the BAH and AAA7 domains of the subunit. Due to a predicted lack of extensive secondary structure organization, this region is expected to exhibit a high degree of conformational flexibility. Phosphorylation might thus induce a conformational change within the region, perhaps by creating a charge-dependent docking site, that either affects the AAA7 domain in DmOrc1 directly or induces conformational changes in the relationships between the subunits, whose specific arrangement within the complex is required for ATP hydrolisys by DmOrc1p. For example, for the budding yeast ORC, ScOrc4 is essential for ATP hydrolysis by ScOrc1p by providing a catalytic arginine finger to the ScOrc1p ATP-binding domain (59). This arginine finger motif is conserved in *Drosophila* and humans, suggesting that a similar mechanism for catalysis is operating in higher eukaryotes. Although Cdk phosphorylation sites are not strictly conserved between yeast, *Drosophila*, *Xenopus*, and mammals, the relative arrangement of potential Cdk sites in subunits Orc1p and Orc2p is similar in all eukaryotes, suggesting that control of the ATPase activity of ORC through Cdk phosphorylation may be conserved in other eukaryotes (supplemental Fig. S5).

Inhibition of the ability of DmORC to bind to DNA in an ATP-bound state provides an obvious mechanism by which Cdns could exert re-replication control in vivo and would be consistent with the requirement for alternating Cdk2-cyclin E activity during *Drosophila* endocycles and alternating Cdk activity during normal mitotic cycles (60–62). The ORCs from all species studied to date can bind to DNA in an ATP-independent mode, but for ScORC and DmORC the ATP-dependent interaction shows higher affinity. Can DmORC remain bound to origins of replication in chromosomes without ATP? Clearly, published cytological evidence discussed above shows that DmORC1p-deficient complexes can be detected with mitotic chromosomes, and this would indicate that at least at some sites this is possible. DmORC might remain bound to chromatin throughout the cell cycle by means of interactions with protein constituents in the chromatin, yet its ATP-dependent interaction with the DNA in chromatin might change during the cell cycle. Cell cycle-dependent changes in the ATP-dependent mode of binding may then be modulated by modifications and may then affect the avidity of ORC for chromatin. Earlier immunostaining studies from our laboratory showed that a fraction of DmORC (as measured by DmOrc2p staining) was constitutively associated with chromatin throughout the cell cycle. However, these studies also revealed that DmORC-chromatin association qualitatively changed throughout the cell cycle. In contrast to other stages of mitosis, at metaphase we found that DmOrc2p staining and detection on chromosomes was sensitive to fixation methods, possibly reflecting changes in the modes of DmORC-chromatin association (55).

Another question is raised if an ATP-ORC-DNA complex is the only mode by which the complex can maintain association with chromatin. Inhibition of the DNA binding activity of DmORC upon Cdk phosphorylation, raises the problem of how DmORC binding to chromatin could be established under conditions of sustained high Cdk activity, as observed during the alternating S and M phases of the syncytialblastoderm stage of the early *Drosophila* embryo or during choriomic gene amplification in *Drosophila* follicle cells, which are both characterized by sustained Cyclin E-Cdk activity (62, 63). Specific localization of phosphatases or their regulatory subunits can be an important mechanism for controlling the dephosphorylation of a given substrate (64). The specific localization of a phosphatase to origins of replication may therefore be one way to overcome Cdk inhibition locally. Alternatively, DmORC might be protected from the inhibitory Cdk activity through developmentally regulated association with co-factors that block access to the subunit. Due to a predicted lack of extensive secondary structure organization, this region is expected to exhibit a high degree of conformational flexibility.
of the Cdk to DmORC. Neither of these ideas is inconsistent with the notion that an ATP-independent mode of ORC association with chromosomes is operative. Thus the tight ATP-dependent contacts that ORC makes with DNA may only be important at one critical time in the replication process.

We found that Cdks phosphorylate ORC to a great degree on residues embedded in minimal recognition context, a situation similar to what has been reported for the human complex (56). This differs from the yeasts, where ORC is exclusively phosphorylated on consensus sites both in vivo and in vitro (14, 21, 65). Perhaps the large number of potential Cdk sites in Orc1 and Orc2 of the metazoan complexes reflects a fine-tuning of a mechanism involving multiple phosphorylations that cooperatively allow for a threshold-regulated function (66).

Phosphorylation of DmORC by Cdk2/cyclin E requires an RXL motif in DmOrc1p. While Cdk1–Clb5 interacts with ScORC via an RXL motif in Orc6 and forms stable complexes with ScORC bound to DNA (19), we did not detect stable complexes between DmORC and Cdk2/cyclin E or Cdk1–cyclin B either by EMSA or in glycerol gradients (not shown). Mutation of the RXL motif of DmOrc1p does affect the phosphorylation efficiency of DmOrc2p, which suggests that the RXL motif in DmOrc1p plays a targeting role in vivo important for the phosphorylation of other pre-RC components.

The stability of the Orc1p subunit, in contrast to the other ORC subunits, fluctuates throughout the cell cycle in both Drosophila and human cells in an APC/C<sub>cdc1</sub>1-dependent or CEP57-dependent manner, respectively, and Orc1p levels are limiting for DNA replication in certain Drosophila cell types (18, 33, 65, 67). It is noteworthy that we did not detect any changes in the stability of the DmOrc1p subunit with DmORC-phosphorylation status. This establishes that neither Cdk- nor CK2-phosphorylation is directly responsible for differential release of the DmOrc1p subunit from the complex in vivo, although the modifications may signal degradation or further functions toward release. In this vein of thought, it is interesting to note that while Cdk-phosphorylation often contributes to the targeting of SCF substrates, for the case of human Cdc6p, Cdk phosphorylation opposes the association of the APC/C<sub>cdc1</sub>1-dependent proteolysis of DmOrc1p is located in the Cdk-site-containing N-terminal half of the protein, and though the Orc1p modifications sites are not embedded in canonical APC/C recognition motifs, the modifications we have mapped may by analogy stabilize the DmOrc1p subunit (33).

DmORC is a substrate for CK2, and unexpectedly it was found that in extracts this is the predominant ORC kinase. In contrast to the effects of the Cdk enzymes, CK2 phosphorylation induces a change in the mode of the DmORC-DNA interaction rather than a substantial reduction of the affinity for DNA DmORC. CK2 phosphorylation does not effect the integrity of DmORC, and our supershift data argue against the possibility that the observed heterogeneity in detected DmORC-DNA species is due to the presence of DmORC sub-complexes.

How CK2 modification may influence ORC function in cells is an open question. Treatment of asynchronously growing Drosophila L2 cells with the CK2 inhibitor TBB almost completely abolished any 32P labeling of DmORC. From these data alone we cannot conclude that CK2 is directly involved in DmORC phosphorylation as CK2 is required for the activation of a large pool of protein kinases in the cell (69). However, supporting a direct phosphorylation of DmORC by CK2 in vivo, we find that CK2 modification of DmORC predominates in embryonic extracts, whereas Cdk phosphorylation of DmORC in these extracts is much more difficult to detect. One reason for these apparent quantitative differences in phosphorylation efficiency of DmORC by CK2 versus Cdk might lie in the fact that Cdk phosphorylation occurs only during a short period of a specific cell cycle stage and detection would require a synchronized cell population, whereas CK2 is potentially constitutively active (70, 71).
